Alternative Testing Approaches in Environmental Safety Assessment

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CONTENTS

SUMMARY

There are many opportunities for applying the principles of the three Rs (replacement, reduction and refinement) when addressing the use of fish for the environmental safety of products and effluents.

When investigating the effects of chemicals to fish a number of clear conclusions and recommendations are made:

- The number of fish used for establishing an effect concentration for environmental risk assessment or classification can be reduced by assessing the effects of chemicals to algae and daphnids and applying the step-down approach. A recent exercise conducted by the European Chemicals Bureau showed a reduction in fish used of over 70% when compared with the original LC_{50} studies conducted on new substances as part of their registration. This exercise should now be extended to existing chemicals and effluents.
- The use of biomimetic techniques (e.g. solid phase micro-extraction) may be helpful when addressing complex mixtures, especially if a common mode of action can be assumed.
- Combining (Q)SAR, grouping and read-across with fish cell lines/fish embryo testing should eventually mean that no fish need be used when addressing the short-term effects of chemicals in the environment. The strategy developed by the Task Force based on these considerations, should be assessed and tested and information on its utility shared to allow for refinement as knowledge increases.
- Modification of existing guidelines to include additional endpoints to assess endocrine activity, e.g. vitellogenin endpoint in OECD 210 test.

When addressing the use of fish for assessing the bioconcentration of chemicals, the following conclusions and recommendations are made:

- OECD 305 should be re-evaluated in terms of clearly identifying the domain for which this test is appropriate and to identify ways in which the same quality of data can be obtained by using fewer animals. For example by running one exposure concentration instead of two, only addressing the uptake phase or by reduced sampling occasions during the exposure and depuration phase.
- Alternative methods, e.g. the dietary bioconcentration factor (BCF) test and the static exposure approach were recognised as reducing the number of animals, while extending the feasibility of assessing uptake (via diet) and leading to a BCF. These alternatives should be further developed and their usefulness explored.
- In considering replacement of fish, the adoption of the strategy identified by the Task Force is recommended. This approach utilises (Q)SAR/groupings and read-across, together with

assessment of the key parameters in the bioconcentration process, absorption, distribution, metabolism and excretion (ADME).
- Biomimetic extraction was recognised as having particular usefulness in addressing freely available chemicals and in some circumstances of being capable of predicting uptake. The usefulness of this method in addressing effluents should be investigated.
- The usefulness of alternative organisms (e.g. invertebrates) to obtain BCF data, albeit as a worst-case value, should also be investigated. These data would then be used in a strategy for assessing bioconcentration and indirect exposure, allowing for refinements to occur depending upon the information and/or risk assessment requirements.

The Task Force organised a workshop on Alternative Testing Approaches in Environmental Risk Assessment, which was held on 7th - 9th July 2004, at Crécy-la-Chapelle and was co-funded by the European Centre for the Validation of Alternative Methods (ECVAM, ecvam.jrc.it/) and the CEFIC LRI. The aim of the workshop was to facilitate an active dialogue amongst industry, regulators and academia on the pragmatic use of alternative approaches. The workshop addressed *in vivo, in vitro* and *in silico* approaches, in line with the 3Rs, for the generation of hazard and exposure information within the context of environmental risk assessment. Thirty-seven scientists with backgrounds in ecotoxicology and environmental fate assessment representing governments, academia and industry attended the workshop. The conclusions and recommendations may be obtained from ECETOC (2004a).

Three research projects were identified by the Task Force and have been funded by the CEFIC-LRI (www.cefic-lri.org/):

- Comparison and evaluation of fish cell line and fish embryo tests - combining daphnid, algae and (Q)SAR with fish embryo and a battery of fish cell tests. The research goal is to provide alternatives that initially reduce the use of fish in the OECD 203 fish acute test and that ultimately will replace the test.
- Establishing a BCF Gold Standard Database - the development of a database holding peer reviewed high quality BCF that would be viewed as a valuable resource for future development of alternative tests.
- Identifying trans-species biotransformation potential - to identify chemicals that rapidly metabolise versus those that do not.

1. INTRODUCTION

European legislation requires that non-animal, alternative approaches of testing should be used in the place of animal procedures wherever possible. The principal EU directive protecting animals used in scientific studies (EEC, 1986) states that 'an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably practically available'.

There are various definitions of what constitutes an animal. Within this report the definition used is that contained in the UK Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK, 1986). This act defines a 'protected animal' as any living vertebrate, other than man [Section 1(1)] and which is then extended to the invertebrate species *Octopus vulgaris* via an amendment (UK, 1993). The protection also extends to certain immature forms of development of mammals, birds and reptiles - from halfway through the gestation or incubation period; and for fish, amphibia and *Octopus vulgaris* - from the time at which they become capable of independent feeding.

Recent developments seeking to implement the principles of reduction, replacement and refinement (the three Rs developed by Russell and Burch, 1959) in mammalian acute oral toxicity testing has led to new guidelines being adopted by the OECD. These include the fixed dose procedure (OECD, 2001a), the up-and-down procedure (OECD, 2001b) and the acute-toxic-class method (OECD, 2001c). Additionally, in response to the sixth and seventh amendments of the European Directives on Cosmetics (EC, 1976, 1993, 2003a), a large amount of research either has been or will need to be performed to replace animal tests. One example is the research carried out to replace the Draize eye irritation test with a suitable *in vitro* based assay (Courtellemont *et al*, 1999).

In comparison to the large amount of research into alternative test methods for warm-blooded animals, alternatives to the use of vertebrates for assessing environmental safety are still at an early stage of development. Therefore the use of alternative approaches in environmental sciences has yet to receive widespread regulatory acceptance. The one exception to this is Quantitative Structure Activity Relationships (QSARs).

In addition, the European Commission has recently adopted a draft legislative text, describing the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH) (DGEE, 2003). Originally published as the *'Strategy for a Future Chemicals Policy'* (EC, 2001), the objective of REACH is to remove the distinction between new and existing substances. All chemicals produced at above 1 tonne per year will be subjected to a registration procedure (see Section 2.2.4). Additionally, it is foreseen that all data for chemicals produced at less than 10 tonnes per year will be based on non-animal procedures. In the document non-animal testing is

promoted, stating that there should be a maximisation of non-animal test methods, that the development of new non-animal test methods should be encouraged and that test programmes should be minimised. However, this document also suggests that all chemicals in use should be assessed to provide information relevant to health and environmental safety. This could mean that approximately 12-13 million animals will be used for the assessment of approximately 30,000 chemicals by 2012 (IEH, 2001).

Strategies for using alternative methodologies within REACH have not been spelled out. Recently, Combes *et al* (2003) described a strategy for how alternative methodologies, *in silico* and *in vitro*, could be used for addressing the human hazard of substances and thus within the conduct of a risk assessment. There are, however, no widely accepted regulatory strategies for using alternative methodologies for assessing the environmental safety of chemicals.

In the context of risk assessment, the Task Force has used the Technical Guidance Document (TGD) for the risk assessment of new and existing chemicals (EC, 2003b).

Additionally, there is a growing trend for the use of whole effluent assessment (WEA) methodologies in the EU. WEA type schemes are generally seen as supporting the hazardous substance strategies of the Oslo Paris OSPAR and the Water Framework Directive (WFD) (EC, 2000). For example, WEA has the potential to contribute to WFD targets for priority substances and it is expected that WEA could be used within Integrated Pollution Prevention and Control (IPPC) (EC, 1996) for the elaboration of emission controls for priority substances. Some European countries also believe that WEA targets will feature in periodical revision of best-available-technique reference documents for certain industrial activities. This could lead to a considerable increase in the number of fish used in effluent assessments. This is also discussed in ECETOC (2004b).

Based on these considerations, an ECETOC Task Force was commissioned with the following terms of reference:

- Review current environmental regulatory endpoints e.g. toxicity and bioaccumulation, but also secondary poisoning and the alternative approaches that provide the required information;
- develop strategies taking account of alternatives including existing data (e.g. read-across and extrapolation), QSARs, *in vitro* and other techniques for implementing the 3Rs;
- propose how the strategies could be implemented within a regulatory context;
- recommend research activities to support these strategies.

2. BACKGROUND

The use of animals for safety testing represents a dilemma between balancing the need to ensure products can be handled and used safely, and legitimate and widely felt concerns about animal testing. A range of product testing is required to provide data for the hazard assessment of products manufactured by the chemicals industry. This is based on current regulations and voluntary industrial initiatives that are designed to protect human and animal health as well as the surrounding environment. Testing for environmental effects in the aquatic environment includes assessment of acute effects such as acute lethality and chronic effects including development, growth and reproduction, endocrine modulation, on aquatic animals, including fish.

2.1 Definitions

2.1.1 Definition of a protected animal

There are a number of legislative instruments, which define a protected animal. Thus alternative methods to tests using such animals would be preferred. For a review of the different regulations related to the protection (and definition) of laboratory animals see: www.publications.parliament.uk/pa/ld200102/ldselect/ldanimal/150/15001.htm).

- The EU Council Directive 86/609/EEC (EEC, 1986) states:
  Article 2 - for the purposes of this Directive the following definition shall apply: 'animal', unless otherwise qualified, means any live non-human vertebrate, including free-living larval and/or reproducing larval forms, but excluding foetal or embryonic forms.

- The UK Act (UK, 1986) defines animals as follows:
  2.6 The Act defines a 'protected animal' as any living vertebrate, other than man [Section 1(1)]. The invertebrate species *Octopus vulgaris* has been added by means of the Animals (Scientific Procedures) Act (Amendment) Order 1993.

  2.7 Protection extends to certain immature forms from the following stages of development: mammals, birds and reptiles from halfway through the gestation or incubation period, fish, amphibia and *Octopus vulgaris* from the time at which they become capable of independent feeding.

- In the US, (USA, 1966) the term 'animal' means any 'live or dead dog, cat, monkey (non-human primate mammal), guinea pig, hamster, rabbit, or such other warm-blooded animal, as may be determined is being used, or is intended for use, for research, testing, experimentation, or exhibition purposes or as a pet'. This definition thus excludes cold-blooded (poikilotherms) animals e.g. fish.

It is important to note that there are potential differences caused by the interpretation of the UK and European regulations, in terms of the subtle descriptions of fish life stages and the term 'independent feeding'. These differences are discussed further in Chapter 3.

2.1.2 Ethical considerations of animal use

To aid researchers in the attempts to reduce, refine or replace animals, there are also definitions of the extent of suffering that relates to animals in regulated procedures. Within the UK system, four bands are defined: unclassified, mild, moderate and substantial. Table 1 gives some examples of the use of these bandings in typical ecotoxicity tests.

Table 1: Examples of the use of the UK severity banding of suffering in typical ecotoxicity tests

Test	Banding
Acute toxicity test	Substantial
Fish growth test	Mild
Bioconcentration test	Mild
Partial or full life-cycle	Moderate/substantial[a]
Reproductive and developmental endocrine effects test	Moderate/substantial[a]
Genotoxicity testing	Substantial
Breeding of amphibia	Mild

[a] Dependent upon Home Office licence holder

2.1.3 The three Rs

Russell and Burch (1959) originally set out the definition of the three Rs (3Rs). 'Replacement' means the substitution of conscious living higher animals by insentient material. 'Reduction' means reduction in the numbers of animals used to obtain information of given amount and precision. 'Refinement' means any decrease in the incidence or severity of inhumane procedures applied to those animals that still have to be used.

The three principles should be applied simultaneously and to the highest extent possible. In the context of refinement, there is clearly a need for judgement relating to the severity of pain, suffering or distress. Examples of this are given in the UK (Scientific Procedures) Act (UK, 1986), discussed above (Section 2.1.2).

Another area for possible confusion is the use of fish for harvesting cell lines, tissues or organs, and whether the subsequent use of these biological materials in a test could be considered as a

replacement or a refinement. Thus in the context of the UK and European regulations, fish that have been held, as though in an aquarium and humanely sacrificed (e.g. schedule 1 procedure, UK, 1986), would not be counted as used in an animal procedure and the test using their organs would be deemed a replacement for the use of fish.

In addition to scientific approaches that will replace, reduce and refine the experimental use of fish (the traditional 3Rs), an additional 3Rs known as the 'Solna principles' (OECD, 1996a) have been identified. These additional 3Rs state that tests for regulatory purposes need to reflect the following:

- Biological relevance;
- reliability/reproducibility;
- regulatory acceptability.

2.2 Information needs

2.2.1 The use of fish in ecotoxicology

Fish represent the oldest and most diverse class of vertebrates. They encompass approximately 48% of the known member species in the subphylum Vertebrata. Fish live in a wide range of aquatic habitats; from fresh to salt water; from the cold polar seas to warm tropical reefs and from shallow surface waters to the ocean depths. The geographical and environmental diversity of fish thus makes them important experimental models, in environmental toxicology.

Certain fish species also possess many specific characteristics that support their use in environmental toxicology such as:

- Fish are in intimate contact with the aquatic environment;
- fish possess specialised functions of higher vertebrates;
- some fish have a relatively short life-cycle;
- they can produce eggs in large quantities;
- eggs can be externally fertilised and are transparent, thus embryonic development can be followed making early life stage assessments easier;
- many fish species are also suitable for field and laboratory experiments, allowing studies to progress from laboratory assessments to 'in-field' tests (e.g. cage monitoring studies).

Historically, these advantages and the economic importance of some fish, both for food and recreation, have made them favoured models in ecotoxicological studies. Consequently, fish have

been used in tests to assess the potential environmental impact of chemicals and effluent, establish water quality criteria and for monitoring water quality in rivers and estuaries.

2.2.2 Numbers of fish used

An idea of the number of animals being used may be obtained from the European Commission's third report (CEC, 2003) on the numbers of experimental animals used in the EU. This indicated that a total of 614,234 fish were used in 1999. This is significantly fewer than for 1996 when the number reported was 1,112,791 (CEC, 1999). According to the figures for 1999, most of the fish (71%) were used 'in experiments for selected purposes' and 29% for toxicological and safety evaluations, including the testing of chemicals and products for or by the aquaculture industry in feed and vaccine development or in studies on diseases. However, bearing in mind that different countries have different definitions, these proportions may vary. It is also apparent that the number of fish used for testing has fluctuated from year to year, for example the data from the United Kingdom showed an increase from 122,438 fish in 1999 to 243,019 fish in 2000 (HMSO, 2000) and then a decrease to 171,092 in 2001 (HMSO, 2001). However, approximately 80% of these experiments represented fundamental research (for example in academic and governmental research laboratories) and not acute or chronic regulatory ecotoxicity testing. This ECETOC report will address the application of the principles of the 3Rs for the use of fish for environmental toxicological and safety evaluation purposes.

2.2.3 Regulatory tests - chemicals

Regulatory testing is established at the national level for wastewater, and at a supranational level (for example, the EU (EEC, 1967, 1992) for newly developed and/or existing chemicals). In the EU, the tests for notification of new chemicals have to be carried out according to Annex V of Council Directive 67/548/EEC, which involves acute toxicity for fish (method C.1), acute toxicity for *Daphnia* (C.2), and the algal growth inhibition test (C.3) (EEC, 1967, 1992).

The current fish tests are designed to evaluate endpoints such as mortality, reproduction, growth rate and toxicokinetic parameters. The fish acute toxicity test (Annex V, C.1) is the most widely used test. In the OECD High Production Volume (HPV) programme, for example, the acute fish test is one of the essential sources of data for the Screening Information Data Set (SIDS) report, as well as the acute *Daphnia* test (Annex V, C.2) and the algal test (Annex V, C.3). Furthermore, in the context of the Globally Harmonised System (UN, 2003) for Hazard Classification and Labelling, the 96-h LC_{50} for fish is applied to designate both acute and chronic toxicity classes by applying an acute to chronic ratio (ACR) or safety factor. These acute tests are discussed in Chapter 3. Other fish tests are used to evaluate chronic effects and effects on specific stages of

development. For the assessment of chemicals with potential endocrine activity, two new screening fish assays (a short-term test and a long-term test for evaluation of reproduction) are currently under consideration at the OECD level (see 3.2.2.3). The objectives of these assays are to evaluate potential endocrine activities in fish by measuring endocrine-related endpoints, such as vitellogenin induction, gonad histology, sex ratio, secondary sexual characteristics and reproductive parameters. Evaluating chronic endpoints by alternatives is also discussed in Chapter 3. The bioconcentration factor (BCF) is determined by using the bioconcentration flow-through fish test (OECD, 1996b). The assessment of bioconcentration is further discussed in Chapter 4.

2.2.4 REACH and its impact on the use of fish

While the testing of substances produced or imported at 1-10 tonnes per year should be limited to *in vitro* tests, base-set testing may be requested for substances marketed at above 10 tonnes per year. It is expected that this new policy will increase the number of fish needed for ecotoxicity testing to at least 4.4 million by 2012 (IEH, 2001).

There is a high reliance in the current chemicals regulations in the EU on *in vivo* data from fish tests. Thus in the base-set acute lethality tests, 210 animals are used, 20% of them being fish, and for level 1 (prolonged toxicity) testing (production volume 100-1,000 tonnes), 602 animals are required, 60% of them being fish (IEH, 2001). If base-set information were required for 30,000 chemicals (1-100 tonnes production), the number of fish required for testing would be 1.260 million, and for level 1 (100-1,000 tonnes production), 2.024 million. However this must be considered as a conservative estimate as often tests will use more than the minimum specified in the OECD protocol in order to satisfy global regulatory demands. Moreover, the testing of these chemicals in *in vivo* experiments would be extremely costly and time demanding. To overcome resource and ethical problems, the proposed legislation, REACH, suggests a change in testing strategy, to use *in vitro* methods for the initial hazard evaluation of chemicals. This will require the establishment and validation of *in vitro* test protocols.

2.2.5 Regulatory tests - effluents

The principle of this approach is that the test species continually respond to the surrounding water quality, integrating its varying state of pollution to give an overall quality assessment of the effluent in question in relative terms or may simply warn that a given standard has been contravened. Consequently, many countries worldwide have established protocols for testing the effects of effluents on fish.

In Canada, effluents from pulp and paper mills must be tested periodically in an acute lethality test involving rainbow trout (Environment Canada, 1989, 1990). A similar approach will soon be applied to mine effluents. Ten fish are exposed for 96 h to a whole-water sample of effluent. If, after 96 h, six or more of the fish have died, the effluent has failed the test. Recently, the possibility of replacing this test with a fish cell test has been explored, with promising results (Dayeh *et al*, 2002). In Germany, the waste water act (Abwasserverordnung, 2004), requires the testing of wastewater effluents by means of a 48-h acute fish lethality test according to DIN 38412-L31 (DIN, 1989), with the golden orfe, *Leuciscus idus melanotus*, as the test species. The test determines the dilution of wastewater that is not lethal to fish within a 48-h exposure period. However, this test has recently been replaced, and wastewater toxicity is now evaluated with a test involving fish eggs, according to DIN 38415-T6 (DIN, 2002). This is discussed in Chapter 3.

Fish testing for environmental effects currently includes assessment of acute effects such as acute lethality and chronic effects such as development, growth and reproduction. Schemes are also being developed to use fish to investigate other potential adverse effects of effluents, notably bioconcentration of contaminants and endocrine modulation.

The influence of using fish for effluent testing on the overall number of procedures used should not be underestimated. For example, a recent survey of three multinational companies revealed that > 90% of the fish used in 2001 were for mandatory effluent biomonitoring, nearly all in the US and Canada, with a small number in Germany. Only 6% of the fish used were for product ecotoxicity testing. Consequently, the inclusion of fish in a routine test for WEA would lead to a dramatic increase of the numbers of animals used for scientific studies (see also ECETOC, 2004b).

2.3 Potential for alternatives

The alternative approaches discussed in Chapters 3 and 4 will generally follow a tiered approach of reduction, refinement and finally replacement. The following sections will briefly describe the main topics addressed.

2.3.1 Reduction

2.3.1.1 Other sources of information (also replacement)

In all cases, the starting point is the available information and the extent to which this enables tests to be redesigned or even not performed. Examples include:

- Read-across or chemical grouping that may enable studies to be designed utilising fewer animals than a full study for each of the chemicals;
- information relating to the potential for biotransformation derived either from biodegradation studies or mammalian studies. Reducing the number of test concentrations or employing the 'step-down' method.

2.3.1.2 Enhancement of existing test methods for assessing endocrine activity of chemicals

Fish screening assays and confirmatory tests are being proposed to assess the endocrine activity of chemicals. The fish full life-cycle and the two generation tests are being proposed at higher tier testing (US EPA, 1998). The design of a partial life-cycle test, based on an existing test guideline that would include the most critical life stage for endocrine activity (sexual differentiation) and relevant endpoints will allow for the assessment of a higher number of chemicals with a limited amount of resources (see Section 3.2.2.5).

2.3.2 Refinement

Refinement includes the use of the fish in tests that may either be altered to improve the environment and thus reduce suffering or in which the test itself is redesigned to concentrate on sublethal/behavioural changes. This also includes using existing guidelines within current methods to limit the suffering of animals.

2.3.3 Replacement

2.3.3.1 In silico methods (also reduction)

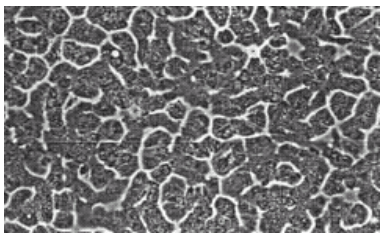
The use of models e.g. (Q)SARs will lead to replacement where the model has been validated and has widespread regulatory acceptance. Such models can also be used to redesign studies to reduce the number of animals being used to confirm an endpoint, in this case reduction. This is discussed further in Chapters 3 and 4.

2.3.3.2 In vitro methods

The use of *in vitro* test systems, particularly those derived from the liver (Blaauboer, 1996) offers the opportunity to study metabolic pathways at the cellular level, thereby playing a key role for understanding the mechanisms of toxicity observed in the whole organism (Fent, 2001). The key differences between *in vivo* and *in vitro* systems are illustrated in Figure 1.

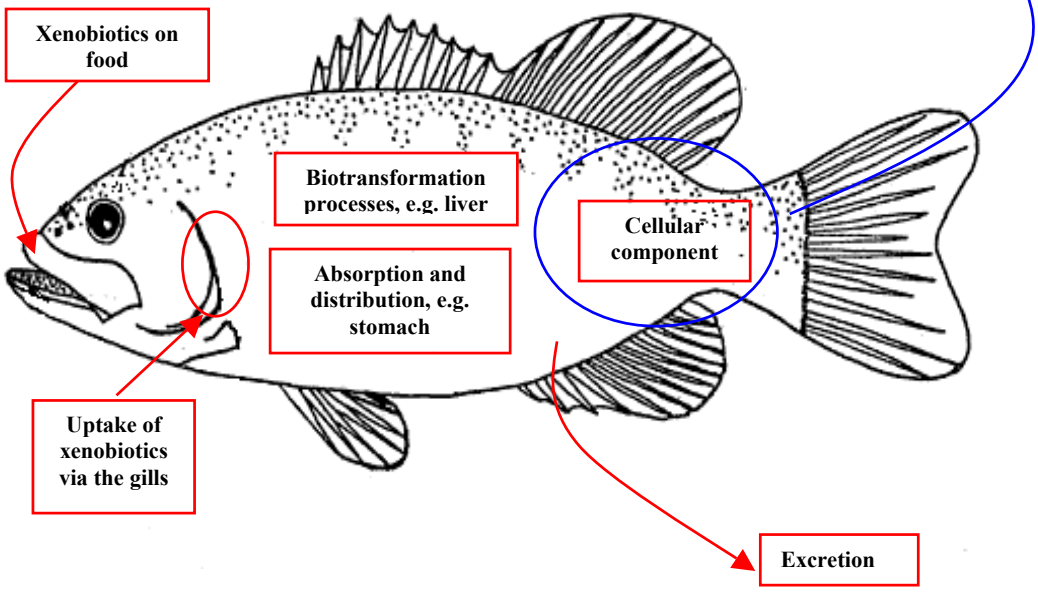
Figure 1: Comparison of in vivo and in vitro assays

In vitro - cellular component isolated from a whole organ (in this case an immortalised cell line derived from a liver tumour).



Fish hepatoma cells

In vivo **-** whole organism



Characterisation of enzyme systems involved in metabolism offers an opportunity for understanding comparative pharmacokinetics between different species and phyla. In turn, this provides valuable support for extrapolation and 'read-across' of data. *In vitro* methods, therefore, offer a potential alternative to using vertebrates to assess aquatic toxicity (see Chapter 3) and also for predicting bioconcentration in whole fish (see Chapter 4).

Fish cell culture is based on the use of three main cell types (Hunt *et al*, 1986):

- Organ (e.g. liver) cultures;
- primary cultures;
- immortalised cell lines.

From these three cell types it is possible to observe different endpoints of toxicity including morphology, inhibition of cell growth, cloning efficiency and inhibition of macromolecular synthesis (Hunt *et al*, 1986), as well as cellular metabolism. Organ (liver) homogenates and subcellular fractions are the simplest and most well-defined *in vitro* methods and are valuable to study metabolism, (see Chapter 4). The use of cell cultures is physiologically more realistic than subcellular systems, as they maintain cellular organisation, as well as cell to cell communication and inducible enzymes. Cytotoxicity can only be studied in intact cellular systems (see Chapter 3).

Most fish cell toxicity assays are based on immortalised cell lines (Isomaa *et al,* 1994; Clemedson *et al,* 1996). Cellular systems can be divided into isolated/primary and immortalised cell cultures. Appendix B provides an overview of the isolation and culture processes. The advantages and disadvantages of these systems (see Appendix C) are described in various key references, notably Pesonen and Andersson (1997), Segner and Cravedi (2001) and Babich and Borenfreund (1991) for primary cultures, and Vrba *et al* (2002), Sandbacka *et al* (1999) and Fent (2001) for immortalised cell lines.

2.3.3.3 Alternative organisms to fish

Alternative organisms may be used to replace fish, as daphnids and algae are frequently more sensitive to chemicals than fish. This is discussed further in Section 3.5.1.

Within a strategy for assessing bioconcentration it may also be possible to use organisms, e.g. mussels, that have minimal biotransformation potential to derive a maximal bioconcentration factor. For example, if there were only a need to demonstrate that the BCF in fish were below a certain value, e.g. < 500 (for GHS) or 2000, in the EU PBT assessment. This is discussed further in Section 4.4.5.

2.4 Report structure

In this report, the principles of the traditional 3Rs (reduction, refinement and replacement) will be examined in detail and recommendations made for alternative test methods to traditional fish

tests. The report will initially address the areas where fish are used for identification of hazardous properties, including the LC_{50} test (see Chapter 3). Next the report addresses the use of fish for assessing the potential for indirect exposure of a chemical through bioconcentration into aquatic species (see Chapter 4). Chapter 5 addresses the scope for using alternative methods for assessing the sediment toxicity of chemicals, while Chapter 6 addresses how indirect exposure is currently assessed, and what alternatives are available to provide the information used in this assessment. The report addresses strategies within which the data and information needs may be systematically obtained depending upon the level of certainty within a risk assessment, and then recommends areas for further research.

Two areas were not considered in the report, because although animals may be used, current assessments rarely lead to new or additional animal testing. These are atmospheric effects and terrestrial hazard assessments. These are briefly discussed in Sections 2.4.1 and 2.4.2.

2.4.1 Atmosphere

For the majority of chemicals there is little need or desire to test their effects on birds. When addressing pesticides, such testing is considered and frequently conducted. The most important tests on birds are those measuring reproductive effects, and there are currently no *in vitro* alternatives (CSTEE, 2004).

Two approaches that may be used, should the question of avian testing arise, are firstly to assess the persistence of a chemical in the atmosphere, e.g. using the Atkinson method (Atkinson, 1987), and secondly to address alternative sources of information including that derived from mammalian studies (see Section 2.4.2).

2.4.2 Terrestrial hazard assessment

When environmental risk assessments (ERA) addressing the terrestrial compartment are conducted for a particular location (e.g. a watercourse, or contaminated site) a number of contaminants may be present and the process differs from that described in the EU TGD (EC, 2003b). Fish, amphibians and other vertebrate organisms can potentially be used in the environmental risk assessment of both aquatic and terrestrial systems. Examples of this for aquatic ecosystems are provided in Chapter 5, where the use of fish to improve the risk assessment of contaminated sediments is described.

In terrestrial systems there is growing pressure to develop ecological risk assessment strategies for contaminated sites and to assess the impact of remediation programmes. For example, in the

UK under Part IIA of the 1990 Environmental Protection Act there is a statutory obligation for local/enforcing authorities to identify contaminated land and the potential impact that this land and any proposed remediation works may have upon the ecological system. To ensure that contaminated site assessments provide an appropriate level of detail, these are often undertaken in a tiered fashion similar to the ASTM Risk-Based Corrective Action framework (ASTM, 1998a). The advantages of using tiers are that the level of detail of the investigation and data required are proportional to the perceived risk. This in itself helps to reduce the number of bioassays, and hence animals, required.

In many cases the bioassays developed for terrestrial ERA use invertebrates and plants. At the highest tiers effects of water runoff may be investigated using fish and amphibians.

There are concerns that other vertebrates could be used for terrestrial ERA because many European countries are developing methods to assess impacts from contaminated sites to protected sites e.g. 'Sites of Special Scientific Interest', nature reserves and areas of special protection for birds. Assessment of impact on these sites generally comprises a combination of bioassays and an evaluation of their potential to cause adverse ecological effects as a consequence of secondary poisoning (see Chapter 6). These are similar to the approach recommended by the US in the management of their Superfund sites (US EPA, 1999).

To assess whether species of concern are likely to be at risk, the first stage is to examine any available existing mammalian and avian toxicity data. Next an extrapolation, using dietary intake to predict an exposure concentration, is undertaken. This information can be used to assess the potential to cause adverse effects. These food chain models work by looking at the particular species to be protected, establishing dietary habits and examining the concentration of specific contaminants in food as described by the US EPA in their Wildlife Exposure Factors Handbook (US EPA, 1993). The EPA Handbook provides a source of information and an analytical framework for screening-level risk assessments for common wildlife species. These screening-level risk assessments may be used for several purposes, and one benefit is that they help to focus research and monitoring efforts and therefore potentially help reduce the amount of animal testing required.

For large sites or those considered to pose a potential risk to a sensitive ecosystem or organism, there may be a requirement to assess effects on raptors and higher predators. If assessment of effects on higher predators is required, these tend to be based on behavioural observations and non-invasive monitoring. Tissue analyses tend only to be conducted on animal corpses found during the monitoring studies. However in some instances (e.g. the US EPA Superfund sites) monitoring of biomarker responses and assessment of contaminant concentrations in fish, amphibians and rodents collected from the wild. In such assessments animals are usually humanely killed prior to tissue analysis.

3. AQUATIC TOXICITY

3.1 Introduction

Aquatic toxicity studies to assess the hazard potential of chemicals are routinely carried out for the assessment of new and existing chemicals. While this represents the major proportion of aquatic species used for testing, aquatic organisms are also used for other purposes. Of the three organisms, fish, algae and daphnids which are used for the evaluation of toxicity as part of the EU 'Base Set' testing for evaluation of New and Existing Substances, fish are the only vertebrates that are used routinely (although amphibians may also be used). Fish have historically always been used for evaluating acute and chronic toxicity and have in the past been considered to be the representative species for the aquatic environment by the scientific community, regulatory bodies, and chemical industry. The fact that they are also an important food source has added weight to this perspective. Within the EU however, the significance of fish being a representative species for the wide and varied aquatic community has been increasingly called into question in recent years (Fentem and Balls, 1993). The use of fish in monitoring of effluents or pollution incidents, is gradually being replaced by the use of invertebrates (e.g. daphnids) or algae. This is on the basis of convenience, cost and sensitivity (see Section 3.5). There is an additional consideration as to the relevance of a precise toxicity value for evaluating potential toxicity in the environment. Schulte and Nagel (1994) also questioned whether the high number of animals used for the determination of acute toxicity for fish and amphibians were justified by the improvement in precision. This is particularly the case as application factors (of at least one order of magnitude) will subsequently be applied to the data at the risk assessment stage.

Furthermore, under the 7th Amendment to the Cosmetics Directive and the proposed REACH legislation, the strategy is that animal (vertebrate) testing should follow the 3Rs principle namely reduction, refinement or replacement, where technically feasible. In comparison to the large amount of research into alternative test methods for warm-blooded animals, alternatives to the use of vertebrates for aquatic toxicity testing are still at an early stage of development. One potential area is the development of the frog embryo teratogenesis assay using *Xenopus* sp. (known as FETAX) to replace mammals in teratogenicity testing. An ASTM Standard Guideline, E-1439-98, has been issued (ASTM, 1998b). However in terms of fish, only preliminary validation has been conducted and has limited acceptance by the regulatory authorities. For these reasons, the development of validated alternative methods to the use of fish are a priority for both regulators and industry.

A confounding influence on the general desire to reduce the number of fish used for ecotoxicity testing is the fact that to obtain the lowest application factor for deriving the Predicted No Effect Concentration (PNEC), the EU TGD recommends the use of three long-term No Observed Effect

Concentrations (NOECs), one of which is derived from studies on fish. This will therefore tend to lead to an increase in the number of fish used in chronic testing.

Care has to be taken when defining what constitutes a protected species. According to the UK Home Office definition, fish are not classed as protected until they are capable of independent feeding. However the stage at which this occurs may vary according to the species. Thus, while for fry it may not be clear as to whether they are protected or not, fish embryos can be regarded as a potential replacement of whole fish. Further discussion of this is given in Section 3.4.

After an initial discussion on the types of tests and current methodology (see Section 3.2) in which fish are used, the chapter takes a tiered approach, dealing first with 'Reduction' in Section 3.3, followed by 'Refinement' (Section 3.4) and 'Replacement' (Section 3.5). In each section, the advantages and disadvantages of the available alternative approaches and methodologies are discussed.

The Task Force has taken the decision to focus primarily on alternatives to acute aquatic toxicity in this chapter since it was considered that alternatives to fish for chronic aquatic toxicity assessment were at an earlier stage of development.

3.2 Aquatic toxicity testing using fish

The types of tests in which fish are used are shown in Table 2 together with information on the number of fish used.

The information in the table for Base Set, Level 1 and Level 2 tests was obtained from the relevant OECD test assessment guidelines. The studies are designed to not only evaluate mortality but also morbidity. A key piece of information required for a risk assessment is the concentration at which a chemical causes an adverse effect (Repetto *et al,* 2001), in particular the NOEC. Such information is used to derive PNECs. In risk assessments, PNECs are compared with predicted environmental concentrations (PECs) to establish whether there is any environmental risk (i.e. PEC/PNEC ratio is > 1). It is not possible to make predictions or establish application factors without this valuable piece of information on effect concentrations. PNECs can be estimated from acute or chronic data. In terms of the risk assessment process, whether acute or chronic exposure data are required currently depends upon the quantity of chemical being placed on the European market.

Table 2: Specific tests with details in which fish are used in ecotoxicity tests

	Number of test groups (minimum)	Number of animals (minimum)	Duration of study	Guideline		Relevant section
Base Set (for 1-10 tpa)				**OECD**	**EU**	
Acute toxicity in fish	5 + control	42	96 h	203 (OECD, 1992a)	92/69 EEC OJ L383A	3.2.1.1
Limit test	1 + control	14	96 h	203 (OECD, 1992a)	92/69 EEC OJ L383A	3.3.1
Level 1 (for 10-1,000 tpa)						
14-d prolonged fish toxicity	5 + control	60	14 d minimum	204 (OECD, 1984)		3.2.1.3
Bioconcentration in fish	2 + control	48	28 d minimum	305 (OECD, 1996b)	98/73/EEC OJ L305 1998	4.2
Juvenile growth test	5 + control	96	28 d minimum	215 (OECD, 2000)	2001/59/EEC OJ L225 2001	3.2.2.1
Level 2 (for 1,000+ tpa)						
Early life stage fish toxicity test	5 + 2 control	60 eggs	28 d minimum	210 (OECD, 1992b)	2001/59/EEC OJ L225 2001	3.2.2.2
Environmental safety programmes						
Effluent monitoring	1-5 + control	14-42	24-96 h	Based upon Guideline 203 (OECD, 1992a)		3.2.1.2

Table 2: Specific tests with details in which fish are used in ecotoxicity tests (cont'd)

	Number of test groups (minimum)	Number of animals (minimum)	Duration of study	Guideline		Relevant section
Methods for endocrine-active chemicals				**OECD**	**EPA**	
Fish screening assay	3 + control	100 minimum	21 d	OECD pre-validation in progress		3.2.2.3.1
Reproduction assay	3 + control	120 up to 10,000 depending on fecundity[a]	28 d (of which 14 are pre-exposure)	In development (see also OECD (2004a))		3.2.2.3.2
Fish sexual developmental test	3 + control	120 minimum	60/90 d	Pre-validation to be initiated		3.2.2.3.4
Life-cycle test	5 + control	350 up to 10,000 depending on fecundity	150 d minimum	Japanese proposal (OECD, 2003a)	EPA 540/9-86-137, July 1986	3.2.2.4
Two-generation test	5 + control	392 up to 10,000 depending on fecundity	180 d minimum	US proposal (OECD, 2003b)		3.2.2.5

[a] Maximum number of fish used in the assays will depend on the fish species and interpretation of regulation regarding the protection of laboratory animals (EEC, 1986)

3.2.1 Fish acute toxicity test

3.2.1.1 Acute toxicity tests for regulatory submissions

The principle of the acute toxicity test (OECD, 1992a) involves exposure of fish to a test substance for a preferred period of 96 h. Observations of gross mortality during a 96-h acute toxicity test are recorded at (3-6), 24, 48, 72 and 96 h. The test requires that a minimum of seven fish be used for each of at least five concentrations plus control(s). Acute toxicity is expressed as the median lethal concentration that kills 50% of the population (LC_{50}) over the given time period. The test environment used should be relevant to the substance tested (i.e. static, semi-static or flow-through). The species of fish used, according to OECD test guideline 203 (OECD, 1992a) (see Table 3), should be selected on the basis of practical criteria, such as their ready availability throughout the year, ease of maintenance, convenience for testing, relative sensitivity to chemicals, and any economic, biological, ecological or geographical factors.

Table 3: Fish species recommended for acute aquatic toxicity assessment (OECD, 1992a)

Recommended species	Recommended test temperature range (^{o}C)	Recommended total length of test fish (cm)
Zebrafish *Danio rerio* (Cyprinidae)	21-25	2.0 ± 1.0
Fathead Minnow *Pimephales promelas* (Cyprinidae)	21-25	2.0 ± 1.0
Common carp *Cyprinus carpio* (Cyprinidae)	20-24	3.0 ± 1.0
Rice fish *Oryzias latipes* (Cyprinodontidae)	21-25	2.0 ± 1.0
Guppy *Poecilia reticulata* (Poeciliidae)	21-25	2.0 ± 1.0
Bluegill *Lepomis macrochirus* (Centrarchidae)	21-25	2.0 ± 1.0
Rainbow trout *Oncorhynchus mykiss* (Salmonidae)	13-17	5.0 ± 1.0

3.2.1.2 Acute toxicity tests for effluent discharge

Monitoring of acute toxicity of effluents to fish is carried out based on OECD guideline 203 (OECD, 1992a). The number of fish used for each effluent is a minimum of seven per

concentration plus control. The number of groups used varies depending upon the endpoint(s) of interest.

3.2.1.3 Fish prolonged toxicity test: 14-day study

The fish prolonged toxicity test (OECD, 1984) may be used in place of the fish acute toxicity test if a longer observation period is considered appropriate, for example if testing highly lipophilic, poorly water soluble substances, and/or the reporting of additional information is considered necessary. The test is based on the fish acute toxicity test (OECD, 1992a) with an additional extended exposure period of at least 14 d. The principle of the test is that threshold levels of lethal and other observed effects and NOEC are determined at intervals during the test period. The test requires at least 10 fish per concentration plus control(s). All other study requirements are as detailed in 3.2.1.1.

3.2.2 Fish chronic toxicity testing

3.2.2.1 Fish juvenile growth test

The fish juvenile growth test (OECD, 2000) looks at the effect of a chemical on the growth of a population of juvenile fish, which are in the exponential growth phase. The fish are weighed prior to commencement of the study and exposed for 28 d at sublethal concentrations of the test chemical. Fish are fed a ration based on the initial weight of the fish, which may be recalculated after 14 days. The fish are reweighed at the end of the test and effects on growth rates determined through regression analysis or through one-way analyses of variance followed by multiple range tests comparisons with control data to determine a NOEC and lowest observed effect concentration (LOEC).

3.2.2.2 Fish early life stage (ELS) toxicity tests

Tests with early life stages of fish are intended to define the lethal and sublethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic and sublethal effects of the substance on other fish species (OECD, 1992b). The test involves fertilised eggs being exposed until all the control fish (as a minimum) are capable of independent feeding. Effects of the chemical (including hatching success and time to hatch, survivorship, deformation, abnormal behaviour, weight and length) are compared with control fish populations to determine a LOEC and NOEC. Normally five concentrations will be employed for each substance, spaced by a constant factor not exceeding 3.2.

The more recent OECD guideline 212 (OECD, 1998) was designed to provide useful information in that it could form a bridge between lethal and sublethal tests. As such it can be used as a screening test for the full early life stage test (OECD, 1992b) or for chronic toxicity. It can be used for fish species where husbandry techniques are not sufficiently advanced to cover the period of change from endogenous to exogenous feeding (OECD, 1998). It was not designed to replace guideline 210 (OECD, 1992b), however the merit of this study as an alternative to guideline 210 is discussed in more detail in Section 3.4.2. The test (OECD, 1998) begins '…by placing fertilised eggs in the test chamber and is terminated just before the yolk sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls'.

3.2.2.3 Methods in development to assess the endocrine activity of chemicals to fish

Currently, there are no internationally validated methods to determine the potential of chemicals to impact the endocrine system, However, a number of methods have been proposed as screening assays to identify the potential endocrine activity as well as confirmatory tests to assess adverse reproductive effects. Candidate protocols are described in a detailed review paper (OECD, 2004a), The non-spawning fish screening assay is currently being validated (prevalidation phase 1B) under the auspices of the OECD. US EPA is proposing a screening assay that includes spawning as an endpoint. This assay is based on Ankley *et al* (2001), where both the pre-exposure and exposure phases have been reduced from 21 to 14 d. The OECD is also considering other protocols, which include exposure during the critical periods when sexual differentiation occurs.

3.2.2.3.1 Fish screening assay

This assay, based on work by Panter *et al* (1998), is designed to identify chemicals that affect androgenic or oestrogenic activity in sexually dimorphic fish exposed during a limited part of their life-cycle, not including reproduction (OECD, 2002). The biological endpoints are gross morphology (appearance, including secondary sexual characteristics and gonado-somatic index (GSI)); plasma or liver vitellogenin levels; and histopathology of excised gonads and are measured in subadult fish after an exposure of 21 d. The levels of vitellogenin in the fish are then measured using an enzyme linked immunosorbent assay (ELISA). Since GSI has recently been found to be an unreliable parameter, the assessment of fecundity has been added in the second phase of the OECD pre-validation. The final test design is still under discussion (OECD, 2004e).

3.2.2.3.2 Short-term reproduction assay

This assay measures the reproductive performance of mature adult fish. It requires a clean water phase (pre-exposure) for the first two weeks, to establish baseline data on fecundity, followed by two weeks of exposure. Two concentrations plus the appropriate controls are used. Four females and two males are used for each of the four replicates per concentration. Observations of, and changes in, spawning behaviour and secondary sexual characteristics (where appropriate) are recorded during 28 d. At the end of the assay, blood is collected for measurement of vitellogenin and sex steroids levels. The gonads are excised for determining the GSI and for later histological examination. Hatching success and post-hatch viability of larvae are monitored for 24 h in control water (OECD, 2004a). A similar protocol, the paired breeding assay, based on research by Harries *et al* (2000), involves adult breeding fish (P) that are monitored for a minimum of six weeks to determine effects on reproduction. The test requires a clean water phase for the first three weeks, to establish baseline data on fecundity, followed by a further three weeks of exposure. Observations of, and changes in, spawning behaviour and secondary sexual characteristics (where appropriate) are recorded during the six weeks. Histological and biochemical endpoints if needed can be determined following the exposure period. If required, progress of the second generation (F_1) can also be assessed.

3.2.2.3.3 Fish sexual developmental test (or extended early life stage test)

This protocol is, in principle, an enhancement of OECD 210, (OECD, 1992b) where the exposure is continued until the fish reach sexual maturity (60 d post-hatch for the zebrafish and medaka and 90 d post-hatch for the fathead minnow). The test is initiated with 60 eggs per replicate and a minimum of two replicates per concentration are used. A total of five concentrations plus the controls are recommended. The endocrine endpoints to be measured are: secondary sexual characteristics, vitellogenin induction, gonadal histology and sex ratio (OECD, 2004a).

3.2.2.4 Fish full life-cycle test (FFLC)

The FFLC test is based on the US EPA guideline for fish full life-cycle toxicity testing (US EPA, 1986). In summary, the test begins with embryos (P) less than 24 h old, which are continuously exposed throughout the development of the fish until the fish are sexually mature. Once the fish achieve maturity, they are assessed for reproductive behaviour and fecundity. During the reproductive phase, embryos (F_1) obtained from the P fish are developed for a minimum of 28 d (post-hatch) to determine in-life biological effects. If required, the development of the F_1 fish can also be progressed to determine histological and biochemical endpoints. Typically, the minimum duration of a FFLC test using fathead minnow is 250 d. Japan has recently proposed to enhance

the fish full life-cycle test with 'secondary mechanistic biomarkers responses' and gonadal histology (OECD, 2003a).

3.2.2.5 Fish two-generation test (proposed guideline)

The fish two-generation test guideline is a proposed redesign of the FFLC. It is intended to establish the effects on reproduction of parent and offspring exposed to a toxicant and to capture any transgenerational effects. The test also enables histological and biochemical endpoints to be determined. The duration of the test is a minimum of 180 d, although it may be considerably longer depending upon the species used, for example, with fathead minnow the test duration would be at least three weeks longer than the traditional FFLC. The principle of the test is that adult spawning fish (P) are exposed for a minimum of 21 d and embryos collected from these fish are used for the development of the next generation (F_1). The F_1 fish are then progressed to maturity and assessed for reproductive behaviour and fecundity. During the reproductive phase, embryos (F_2) obtained from the F_1 fish are developed for a minimum of 28 d to determine in-life biological effects.

3.2.2.6 Amphibians

Currently, fish are the principal vertebrate animals used for assessing hazards to aquatic organisms. However, in the future it is probable that amphibians will also be used, especially for the detection and characterisation of endocrine disrupting chemicals.

OECD member countries are currently developing methods that could cover effects of chemicals on the reproductive system (estrogen agonists/antagonists and androgen agonists/antagonists) and on the development system (thyroid).

The three classes of amphibians (anurans, urodeles and caecilians) undergo metamorphosis, although in reality, not all species within each class of amphibians metamorphose. Obligatory neotenic urodeles do not metamorphose since they have the unique ability to reproduce as an aquatic adult larva. This phenomenon is called neoteny.

Metamorphosis in amphibians is a period of considerable physiological and morphological changes, mostly marked in anuran species, which develop through the larval stages of development. Metamorphosis has been reasonably well studied in three anuran species: *Xenopus laevis* (South African clawed frog), *Rana catesbeiana* (bull frog), *and R. pipiens* (northern leopard frog).

Most of the metamorphic events are triggered by the thyroid hormone (TH) and tissue responsiveness is based on selective TH interaction with thyroid receptors (TR).

Several test protocols have been proposed at the OECD level to evaluate the potential impact of xenobiotics on the amphibians' metamorphosis and to provide indications of whether a chemical substance acts as a thyroid disruptor in amphibians. The genetic similarities within the vertebrate thyroid system may make it possible to use amphibians for evaluating thyroid disruption in other vertebrate species, including humans. This rationale has been included in a detailed review paper (OECD, 2004b) that provides the current state-of-the-knowledge in the area of amphibian metamorphosis. Amphibian metamorphosis assays require the exposure of larvae (tadpoles). As larvae comply with the regulatory definitions of 'protected animals', the evaluation of alternative methods such as the 'Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX)' to the amphibian metamorphosis assay should be considered.

Other tests using *Xenopus* are still being evaluated for their ability to identify substances that may disrupt endocrine function or affect reproduction and development (e.g. tail resorption assay, vitellogenin assay, limb bud assay) (Opitz *et al*, 2005). For the purpose of this report, the following discussions will address the amphibian metamorphosis assay and the FETAX.

3.2.2.6.1 The Xenopus metamorphosis assay (XEMA)

The test is a 28 d morphological assay, with exposure of *Xenopus* sp. larvae in a static renewal system and assessment of length (total and tail) and developmental stages at days 7, 14, 21 and 28.

Principal candidate species for the amphibian metamorphosis assay are *X. laevi*s and *X. tropicalis*. While these native African species may have limited ecological relevance to temperate northern hemisphere environments, they are highly suited to laboratory testing. Higher tier hazard characterisation of chemicals may however require the use of other, more ecologically relevant species.

The exposure period starts with larvae at the developmental stage 51-52, with control larvae reaching approximately stage 58-59. Developmental stages are determined according to Nieuwkoop and Faber (1994) by using a dissection microscope. As currently designed, the assay may have sensitivity to weak agonists as it incorporates a significant window in which endogenous TH levels are low, but larvae may respond to exogenous thyroid active chemicals.

Developmental stage, whole body length and tail length are determined for each tadpole at the beginning of the exposure (day 0) and again at day 7, 14, 21 and 28, during the changeover of test

solution. Test design typically incorporates 5 test chemical concentrations, a negative control (75 mg/l 6 n-propyl-2-thiouracil), and a positive control (1 µg/l thyroxin) (OECD, 2004b).

Water exposure appears to be the most common route to expose larval and metamorphic amphibians to chemicals suspected of disrupting endocrine systems.

3.2.2.6.2 Frog Embryo Teratogenesis Assay Xenopus (FETAX)

FETAX, according to the standard described by the American Society for Testing and Materials (ASTM, 1998b), is designed to use *X. laevis* embryos. Song *et al* (2003) proposed *X. tropicalis* as an alternative species. FETAX is a 96 h whole-embryo test developed to determine the teratogenic and developmental toxicity potential of chemicals and complex mixtures. The FETAX is relatively easy, rapid, and uses early developmental stages of a vertebrate species before the regulations apply to them.

FETAX does not specifically address endpoints indicative of endocrine disruption activity. Nonetheless, FETAX might be useful for the work on endocrine disruption testing in amphibians, as it provides useful technical information for the development of a frog metamorphosis assay:

- For the ecotoxicological evaluation of chemicals, significant embryo toxicity in a FETAX test negates the need for further testing of larvae;
- using FETAX as a screening test may be helpful to refine the concentration range that will be applied in a subsequent 3 week metamorphosis assay.

The primary endpoints include mortality, malformations, and growth inhibition. Three criteria have been developed to identify teratogens using FETAX:

- The teratogenic index (TI) - based on mortality and malformation data obtained over a range of dose levels, the 50% lethal concentration (LC_{50}) (i.e. the concentration estimated to induce lethality in 50% of exposed embryos) and the 50% effective concentration for malformations (EC_{50}) (i.e. the concentration estimated to induce malformations in 50% of exposed embryos) are calculated. The TI is equal to the LC_{50} divided by the EC_{50}. TI values > 1.5 signify a greater potential for embryos to be malformed in the absence of significant embryo mortality;
- Growth inhibition - is ascertained by measuring the length of the embryos. The minimum concentration to inhibit growth (MCIG) (i.e. the lowest effective concentration for growth inhibition) is determined by statistically comparing the mean 96-h head to tail length of the treated embryos at each treatment concentration to that of the control embryos. Teratogenic

hazard is considered to be present when growth is significantly inhibited at concentrations below 30% of the 96-h LC_{50} (i.e. when the MCIG:LC_{50} ratio is less than 0.30);
- Severity of malformations - teratogens generally cause moderate to severe malformations at the 96-h LC_{50}, whereas non-teratogens only cause slight malformations (as defined by the guideline).

Any one of these three criteria (TI, growth inhibition, or severity of induced malformations) is used to identify a teratogen. Due to the nature of the endpoints assessed, FETAX does not provide information on substances that may induce functional developmental defects in mammals. As *Xenopus* embryos are deficient in mixed function oxidase-dependent metabolic activation processes, the addition of an exogenous metabolic activation system (MAS) to the assay has been proposed to assess the developmental toxicity of metabolites in addition to the parent substance.

As a screening test, a chemical substance giving a positive FETAX response would indicate a potential human hazard and, in the absence of other data, would be considered a presumptive teratogen/developmental toxicant. Such information could be used to prioritise chemicals for more definitive testing.

A major peer review of the FETAX protocol was published by the National Toxicology Program (NTP, 2000) and National Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). This review recommended improvements to the methodology.

3.3 Reduction

The concept of reducing the number of fish used per experiment has been discussed for many years. Douglas *et al* (1986) first suggested that with only a minimal loss of precision, the number of test concentrations could be reduced from five to four, which would equate to a reduction of 35 fish per substance tested. This proposal was, however, based on limited non-validated data and has therefore not been adopted by regulatory authorities. At one time, 10 fish were used per concentration, but in 1992, OECD test guideline 203 was adopted, which requires the use of only 7 fish per concentration. This has effected a reduction of 18 fish per test substance.

In contrast to the method modifications outlined above, the US EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS) most recent guideline regarding acute toxicity to fish (OPPTS, 1996) requires that two replicates per concentration are tested with 10 fish per replicate. Consequently, nearly three times as many fish would be used for studies following this US guideline compared with OECD test guideline 203 (OECD, 1992a).

While it is evident that at present there is no clear consensus on reducing the number of fish used in a conventional test, there are a number of alternative approaches which are either already accepted by regulators or there is sound scientific evidence to support their use and thus could be applied to a much wider range of studies than at present.

3.3.1 Limit test

The concept of a limit test for testing poorly water soluble substances is outlined in Annexe VI of Directive 67/548/EEC (EEC, 1967) for acute studies. The test is conducted at a single concentration of 1 mg/l and if no toxicity is observed, testing of additional concentrations is not required. A similar limit test approach may be taken for the testing of highly soluble substances. In this case, the concentration selected is the upper threshold for EU classification and labelling purposes, namely 100 mg/l. If no toxicity is observed, no further testing is required.

Limit tests, therefore, may significantly reduce the number of fish used in ecotoxicity tests, and as discussed in OECD 210 are also extended to chronic test procedures where an upper limit of 10 mg/l is recommended.

3.3.2 Acute threshold (step-down) approach

The acute threshold (step-down) approach was proposed by Hutchinson *et al* (2003) as a strategy to reduce the number of fish used in acute ecotoxicity testing of pharmaceuticals. The objective is to reduce the number of fish used (from ≥42 to ≥10 fish per substance) and to determine an LC_{50} by applying comparative threshold data obtained from the most conservative data from algae and/or daphnid acute tests (see Section 3.5 for further details regarding the use of invertebrates in ecotoxicity testing). To date, this principle has been partially substantiated for 91 active pharmaceutical ingredients (API). Approximately 80% of the APIs had an LC_{50} value equal to or higher than the most sensitive algae or daphnid test. Hutchinson *et al* (2003) proposed that for the further 20% of the APIs, it would be possible to extrapolate a comparable LC_{50} value for the fish by employing a step-down factor of 3.2 to the EC_{50} value derived from the most sensitive species.

The acute threshold approach illustrates the importance of taking a tiered approach to testing, i.e. algae and daphnids should be tested first. This information could then be used to either remove the need for fish testing, for example if there was clear evidence of high toxicity or to reduce the number of fish used, for example by subsequently performing either a limit test or using fewer test concentrations to determine the LC_{50} value.

As part of ongoing work, the ECVAM Task Force on Ecotoxicology and the European Chemicals Bureau (ECB) identified full data sets, containing specific EC_{50} and LC_{50} for algae, daphnids and fish, for over 1,400 chemicals from the New Chemicals Database. Jeram *et al* (2005) demonstrated that a reduction between 53 and 71% of the fish used to establish the LC_{50} value would have been achieved by using the step-down approach. As part of a joint exercise in 2005 between ECVAM and ECETOC, company data for existing substances were assessed. Data for nearly 600 chemicals gave very similar results to those obtained by Jeram *et al* (2005).

3.3.3 (Quantitative) Structure Activity Relationships

The use of (Q)SARS to evaluate toxicity is another way in which non-animal range finding could be performed. For example, predictions obtained from (Q)SARs can be used in place of range-finding studies or to enable studies to be redesigned to reduce the number of animals used. The use of (Q)SARs will be discussed more thoroughly in the Replacement section of this chapter (Section 3.5.4). For a comprehensive review of the (Q)SARs that are currently available, see ECETOC (2003a).

3.3.4 Read-across

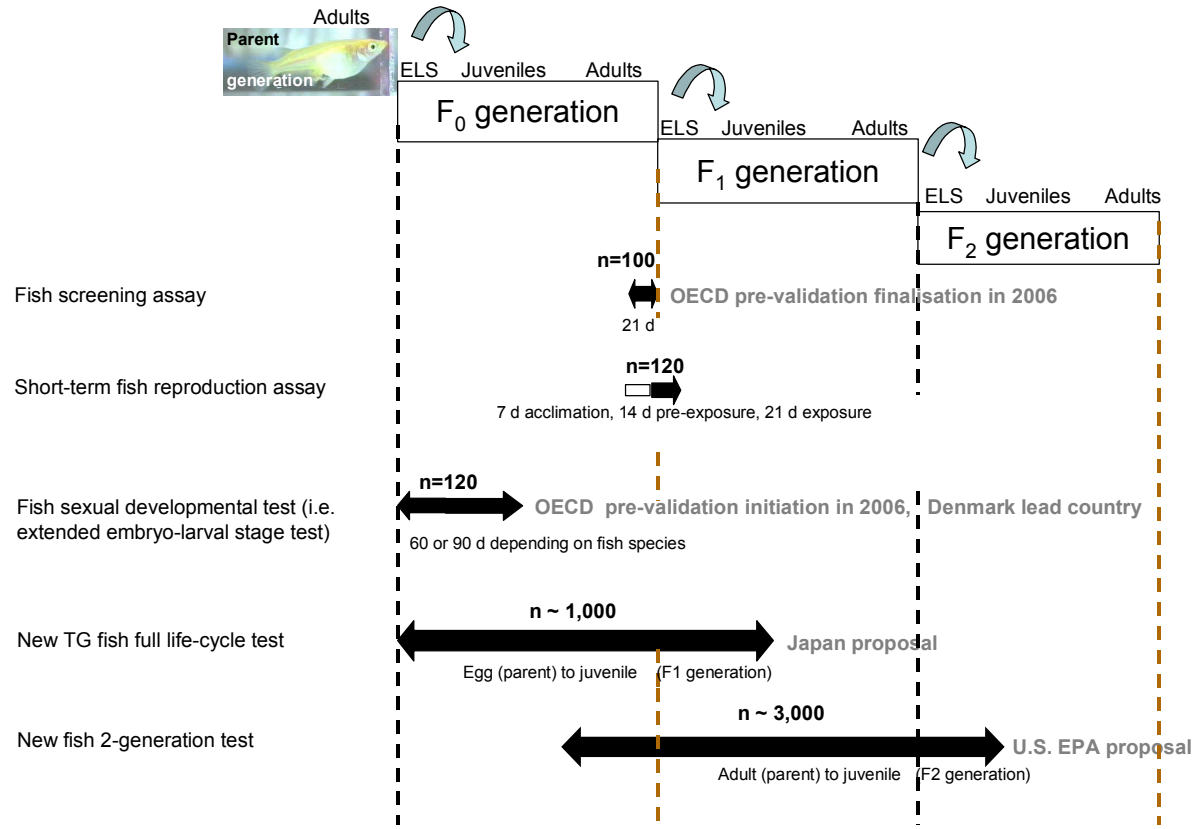
Where chemicals display similar physico-chemical properties in terms of log K_{ow} and/or partition coefficients, and ecotoxicity data already exists for example, for some members of a homologous series, but not others, the possibility of 'read-across' of ecotoxicity data may be considered. Information on structurally analogous chemicals may also be used. The read-across approach is generally accepted by regulatory authorities on a case-by-case basis (Barratt, 2003). As part of the High Production Volume Challenge programme, the US EPA has identified a number of structurally similar chemicals as a group or category. Testing is then conducted on representative members of a specific category, with the objective of read-across to other members. Further information can be found on the EPA web site (www.epa.gov/chemrtk/sarfinl1.pdf). In addition a recently published draft report (OECD, 2004c) gives guidance on the information and use of chemical categories, and outlines the difference between read-across, interpolation and extrapolation.

3.3.5 Use of the most sensitive life stages for testing endocrine active chemicals

The enhancement of existing OECD guidelines with specific endocrine endpoints would allow the development of test methodologies for endocrine active chemicals that are resource-effective, minimise animal testing and deliver timely results (Huet, 2000). The OECD has developed an 'enhanced TG 407' protocol for detecting endocrine active chemicals by adding specific

endpoints (sperm evaluation, female cycle), which has gone through a validation process (Cho *et al*, 2003). The enhancement of existing OECD fish guidelines has been discussed (OECD, 1998) based on research from Gimeno (1997) and Panter *et al* (1998). Alternatives to the fish full life-cycle test are now being considered by addressing sensitive windows of exposure, e.g. the period of sexual maturation (an annual or continuous process in adults), and the period of sexual differentiation (first development of primary and/or secondary sexual characteristics in embryos or young individuals). Yamamoto (1969) showed that sexual differentiation in fish is dependent on sexual steroids, and as such it is a very sensitive life stage to endocrine active chemicals. A comparative study with male carp exposed either to a weak or potent oestrogenic chemical showed that the period of sexual differentiation is more sensitive than the maturation stage (Gimeno *et al,* 1998a,b). Similar comparative studies being conducted with fathead minnows exposed to the same weak oestrogenic chemical and an androgenic active chemical support this finding (Gimeno *et al*, 2004; Panter *et al*, 2005; Bogers *et al*, 2005). Results from these studies may indicate whether or not short-term reproductive assays can be used as an alternative to the fish full life-cycle test (see Figure 2).

Figure 2: Comparison of exposure durations and approximate number of animals used for assessing endocrine activity



3.4 Refinement

3.4.1 Use of non-lethal endpoints

3.4.1.1 Animal welfare considerations within a test

As part of the existing methods addressing the effects of chemicals, all tests should be conducted within the terms of a licence (for example see UK, 1986). Within these licences, it is noted that there are several approaches that may be used to reduce animal suffering, while still obtaining the necessary information. This potential to refine tests to reduce suffering, without affecting the acceptability of the method to regulators, is part of the way that all responsible testing laboratories operate. Some of these are shown in Table 4.

Table 4: Animal welfare considerations in toxicity tests

Test	Refinement	Comments
Acute toxicity test (including range finders)	Fish exhibiting toxic symptoms and considered unlikely to survive to the next observation period will be humanely killed.	This may be difficult, since it is not always possible to determine if an organism is going to die by the next observation period.
Fish growth tests	Anaesthesia is used during the branding of the fish to minimise the stress of this procedure.	This is good standard practice.
	Exposure concentrations that cause marked signs of toxicity are not expected in this test. If such effects are observed the fish in that exposure concentration will be humanely killed.	While unexpected mortality can occur in any test, fish that exhibit major symptoms of toxicity could suffer needlessly. Hence the purpose of this step.
Fish early life stage test	Daily observations of the eggs and larvae made and any damage or deformed larvae recovered and killed humanely.	There are instances of damaged or deformed organisms surviving in the wild and it cannot automatically be assumed that the deformities are responsible for pain/suffering.
Fish bioaccumulation/metabolism tests	Choice of exposure concentrations. If effects are seen, then the group should be killed humanely without delay.	The purpose of the test is not to address effects, thus if any are observed, this would invalidate the study.

3.4.1.2 Refining lethality tests

A clear refinement in acute toxicity testing would be to avoid using lethality as an endpoint (Russell and Burch, 1959). This could involve the determination of a NOEC and a LOEC or an

EC_5 and an EC_{10} instead of an LC_{50} value. It should be noted, however, that Weyers *et al* (2002) suggest that 'NOECs, due to their dependence on test design and variability, are an unsatisfactory measure of 'no-toxicity' for regulatory purposes'. In addition, it is also regarded that NOEC and LOEC values are not as statistically robust as EC_x values since they do not have any measure of precision (e.g. 95% confidence intervals).

However, in the context of effects in the environment, non-lethal endpoints would be more representative and more relevant for hazard evaluation (Fentem and Balls, 1993). Furthermore, an LC_{50} result only provides information on the percentage of a population that will be dead at a certain concentration and after a certain period of time. It does not distinguish, or differentiate, the percentage of the population, which is technically alive but 'ecologically dead' (e.g. fish that are alive lying still on the bottom of the test vessel, or those that are functionally impaired).

No ring test using fish has been conducted by OECD to compare the statistical robustness of non-lethal endpoints (e.g. NOEC, LOEC or EC_{10}) with that of lethal endpoints (LC_{50}).

It can also be difficult to analyse sublethal and behavioural effects in fish. To overcome this, Kane *et al* (2004) suggested a video analysis system to investigate stress and contaminant induced behavioural alterations in mummichog (*Fundulus heteroclitus*). The behaviour of the fish, exposed to sublethal concentrations of a chemical, was monitored at multiple and discrete intervals. The data was then digitised and transformed into relevant behavioural endpoints using software designed for tracking fish movement combined with specific algorithms. The endpoints included velocity, total distance travelled, angular change, percent movement, space utilisation, and fractal dimension (path complexity). According to the authors, the data can be used to calculate a more sensitive LOEC or NOEC for sentinel fish species.

3.4.1.3 Reducing behavioural stress

Hoffmann *et al* (2004) have suggested the use of optical barriers to further refine and minimise stress, induced by the dominant behaviour of certain fish, in aquatic toxicity testing schemes. The authors suggested that the aggressive behaviour of rainbow trout (*Oncorhynchus mykiss*) could bias or even invalidate test results through test substance independent mortalities. With the addition of optical barriers, the number of aggressive attacks by the dominant fish was significantly reduced without affecting the growth of juvenile fish. The authors also proposed that optical barriers could reduce control mortalities and make acute and chronic tests, using rainbow trout, more reliable and reproducible.

3.4.2 Refinement of the fish, short-term toxicity test on embryo and sac-fry stages

The short-term toxicity test (OECD, 1998) is part of a tiered process and is used as a screening test to form a bridge between lethal and sublethal tests and not as a replacement for the early life stage test (OECD, 1992b). The test involves newly fertilised embryos that are exposed following hatching for a period of time depending on species. During the test, the hatched fry do not receive food and rely purely on endogenous food reserves. For some species this is not a problem, for example, rainbow trout are able to feed off their yolk sac for up to 30 days and are not actually capable of exogenous feeding until approximately 15 days post-hatch (Love and Williams, 2000). Conversely, most warm water species have a reduced yolk sac period and are generally capable of exogenous feeding soon after hatching (see Table 5). This transition from endogenous to exogenous feeding can be affected by abiotic (e.g. temperature) and biotic (e.g. disease) factors (Kamler, 1992). Furthermore, the exact determination of exogenous feeding can be relatively subjective and relies on observable, behavioural cues such as swim-up. In general, warm water species of fish (e.g. fathead minnow or zebrafish) are capable of independent feeding soon after hatching occurs and are classed as protected from this point according to the UK Animals, Scientific Procedures, Act (UK, 1986).

Table 5: Fish embryonic durations and recommended feeding time for survival, according to OECD 212 (1998)

Fish species	**Temperature**	**Durations of embryonic stages**		**OECD recommended period of time to feeding[a]**
		Embryo	**Eleutheroembryo (sac fry)**	
		***(Days from fertilisation until hatch*)**	***(Days from hatch until resorption of yolk sac)***	**(*Days post-hatch*)**
Rainbow trout *Oncorhynchus mykiss*	11±1 °C	30-35	25-30	20
Common carp *Cyprinus carpio*	21-25 °C	5	> 4	4
Zebrafish *Danio rerio*	25±1 °C	3-5	8-10	5
Medaka *Oryzias latipes*	23±2 °C	8-11	4-8	5
Fathead minnow *Pimephales promelas*	25±2 °C	4-5	5	4
Bluegill sunfish *Lepomis macrochirus*	21±1 °C	3	> 4	4

[a] The time when fish become capable of feeding might occur much sooner, depending upon several parameters specific to the experimental conditions, such as temperature, metabolic rate, parents' reproduction frequency, parents' health and feeding status, and hatching rate. For example, in zebrafish several authors have reported different durations for the eleutheroembryo stage, varying from half a day up to 6 days (Piron, 1978; Axelrod, 1987; Andrews, 1986; Rundle, 1996) and fathead minnows may move to independent feeding earlier than 4 days post-hatch (Hutchinson and Williams, 1994).

It has been recommended that salmonid fish should be fed from the point when they are able to consume food to avoid any possibility of stress from starvation (Environment Canada, 1998). Furthermore, it is well documented that underfed fish are more susceptible to environmental pollutants due to reduced immunological functions (Blaxter, 1969; Dave, 1981). In addition, the biochemical components (e.g. DNA:RNA ratio, water, triglyceride, carbohydrate, carbon and nitrogen content) of the larvae of several species have been shown to be affected by starvation (Blaxter, 1988). These considerations should be taken into account especially when testing warm water species of fish. Based upon optimum laboratory conditions (e.g. high temperature), yolk resorption might occur sooner than indicated in the guideline, which might justify a need to feed the fish. However, refinement encompasses the decrease in the incidence or severity of procedures.

In conclusion, if during the test no feeding occurs, the test can be considered as a replacement. If, however, feeding occurs due to species needs and/or speed of development due to water temperature then the use of the early life stages of fish would be considered as a refinement.

3.5 Replacement

Four strategies are identified in terms of replacement:

- Use of non protected organisms e.g. embryos, invertebrates, algae or bacteria, as an alternative to fish or amphibians;
- use of fish cell lines to replace whole fish;
- toxicogenomics;
- *in silico* techniques.

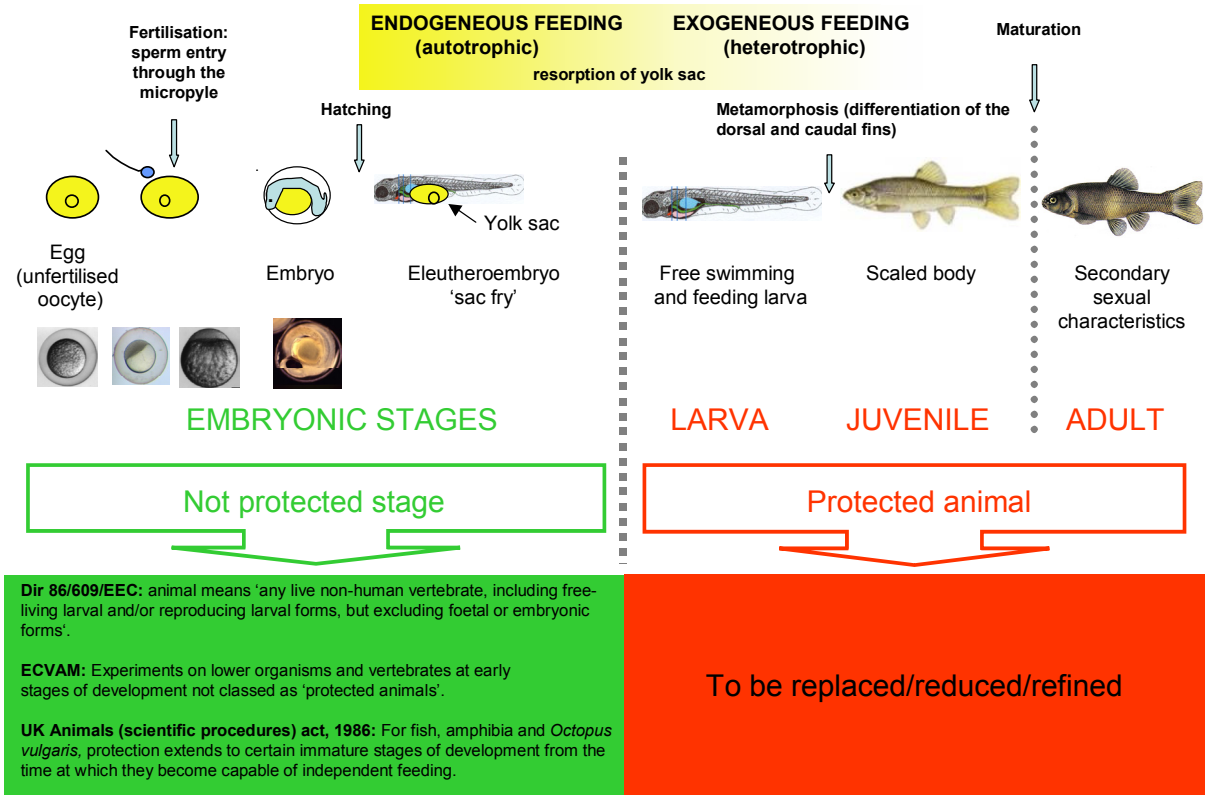
3.5.1 Replacement by non-protected organisms

3.5.1.1 Use of embryos (fish and amphibians)

For the purpose of this report, the term fish embryo refers to the fertilised egg before hatching. However, it is important to note that the embryonic period also extends past the point of hatch (see Figure 3). Fish at this early-hatched stage of development are referred to as eleutheroembryos and still rely on endogenous yolk reserves for survival. According to Blaxter (1988), many fish species hatch with under developed mouthparts, and the development of the mouth occurs when fish are able to feed exogenously. The transition from endogenous to exogenous feeding varies depending on the different species of fish (see Table 5). Fish that are capable of feeding exogenously are regarded to be feeding independently from their yolk reserve irrespective of how much yolk is remaining. According to the UK Animals (Scientific

Procedures) Act (UK, 1986), fish that are capable of independent feeding are classed as protected organisms. Fish embryos are not capable of independent feeding; hence, they are not classed as protected.

Figure 3: Fish life stages



Fish embryos are less sentient as they have less developed neuroendocrine and olfactory systems than juvenile and adult fish. Therefore the use of embryos could be regarded as an ethical alternative to the use of whole fish. Additional benefits of using fish embryos include:

- Single fish embryos can be maintained in small volumes of test solution;
- fish embryos can be cultured in microtitre wells and processed automatically on a standard microtitre plate reader. Hence, they can be used as a high throughput screening tool;
- most fish embryos are inexpensive to maintain and easily bred in large numbers (e.g. single mating pairs of zebrafish produce between 100 and 200 eggs in one spawning);
- the majority of fish embryos are completely transparent and therefore development can be easily observed, enabling the possibility of determining specific organ toxicity, such as to

the liver and the kidneys, as well as developmental teratogenicity through immunochemical techniques;
- fish embryos can be used as a promising tool for determining the genotoxicity of a substance since approximately 90% of the genome is active during embryogenesis whilst only approximately 10% is functional during adult life.

A large amount of work has been undertaken in the field of fish embryo ecotoxicology predominantly using zebrafish (*Danio rerio*), although other OECD species have also been investigated (Cairns *et al*, 1965; Laale, 1977; Birge *et al*, 1979; Ensenbach *et al*, 1989; Schulte and Nagel, 1994; Lange *et al*, 1995; Canaria *et al*, 1999).

The effect of chemicals on fish embryos can be evaluated, not only in terms of overall lethality but also other endpoints such as coagulation of the egg, gastrulation, number of somites, movement, development of organs, pigmentation, heartbeat and circulation (Schulte and Nagel, 1994) which provide guidance in terms of the potential mechanisms and biological significance of the effects. These parameters have been further characterised into two groups of EC_{50} classifications, EC_{50} I and EC_{50} II:

EC_{50} I endpoints (time taken for observation of each parameter shown in parentheses):

- No completion of gastrulation (12 h);
- no somites (16 h);
- no movement (48 h) and no heartbeat (48 h);
- coagulated egg.

Evidence of deficiencies in any of these parameters will result in a negative hatch or survival problems.

EC_{50} II endpoints are summarised as arrested development, developmental deficiencies, reduced motility, growth and fitness and can be regarded as indicators of toxicity. Schulte and Nagel (1994) used this approach to evaluate six chemicals with different fish acute toxic syndromes (FATS) including organic herbicides, pesticides and phenolic-based compounds. Using zebrafish, the EC_{50}s and the LOECs were found to be comparable to acute LC_{50} data with the same chemicals (i.e. the results fitted into the same EU classification tier). However, in some instances, the EC_{50}s differed by two to three orders of magnitude. Earlier work (Ensenbach *et al,* 1989), based only on mortality data, did not find fish embryos as sensitive as juvenile/adult fish. Metabolic capability may not be well developed in embryos (Lange *et al,* 1995) and as a result they are not as sensitive to toxins requiring initial metabolic activation. This may have implications if toxic metabolites are not synthesised within the 48-h exposure period of the embryo assay. Another reason for reduced sensitivity is that once the micropyle closes, following

fertilisation, the embryo is less sensitive as the embryonic chorion acts as a protective barrier to certain toxicants. Therefore embryos used for acute toxicity tests will tend to be less sensitive than embryos used in long-term chronic toxicity tests, for example in life-cycle studies where eggs and embryos are exposed continuously. This may be overcome by a pre-treatment with a dilute solution of pronase to remove the chorion (Westerfield, 1995).

Canaria *et al* (1999) have proposed a method for a seven-day embryo toxicity test using rainbow trout (*Oncorhynchus mykiss*) where the fertilisation success, embryo mortality and morphogenesis of the embryos are assessed. Morphological development can be observed on the developing embryos after 7 days, when they have reached stage 11 of development, as described by Ballard (1973). At this stage of development the embryos are clearly visible once they have been cleared with a clearing solution (e.g. acetic acid). Any embryos not at this stage (stages 1 to 10 as described by Ballard (1973)) are recorded as non-viable. The 7-d embryo test design using rainbow trout offers advantages over some traditional fish early life stage (ELS) protocols. Fish ELS studies are relatively long in duration (30-d embryo phase followed by a 90-d grow-out phase) and consequently are expensive. ELS studies are also carried out under flow-through conditions whilst the 7-d embryo test is carried out under semi-static conditions. Water hardening[a] can take place in rainbow trout embryos whilst being exposed to the test chemical for up to 30 minutes post-fertilisation. During this 30-minute period the micropyle will still be open and the embryonic chorion will still be relatively permeable. An ethical issue involved with such a study is the collection of milt from the gonads (which is usually obtained from altered sex females that do not have sperm ducts). This is a terminal but not regulated procedure. Therefore provided that the milt is obtained from a fish farm, it is not covered by the UK Home Office license. The milt can also be cryopreserved and stored for use in future studies (Billard *et al*, 2004).

A short review of the use of fish embryos in acute aquatic toxicity assessment is given in Table 6 and a summary of the results shown in Appendix A.

A review of the ECETOC Aquatic Toxicity (EAT III) database (ECETOC, 2003b) highlighted the importance of the developmental stages of fish in determining their sensitivity to different chemicals. From the data analysed, it was concluded that there were no apparent differences between the sensitivity of embryos and larvae for the chemicals that were tested. Furthermore, larvae were not more or less sensitive than juvenile fish. However, juvenile fish are reported to be more sensitive than adult fish in acute toxicity tests. This is in contrast to previous findings by Hutchinson *et al* (1998), who compared results obtained from the original EAT database (ECETOC, 1993) for fish at different stages of development. They concluded that larvae are more sensitive than embryos for 68% of substances and, compared to juvenile fish, were more sensitive for 83% of the substances tested. Consistent with the current EAT III report, Hutchinson *et al*

[a] The process of water hardening is the closure of the micropyle following fertilisation. The micropyle is the opening in the protective chorion created by the penetrating sperm.

(1998) found that juvenile fish are more sensitive than adult fish for 92% of the substances examined. The increased sensitivity of juvenile and larval fish has been attributed to the surface area:volume ratio (Hutchinson *et al*, 1998). In addition, young fish have also accumulated less fat than adult fish and, therefore, have less capacity to store lipophilic substances.

Table 6: Examples of the use of fish embryos in acute aquatic toxicity assessment

Test substances	Comments	Reference
Zinc chloride, potassium cyanide, potassium dichromate and naphthenic acid	Adult bluegill sunfish and zebrafish embryos more sensitive to all chemicals tested except naphthenic acid.	Cairns *et al* (1965)
Phenol, aniline and chlorobenzene	Rainbow trout embryos and larvae more sensitive to the chemicals tested and suggest that embryos are acceptable as a replacement to acute toxicity tests with adult fish when toxicity data is required on organic chemicals.	Birge *et al* (1979)
Methanol, acetochlor, acrylamide, benzene, colchine, diethylene glycol, diethyl-nitrosamine, Triton X-100	Embryos exposed to methanol proved to be seventy seven times more sensitive than adult fish. LC_{50} values for zebrafish embryos and adult fish, for the other seven chemicals tested were comparable even though sensitivity varied with each substance.	Kovrižnych and Urbančíková (2001)
Sodium dodecyl sulphate	The 7-day old rainbow trout embryo toxicity test was more sensitive than the 7-day old fathead minnow survival and growth test. Furthermore, the 7-day embryo toxicity test was more sensitive by almost an order of magnitude than the 96-h acute toxicity test with juvenile rainbow trout.	Canaria *et al* (1999)
Cadmium and copper, sodium pentachlorophenol and malathion, chromium and malathion	The chemicals tested effected morphological differences in embryos such as reduction in pigmentation, yolk sac oedema and deformation of the notochord. Following quantification, sensitivity was summarised as growth>abnormality>larval survival>embryo survival>hatchability. Results from the 5-d embryo larval studies with African catfish were similar to the sub-chronic results obtained from a 12-d ELS using zebrafish. They were also comparable with data from ELS studies conducted on other OECD species.	Nguyen and Janssen (2002)

In conclusion, fish embryos offer many benefits as an alternative approach including costs, resource and various endpoints independent of lethality (e.g. genotoxicity or teratogenicity). They may also provide a longer-term alternative to the use of juvenile or adult fish in acute LC_{50} testing. However, this very much depends on the sensitivity of fish embryos to different classes of chemicals (i.e. domain of applicability). One approach that warrants further research is to evaluate the removal of the protective chorion that acts as an effective barrier to many

hydrophilic and ionisable chemicals. An area of concern with the use of fish embryos is the fact that some toxic metabolites may not be synthesised during early stages of development. A further consideration in the use of fish embryos is that it may not be possible to maintain the concentration of certain test compounds in small volumes of test solution. The small volumes may also be problematic for the purpose of chemical analysis.

3.5.1.2 Replacement of vertebrates by invertebrates

The use of invertebrates is not included in animal welfare laws and thus raises considerably less concern among the public than the use of vertebrates. Aquatic invertebrates have been used for some time in acute and chronic ecotoxicity testing for hazard identification. Procedures using cladocerans (i.e. daphnids), chironomids and *Hyalella* sp. are currently the most frequently used freshwater invertebrate tests to be endorsed by international organisations. Other organisms such as *Hydra* sp. and protozoa are also being evaluated. However their applicability as possible alternatives in aquatic toxicity testing is hampered by limitations arising from species specificity of individual responses due mainly to differences in metabolism, physiology and anatomy (Lagadic and Caquet, 1998). With this caveat, these non-protected organisms offer potential benefits to the use of fish including:

- Maintenance and handling: many invertebrate species are receptive to easy culturing and maintenance in the laboratory due to their small size and ease of handling. High reproduction rates and short life-cycles also facilitate the performance of chronic as well as acute toxicity tests;
- genetics: some invertebrate species are parthenogenetic, which reduces genetic variability in test organisms. However, this is not the case for all invertebrates;
- ecological relevance: invertebrates occupy key positions in the aquatic food web;
- species richness: the diversity of invertebrate communities coupled with their widespread distribution also complements their use as potential bioassay organisms.

A recent development to the use of invertebrates in ecotoxicity testing has been the development of small-scale microbiotests. These involve dormant stages of invertebrate organisms, which can be hatched prior to use. Microbiotests do not require specialised culture conditions and can, as a result, be used for environmental monitoring in situations where other types of testing may not be feasible. An inter-laboratory comparison with other standard invertebrate acute toxicity methods showed that each assay had advantages and disadvantages (Daniel *et al*, *in press*). The respective microbiotests for *Daphnia magna*, algae, etc. will be discussed in the relevant sections below.

3.5.1.3 Daphnids

Acute toxicity testing of daphnids is a standard requirement for testing of new chemicals under Directive 79/831/EEC. In the standard test method, the EC_{50} value, which is based on immobilisation of the test organism, is calculated after exposure to treated water, under laboratory conditions, for a preferred maximum period of 48 h. A large database now exists for *Daphnia* sp. acute toxicity, and it has been shown to be sensitive to many different chemicals. Chronic testing on *Daphnia* sp. may also be required, to evaluate the potential effects of a chemical in the longer term (Fentem and Balls, 1993).

Several species of daphnid have been used extensively over the years in aquatic toxicity testing. *D. magna* is used in the majority of the standardised tests but is restricted to hard water habitats, whereas *D. pulex* has a widespread distribution and is also often used. An overview of the acute and chronic standard test procedures with daphnids is given by Persoone and Janssen (1994).

Walker *et al* (1991) have suggested, based on evidence from a large number of industrial chemicals, that where toxicity is observed both fish and daphnids show a similar order of toxicity. A good correlation between the sensitivity of *Daphnia* sp. and guppies to a range of organic chemicals has been reported and the experimental data also agrees quite closely with predicted toxicities based on QSAR studies (Fentem and Balls, 1993). A similar study has been conducted by ECETOC (2003b). Their EAT III database was prepared based on peer reviewed published data on fish, daphnids and algae. Interspecies comparisons of these data suggest that freshwater daphnids are more sensitive than combined fish species for 37% and equally sensitive for 44% of the chemicals listed (n=84). In addition, daphnids were more sensitive than algae for 41% of the chemicals tested and equally sensitive for 18% of the chemicals (n=22).

In a study of acute data from the New Chemical database, Weyers *et al* (2000) examined the relative sensitivities and relationships of algae, *Daphnia* sp. and fish. Their results showed a good correlation between the EC_{50} for daphnids and LC_{50} for fish. Algal growth inhibition was the most sensitive endpoint in the database. This supports the step-down approach discussed in Section 3.3.2.

It also suggests that, if a substance has physico-chemical properties in common with others in a homologous group, and if the data for daphnid toxicity agree with predictions based on QSAR studies, then the fish-daphnid sensitivity correlation established for one chemical in the group will apply to all other members of the group (Fentem and Balls, 1993). Such a proposal would result in the replacement of a significant number of fish acute lethality tests with a testing strategy involving physico-chemical data, (Q)SAR and daphnid acute toxicity studies (see Section 3.7).

3.5.1.4 Algae

Many studies have been concerned with the effects of pollutants on phytoplankton growth because of their ecological importance in the aquatic environment. Algal toxicity tests on chemicals are also mandatory as part of the base set tests for classification of chemicals in the EU. They also offer the advantage that they represent both an acute and a chronic test at the same time.

In standard algal toxicity test methods, a rapidly growing algal population in nutrient-enriched medium is exposed to the toxicant for 3-4 days. The most frequently used species are *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) and *Scenedesmus subspicatus* although other algal species have also been used. Published data shows the sensitivity of algae to chemicals varies widely depending on both the species and the nature of the environmental conditions, However, ECETOC (1993) found that algae were more sensitive than fish for 85% of the chemicals tested (r^2=0.99). This was also reported by Weyers *et al* (2000). Algal tests therefore appear to have good predictive value, but may be over conservative. Nevertheless, it is recommended that toxicity tests using algae be included in a test battery on the basis that they evaluate effects on photosynthetic capabilities of primary producers (Isomaa *et al,* 1994; Repetto *et al*, 2001; Rojickova-Padrtova *et al,* 1998).

There has been a trend in recent years to try to enhance algal toxicity tests by increasing the simplicity and cost-effectiveness (Radetski *et al,* 1995; Persoone, 1998). A miniaturised version of the conventional flask method with *S. capricornutum* was developed by Blaise *et al* (1986), whereby algae are exposed to the toxicant in 96 well microplates. Algal cell density is usually determined using a haemocytometer or an electronic particle counter. The main advantages of the microplate assay, relative to the conventional flask method are:

- The small sample volumes (thereby enabling more replicates to be included while at the same time reducing the amount of laboratory space required;
- use of disposable materials;
- increased number of replicates;
- the potential for automation of the test (Blaise *et al*, 1986). St-Laurent *et al,* 1992, reported that a good correlation exists between results of the conventional flask assay and the microplate test for phenol, metals and herbicides.

A microbiotest has also been developed with *R. subcapitata* (Persoone, 1998). This has the advantage of eliminating the need for pre-test culturing of algae. This is possible since the algae are supplied in the form of algal beads, which can survive storage for up to several months.

In conclusion, the evidence supports that the use of algae as part of a battery of tests, including daphnids may provide viable alternatives to the use of acute fish for aquatic toxicity testing. This is described further in Section 3.7.

3.5.1.5 Toxicity tests with a freshwater Cnidarian (Hydra) model

Hydra attenuata is a primitive invertebrate that is made up of complex tissues and organs, but still has the capability for whole body regeneration. Its wide distribution in freshwater environments, (which makes it suitable as a representative model for conducting hazard assessment), ease of maintenance and handling, and the possibility to observe morphological changes during the exposure phase, offer certain advantages to the use of *Hydra* sp. in toxicity testing. It has been used for effluent testing in Japan (Fu *et al,* 1994; Blaise and Kusui, 1997). Published data evaluating the use of *Hydra* sp. as a screening tool are shown in Table 7. The limited database available indicates that while *Hydra* sp. may offer some advantages in terms of handling, it is less sensitive compared with other invertebrate species.

3.5.1.6 Protozoa in toxicity tests

Protozoa are eukaryotic unicellular organisms that are ubiquitous in both the aquatic and terrestrial compartments (Twagilimana *et al,* 1998). They are primary consumers and are an important link in the aquatic food chain, as well as playing a critical role in wastewater treatment. Other advantages of using ciliated protozoa for evaluating the toxicity of individual chemicals or complex effluents include their short life-cycle, which allows easy, cost-effective cultivation and the fact that they are eukaryotic cells with a plasma membrane resembling the membrane of cells of higher organisms (Isomaa *et al,* 1994; Twagilimana *et al,* 1998). The most common protozoan model used in toxicological studies is the ciliated protozoa *Tetrahymena pyriformis* (Pauli and Berger, 2000). *T. pyriformis* is the first protozoan to be cultivated axenically. Other tests integrating protozoan tests and (Q)SAR with biomarkers have also been carried out (Bogaerts *et al,* 1998, 2001). The sensitivity and applicability of the species *T. pyriformi* has been compared with those of other *in vivo* and *in vitro* models such as algae, fish and mammalian cells.

Table 7: Examples of the use of Cnidarian (Hydra) toxicity tests

Test substances	Assays employed	Comments	Reference
Cadmium, copper and zinc	Use of freshwater coelenterate *H. vulgaris* for measuring acute toxicity using two methods: the conventional determination of LC_{50} and evaluation of progressive changes in structure using a scoring system.	Relative toxicity is Cu>Cd>Zn for both methods. Assessment using progressive changes has several advantages over conventional recording of death: it reveals more detail of the pattern of response and concentrations causing a particular stage of toxicity: is a more sensitive indication since morphological changes can be detected at lower concentrations than those causing mortality in the same timescale: provides a means for studying the ability of animals to recover: ease of culturing: simplicity and lower cost.	Karntanut and Pascoe, 2000
14 wastewater sample from the Krakow region (Poland)	A microplate-based *H. attenuata* bioassay. The test monitors morphological changes in adult *H. attenuata* exposed continuously during 96 h. This was compared to the Microtox® test.	Results indicate that toxicity to *H. attenuata* increases when ammonia concentration increases. Comparisons with the Microtox® assay show *H. attenuata* to have a higher sensitivity to whole water samples and therefore may prove useful in the detection of environmental contaminants. However, the impact of natural water properties should be studied to fully understand the usefulness of *H. attenuata.*	Pardos *et al*, 1999
4-chlorophenol, endosulfan and copper	Assessment of the acute and subchronic toxicity of endosulfan and copper to *Hydra* sp. using mortality and population growth assessment.	*Hydra* sp. were less sensitive to the toxicant compared with other invertebrate species. Findings are in agreement with previous studies. *Hydra* sp. cannot be considered a good model species for invertebrates and would have limited applicability for toxicity testing.	Pollino and Holdway, 1999

Table 8: Examples of the use of protozoa toxicity tests

Test substances	Assays employed	Comments	Reference
Tri-substituted benzenes	The Spirotox test utilising the ciliate protozoan *Spirostomum ambiguum.*	Results were compared to Microtox®, *T. pyriformis*, and *D. magna* standard tests. Microtox® was more sensitive than the Spirotox test for almost all toxicants tested, as was the *D. magna* study, but has a rather low sensitivity to organic compounds and calls to have it included in a test battery are unsupported as yet.	Nalecz-Jawecki and Sawicki, 2002
13 inorganic substances in the form of metal salts and 21 organic substances	The *T. pyriformis* fluorescein diacetate (FDA)-esterase test using fluorescein dye to estimate the activities of non-specific esterases of a cell for screening and assessing cytotoxicity of xenobiotics. The *T. pyriformis* population test and the Microtox® test were evaluated in relation to these chemicals. The relationship between the toxic effects of the test substances and the ion characteristics of the metal ions or the hydrophobicity (quantified by log K_{ow}) of the organic compounds was investigated.	FDA test allows xenobiotics to be classified according to their mode of action. Main advantage of *T. pyriformis* FDA test is that information can be obtained in 1 h rather than the 9 h required for the standard growth test, making it a cost-effective method for monitoring toxicity. The two tests involving *T. pyriformis* have sensitivities comparable to that of Microtox®. Relative toxicity of metal ions and organic compounds is predictable using two ion characteristics and log K_{ow} respectively. *Tetrahymena* sp. is possibly a suitable model for ecotox studies although testing conditions remain to be standardised.	Bogaerts *et al*, 2001
10 metal salts and 8 organic substances	The *T. pyriformis* FDA test. Comparisons with conventional *T. pyriformis* proliferation rate 9-h median inhibitory concentrations, Microtox® 30-min EC_{50} and the *D. magna* galactoside 1-h EC_{50}.	FDA test offers high sensitivity, speed and ease of toxicological analysis as well as ability to assess the toxicity of natural samples. Application of the microplate technique should be considered to increase speed and numbers of xenobiotics that can be assessed simultaneously. A highly significant correlation is found between this method and those obtained with the growth inhibition and results from Microtox® tests. Proposed as a sublethal biotest with possible use of other fluorogenic substances to provide a battery of microtests.	Bogaerts *et al*, 1998

An international inter-laboratory study by Larsen *et al* (1997) has helped to standardise test conditions and improve the validity of using *T. pyriformis*. However, standardisation of microbiotests using freshwater ciliates is complicated by the fact that many species do not form cysts, such as *Spirostomum teres* (Twagilimana *et al,* 1998). This complicates inter-laboratory bioassays and maintenance of strains. While some studies have demonstrated the validity of protozoa as a model species, and concluded that protozoa testing may be considered as a complement or alternative to animal models in toxicological research (Sauvant *et al,* 1999), more recent testing by Nalecz-Jawecki and Sawicki (2002) and Bogaerts *et al* (2001) suggest that it offers no advantage over bacterial microbiotests (see Section 3.5.1.7) or the daphnid test. Thus, while protozoa testing may be useful in some circumstances (see Table 8), e.g. for monitoring effluent, it is unlikely to be suitable as a part of a non-vertebrate test battery.

3.5.1.7 Toxicity tests using bacteria

Bacteria are an important component of the aquatic and terrestrial ecosystem and play an essential role in the activated sludge in municipal and industrial wastewater treatment plants. Bacteria lack many of the intracellular structures of eukaryotic cells and they also have (in addition to a plasma membrane) a cell wall that may have a protective effect. Bacteria also demonstrate a high adaptive ability. It is for these reasons, that bacteria may be less sensitive to chemical toxicants than many other biological assays. Isomaa *et al,* 1994 suggest that their usefulness as models in toxicity assessments may be limited. However, bacteria have received increasing attention, recently, as models for toxicity assessments.

National/international standardisation and/or regulatory bodies have proposed several microbial tests. These are reviewed by Mayfield (1994). In these assays, bacteria are inoculated under appropriate growth conditions into a nutrient medium, the toxicant is added at various concentrations, the flasks are incubated and samples are removed at regular intervals for determination of the relevant endpoint. One of the major advantages that most of these bacterial microbiotests have over conventional assays is that no culturing is required as the test organisms are stored in a lyophilised form. Bacterial microbiotests are also amenable to automation, have a rapid growth rate and a short exposure time. Bacterial bioassays can be grouped into the following categories, according to the kind of parameter used to assess the toxic effect (adapted from Isomaa *et al,* 1994):

- Bacterial growth;
- cell energy;
- substrate consumption;
- respiration;
- bioluminescence.

Bacterial bioluminescence has received increasing amounts of attention as a parameter in toxicity assessments in recent decades. In this procedure, the enzyme luciferase, which is intrinsically linked with the metabolism of bacteria, catalyses a light-emitting reaction. The microbiotest bioluminescence test with the marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*), referred to as Microtox® is probably the most widely known. The assay is based on the change in the light output of the luminescent bacteria and this is quantified photometrically. It has the key advantage that the bacteria are available in a freeze-dried form, which eliminates the need for culturing. The test is rapid and easy to conduct. One disadvantage may be that the very short incubation time is too brief for some chemical compounds to equilibrate between the external medium and the cytoplasm, and as a consequence cellular damage may not be observed (Isomaa *et al,* 1994).

Despite this, a good correlation has been found with many aquatic species including fish species (fathead minnow, bluegill, catfish, goldfish, golden orfe, guppy, killifish, rainbow trout, sheepshead minnow and zebrafish), invertebrates (*Daphnia* sp. *Artemia* sp.), protozoa (*T. pyriformis)* and algae (Kaiser, 1998). Correlation of Microtox® EC_{50} values with LC_{50} values for fish and daphnids is high for soluble organic compounds and the sensitivity of the test is approximately equal to that of *Daphnia* sp., rainbow trout and fathead minnow bioassays.

The Microtox® bioassay has been used extensively to generate toxicity data for the assessment and prediction of aquatic toxicity of chemicals through mathematical modelling using QSAR (Fentem and Balls, 1993). It is also often used in comparative studies involving other test systems: microbiotests (Jung and Bitton, 1997; Toussaint *et al,* 1995); TETRATOX (Bogaerts *et al,* 1998 and 2001); Spirotox (Nałęcz-Jawecki and Sawicki, 2002) and *Hydra* sp. (Pardos *et al*, 1999). Microtox® has also been proposed for inclusion in several test batteries (Rojickova-Padrtova *et al,* 1998 and Repetto *et al*, 2001) and as a chronic test (Radix *et al,* 2000). In terms of screening new or existing chemicals, extrapolation of data from bacteria to fish and other organisms can be done with varying degrees of confidence (Kaiser, 1998).

Other bacterial microbiotests are discussed by Janssen (1997) and Janssen *et al* (2000) and examples of their application are shown in Table 9. Unlike the Microtox® test system, these other microbiotests, while potentially attractive for the rapid toxicity screening of chemicals and environmental wastes, do not meet the criteria for a good assay set out by Tebo (1995) and many have not progressed significantly beyond the conceptual stage.

Table 9: Examples of the use of Toxkits

Test substances	Assays employed	Comments	Reference
50 industrial chemicals	A ciliate toxkit microbiotest using *T. pyriformis* and *T. thermophila*. This is compared to toxicological literature data from the IUCLID (International Uniform Chemical Information Database).	Despite evolutionary distance between the species, these results support the hypothesis of a comparable toxicological susceptibility. Results suggest both systems complement each other with regard to the estimation of toxic effects in wastewater treatment. Test does not require sophisticated sample preparation and a multigenerational (chronic) test can be conducted by untrained personnel without expensive equipment. Ciliate tests could hence serve as a powerful tool for the prediction of possible hazards to sewage treatment processes.	Pauli and Berger, 2000
Industrial effluents from Florida	A comparison of an acute 1-h *C. dubia* toxicity test (CerioFAST™) with Microtox® and the standard 48-h *C. dubia* acute toxicity test.	EC_{50}s of CerioFAST™ correlated well with the EC_{50}s of the 48-h test. Therefore, feeding behaviour of daphnids may be a suitable criterion for short-term acute toxicity testing. CerioFAST™ showed a comparable sensitivity to pure compounds and industrial samples with the standard 48-h *C. dubia* toxicity test. CerioFAST™ may thus be used as an alternative to the standard assay. This tool could be useful for range-finding tests and screening tests.	Jung and Bitton, 1997

3.5.2 *In vitro* toxicity

As outlined in Section 2.3.3.2, *in vitro* methods using cell cultures offer an opportunity to develop acute toxicity testing methods as an alternative to testing in the whole organism. Many of these approaches rely on the use of cytotoxicity testing in cultured fish cell lines. The following sections outline the various methods and how these have been evaluated in various fish cell lines. For a recent comprehensive review see Castaño *et al* (2003).

3.5.2.1 Cytotoxicity assays

Cytotoxicity is a measure of toxicity to living cells as a result of a toxic exposure. Estimation of cytotoxicity is generally based on the uptake or exclusion of a marker chemical that labels cells as being dead or alive. For a population of cells (e.g. a microtitre-plate well, test tube), toxicity will be determined as a test chemical concentration versus marker chemical concentration response. *In vitro* cytotoxicity for the toxicity ranking of chemicals has already been suggested as a possible alternative or supplementary bioassay for the conventional acute toxicity test utilising fish (Babich *et al,* 1991; Segner and Lenz, 1993; Brüschweiler *et al*, 1995; Castaño *et al,* 1996; Fent and Hunn, 1996). Neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays remain among the most frequently employed endpoints for cytotoxicity measurements in monolayer cultures (see Table 10). Other, less frequently used cytotoxicity assays include the release of lactate dehydrogenase, crystal violet (CV) staining, trypan blue, decrease in adenosine triphosphate levels (Castaño *et al,* 1996) and cell proliferation assays (Kohlpoth and Rusche, 1997). Cell proliferation assays, in which a thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU) is incorporated into replicating DNA and subsequently localised by a specific monoclonal antibody (Gratzner, 1982), have also been used.

3.5.2.2 Fish cell culture

Fish cell culture has been widely documented since the 1960s and progress using *in vitro* techniques has led to the culture of two main cell types being developed. These cultures comprise of primary cultures and immortalised cell lines. From these cell types it is possible to observe different endpoints of toxicity including morphology, inhibition of cell growth, viability, cloning efficiency and inhibition of macromolecular synthesis (Hunt *et al*, 1986).

Table 10: Commonly used cytotoxicity assays

Cytotoxicity assay	Endpoints measured	Advantages or disadvantages	References
Neutral red release (NRR)	Membrane integrity. Toxicity represented as the concentration that causes 50% of the dye to be released.	More rapid than cell viability and cell growth inhibition. Not appropriate for all chemicals, e.g. surfactants and products containing fixative (Zuang, 2001).	Zuang (2001)
Neutral red uptake (NRU)	Cell viability, determined as inhibition of the cell cultures to take up the NR dye into active lysozomes, toxicity represented as the concentration that causes 50% inhibition of the cells to take up the dye.	Good correlation observed with acute LC_{50} in golden orfe (Brandão *et al* (1992). Found to be highly reproducible (Magwood and George, 1996). Excellent agreement of cell burden-based toxicities versus whole body burdens (Bernhard and Dyer, 2005).	Brantom *et al*, 1997; Brandão *et al* (1992); Magwood and George (1996)
Mitochondrial reduction of tetrazolium salts (MTT)	Cell viability based on colorimetric evaluation of the reduction of a tetrazolium salt (MTT) by mitochondrial dehydrogenases to from a blue formazon product. Toxicity represented as the 50% reduction in MTT reduction quantified by absorbance.		Brantom *et al*, 1997

3.5.2.2.1 Primary cultures

Primary cultures are made from isolated cells that have been directly removed from the organism, disaggregated and then placed in culture media (suspension or plates). Appendix B provides an overview of the isolation and culture processes. Isolated fish cells have been used in toxicity research in the area of mechanistic toxicity (peroxisome proliferation and endocrine disrupting mechanisms), xenobiotic metabolism and genotoxicity (Baksi and Frazier, 1990; Pesonen and Andersson, 1997). It has been suggested that the mechanisms involved in chemical-induced toxicity can be determined in more detail *in vitro* than would be possible *in vivo* (Pesonen and Andersson, 1997), and that the metabolism of xenobiotics by fish primary hepatocytes is generally similar to that seen *in vivo* (Segner and Cravedi, 2001). However, a disadvantage of using primary (fish liver) cells is that they require specialised isolation and culture procedures and typically do not replicate, and hence cannot be passaged. Consequently, newly prepared cells are required for each piece of work (Babich and Borenfreund, 1991).

3.5.2.2.2 Immortalised cell lines

Immortalised cell cultures are based upon cells that have been removed from different tissues (either normal or derived from tumour tissue, e.g. the PLHC-1 cell line, see Section 3.5.2.3) and as such they can replicate in culture and be maintained indefinitely. This advantage must be balanced, since there is evidence to suggest that in some cases chromosomal aberrations can occur in immortalised cell lines and that immortalised cell lines may become dedifferentiated (Vrba *et al*, 2002; Sandbacka *et al,* 1999), retaining only part of the characteristics of the tissue of origin. This is of importance when conducting biotransformation studies, since immortalised cell lines tend to have a reduced metabolic activity. However, while Hunt *et al* (1986) mentioned that immortalised cell lines are the least representative of *in vivo* situations, they do have the advantage to be a convenient and easy to handle system with relatively low variability (see Chapter 4).

Most fish cell assays, based on basal cytotoxicity, use immortalised cell lines (Isomaa *et al,* 1994; Clemedson *et al,* 1996) and are discussed in Sections 3.5.2.3 to 3.5.2.6.

3.5.2.3 PLHC-1 cell line

The PLHC-1 cell line is an immortalised cell line developed from the desert top minnow (*Poeciliopsis lucida*) hepatocellular carcinoma after exposing the minnow to the carcinogen 7,12-dimethylanthrabenzene (Hightower and Renfro, 1988). The benefits of using PLHC-1 cell lines include ease of culture; they contain an aryl hydrocarbon receptor (AHR), inducible and stable cytochrome P450 (CYP) enzymes and have certain metabolic activity (Fent, 1996). The function of metabolism and the presence of CYP enzymes are important to assess xenobiotic activation or deactivation. However they may be affected by temperature (Babich *et al*, 1991; Denizeau, 1998). The relevance of PLHC-1 cell lines for ecotoxicity testing has been evaluated through the use of the NRU assay and the MTT assay (Brüschweiler *et al*, 1995; Fent and Hunn, 1996). In these studies there was correlation shown between the two assays for all the chemicals tested which included organotin compounds, chloro- and nitro-phenols, sulphonic acids and alkylphenols (Fent, 1996). The assays also showed correlation between the *in vitro* and *in vivo* data for organotin compounds and substituted phenols (Fent and Hunn, 1996). However, no *in vivo* data was obtained for the sulphonic acids and the data that was referenced was obtained from an external source. Recently, Bernhard and Dyer (2005) showed that toxicity of surfactants to PLHC-1C cells based on cellular burdens (ED_{50}) were nearly identical to those found for the same materials where organism toxicity was expressed as critical body burdens.

3.5.2.4 RTG-2 cell line

The RTG-2 cell line is derived from rainbow trout gonadal tissue. This cell line has been used to compare cytotoxicity using the NRU and MTT assays, with acute toxicity to embryos and adult zebrafish (Lange *et al*, 1995). In these studies, the toxicity of ten different chemicals with different modes of action was analysed. The authors found that for that for 4 of the chemicals there was no difference in the observed toxicity with the two assays, and concluded that the toxicity of chemicals with different modes of action can therefore be assessed using cell-based techniques. In contrast, the embryo test was, in most cases, more sensitive than the acute LC_{50} test in adult fish and in all cases more sensitive than the RTG-2 cell test (Lange *et al*, 1995). The authors suggest that the RTG-2 cell test has a great deal of potential.

Castaño *et al* (1996) have also considered the use of the RTG-2 cytotoxicity test and have compared data, obtained from 96-h LC_{50} assays performed on rainbow trout *in vivo*, with the cytotoxicity assays for 16 different chemicals. The authors employed 3 different cytotoxicity assays, the NRU assay, to assess cell viability, intracellular adenosine triphosphate content, based on a luminescence assay, and the detachment of cells from a substrate, through the use of the kenacid blue protein (KBP) assay. The results indicated good correlation between the three different assays although absolute sensitivity did vary. The EC_{50} values from the cytotoxicity assays showed good correlation with the *in vivo* LC_{50} data, but in some cases were 200 times less sensitive. The difference in the duration of the *in vivo* and *in vitro* assays (96 h versus 48 h) may account for some of these differences. Gagné and Blaise (1998) compared the sensitivity of the RTG-2 cytotoxicity test with rainbow trout hepatocytes exposed to more than 30 complex mixtures of industrial and municipal wastewaters. In each case the NRU assay was used and compared with concurrent 96-h LC_{50} data performed *in vivo*. The results indicated that the two assays, performed *in vitro*, had a weak, but significant correlation (R=0.509) (Gagné and Blaise, 1998). The comparison between the *in vitro* and *in vivo* data suggests that cytotoxicity assays with hepatocytes showed a better correlation with the *in vivo* data than the RTG-2 cell line.

3.5.2.5 R-1 cell line

The R-1 cell line is isolated from rainbow trout liver. Cytotoxicity assays with this cell line can be assessed through the use of trypan blue to determine cell viability (Ahne, 1985). Ahne tested various wastewater samples and found that when compared with parallel *in vivo* studies the R-1 cytotoxicity test was generally more sensitive. Rusche and Kohlpoth (1993) also used this cell line to evaluate the cytotoxicity of 362 different wastewaters. They compared the results with *in vivo* test data performed concurrently on golden orfe (*Leuciscus idus melanotus*); good correlation was observed for 86.8% of the wastewaters. Lenz *et al* (1992) evaluated the response of the R-1 cell line with three cytotoxicity assays; the CV assay, (which evaluates the ability of

cells to attach to a substrate), the NRU assay and the MTT assay. Fifteen test substances were evaluated. These 3 methods involve different endpoints. A good correlation between the three different methods was observed when ranking chemicals in order of sensitivity, although there was a large range in the actual sensitivity. The results were also analogous to *in vivo* data obtained using golden orfe. The three cytotoxicity endpoints were also evaluated by Halder and Ahne (1990). The CV assay was found to be the most sensitive and appeared to show greater correlation with data conducted *in vivo* on golden orfe, although the data from the assays performed *in vivo* were obtained from an external source. The data also indicated that the CV assay was more sensitive than the NRU and MTT assays. However as all of these studies were conducted with wastewaters, which contain a range of chemicals, further work would be necessary to understand the differences in sensitivity.

3.5.2.6 BF-2 cell line

The BF-2 cell line, derived from caudal trunk of bluegill sunfish, has been evaluated using the NRU assay (Babich and Borenfreund, 1987). A total of 18 chemicals were tested of which 11 had corresponding *in vivo* data sourced from published literature. Good correlation (r=0.98) was observed for all the chemicals except for the uncoupling agent 2,4-dinitrophenol (Babich and Borenfreund, 1987). The authors concluded that this cell line offers potential as a replacement for *in vivo* fish testing, although further evaluation is still needed, for example for chemicals which uncouple oxidative phosphorylation. The sensitivity of the BF-2 cell line has also been compared against the RTG-2 cytotoxicity test using the NRU assay to assess the toxicity of four metals (cadmium, zinc, copper and nickel) (Babich *et al*, 1986). The BF-2 cell line was found to be more sensitive than the RTG-2 cytotoxicity test and also has the advantage of less stringent temperature requirements for growth and a quicker doubling time (Babich *et al*, 1986).

The BF-2 cell line has also been used in a novel *in vitro* assay where the cells were transformed with a recombinant bacterial plasmid containing the *luc* gene, coding for luciferase, and the neomycin resistance marker for identification of transfected cells (Thompson, 2001). Cytotoxicity using the neutral red assay was compared with *in vivo* data for a range of toxicants. These results suggest that the *in vivo* assay was more sensitive than the transformed BF-2 cells for 65% of the chemicals tested and that the transformed BF-2 cell line was more sensitive than the neutral red assay for 53% of the chemicals tested. However, because of the different exposure periods used with the various tests, no overall conclusions can be drawn and further work on the transformed cell lines would be needed if these tests were to be used as acceptable alternative methods.

3.5.2.7 Suspension-cultured fish cells

The cells discussed above are cultured and exposed attached to a substratum; however, it is also possible to culture the cells in suspension. The exposure of any fish cell line can be performed in suspension for a limited period of time, usually only a few hours. Expression of cytotoxicity can be measured from the most simple and early response such as the uptake of a vital die or the efficiency to attach to a substratum, to other classical viability endpoints. For example the use of suspension-cultured fish cells (CHSE-sp) obtained from chinook salmon (*Oncorhynchus tshawytscha*) has been investigated by Okamura *et al* (2002) by means of a cell viability assay using the dye Alamar Blue™ (Voytic-Harbin *et al*, 1998). The chemicals tested were six different antifouling compounds. The results from these experiments (expressed as 24-h EC_{50} values) were correlated with chronic *in vivo* (LC_{50}) data conducted on juvenile rainbow trout. These data indicated that for all the test materials, the CHSE-sp was up to 100-fold less sensitive than the assays performed *in vivo* although this may be attributed to the much shorter exposure period of 24 h for the *in vitro* assays. In spite of this, the assays performed *in vitro* did show agreement with the order of decreasing toxicity observed in the assays performed *in vivo*.

The use of suspension cultured fish cells has been compared with the use of primary cultures and epithelia cultured on filter papers in an experiment where the different culture systems were exposed to 30 reference toxicants derived from the multicentre evaluation of *in vitro* cytotoxicity (MEIC) project (Sandbacka *et al*, 1999). The three culture systems were all obtained from rainbow trout gill epithelium tissue and were evaluated using the fluorescent viability probe calcein-AM. The experiments showed that there was no significant difference between the three systems for any of the chemicals tested, although the epithelia cultured on filters were more variable. Mori and Wakabayashi (2000) have also compared the CHSE-sp cell line with a monolayer cell line from the chinook salmon (CHSE-214). Eleven test chemicals, including organic chemicals and pesticides, were evaluated. The authors also exposed five different monolayer cell lines to four different metal salts. Cell viability in both comparisons was assessed via the NRU assay. The results for the monolayer comparisons indicated that the five different cell types all had similar EC_{50} values based on NRU. Similarly, the comparisons between the CHSE-sp and CHSE-214 cell lines showed that the two assays were highly correlated ($r=0.98$). Therefore, the CHSE-sp cell line could be a good candidate cell line for alternative ecotoxicity testing since suspension-cultured fish cells are simpler and the assays can be performed more rapidly than monolayer-cultured fish cells (Mori and Wakabayashi, 2000).

3.5.2.8 Summary of in vitro test systems

Each test cell type offers advantages and disadvantages (see Table 11).

Table 11: Comparison of advantages and disadvantages of cell line in vitro assays

Type of cell line	Advantages	Disadvantages
PLHC-1	Derived from liver cells; retain a certain metabolic capability. Immortalised cell lines and therefore easy to culture.	Repeated cell division may lead to DNA damage and therefore not suitable for evaluating genotoxicity.
RTG-2	Immortalised cell line, therefore easy to culture. Reproducible results. Good correlation with *in vivo* assays. Same DNA fingerprint as *in vivo* individuals, so very good for genotoxicity studies. Retain certain metabolic capability. Used to develop oestrogen-responsive reporter gene system.	Low cell cycle. Less sensitive to exogenous chemicals than fish hepatocytes, juvenile fish and fish embryos. Less metabolic activity than hepatocytes.
R-1	Derived from rainbow trout liver. Immortalised cell line and therefore easy to culture. Good correlation with *in vivo* test results when testing wastewaters.	Less sensitive to exogenous chemicals than fish. Less metabolic activity than hepatocytes.
BF-2	Immortalised cell line, therefore easy to culture. Reproducible results. Showed good correlation with *in vivo* test results for metals, anions and thirteen phenolic derivatives.	More sensitive than RTG-2 cell line for some chemicals. No metabolic capability.
Suspension cultured fish cells	Simpler and more rapid test procedure than monolayered cultured fish cells. No significant difference in response to primary and epithelial cell lines.	May be less sensitive than *in vivo* tests. No metabolic capability. Less reproducibility than monolayer cultures. Much more testing required to explore the advantages/disadvantages.

Currently, based on the previous discussion, *in vitro* tests with a single cell type line do not appear to represent a suitable alternative to acute testing *in vivo,* and if incorporated in a battery approach it may be that a more accurate prediction of the effects *in vivo* would be achieved. The strategy on how to use *in vitro* and other test systems is discussed further in Section 3.7.

However, the other issue that requires some attention is the quantifying of exposure. This will be further discussed in Section 3.8.4.

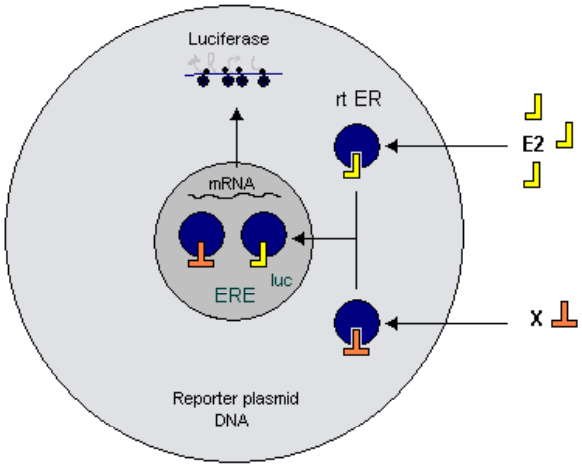
3.5.3 Toxicity evaluation using subcellular biomarkers

A subcellular biomarker was defined by van der Oost *et al* (2003) as 'a biological response, which can be related to exposure to or toxic effects of environmental chemicals'. There are many different biomarkers which can be indicative of exposure, effect or susceptibility but for the purpose of this review, the three areas of endocrine activity, cytochrome P-450 induction and heat shock proteins, will be considered.

3.5.3.1 Replacement of the fish screening assay for endocrine activity

In vitro assays using cells rich in specific estrogen and or androgen receptors are available to detect chemicals binding to the estrogen and androgen receptor (Zacharewski, 1997). These are expected to be used in screening batteries to complement *in vivo* assays (Zacharewski, 1997; Hornung *et al*, 2003). Hepatocytes isolated from rainbow trout (Jobling and Sumpter, 1993) and male carp (Smeets *et al*, 1999) are able to produce vitellogenin (VTG), the standard biochemical endpoint to detect an estrogenic response. In addition, Fent (2001) also developed an RTG-2 cell line, with an estrogen responsive element fused to a luciferase gene that is able to detect estrogenic active chemicals (see Figure 4).

Figure 4: Schematic design of the RTG-2 cell reporter gene system (adapted from Fent, 2001)



E2 = 17 β-estradiol
X = xenoestrogen
rt ER = rainbow trout oestrogen receptor
ERE = oestrogen responsive element

The principle of this system is that an estrogenic chemical binding to the estrogen receptor causes regulation of gene expression by binding to the estrogen responsive element. Subsequently, there is up regulation of the luc gene and hence luciferase is produced. Luciferase activity can then be measured spectrophotometrically. This system allows detection at very low levels and to a greater sensitivity than most mammalian or yeast cell systems and the rainbow trout primary hepatocyte

vitellogenin assay (Fent, 2001; Ackermann *et al*, 2002). Schmieder *et al* (2000) incubated male rainbow trout liver slices in the presence of ethynylestradiol to detect VTG induction. To detect potential androgenic-active chemicals in fish, Katsiadaki *et al* (2002) developed a novel method based on ELISA for spiggin, which could be potentially applied to isolated kidney cells, avoiding therefore the exposure of sticklebacks *in vivo*. It should be noted however, that as with other biochemical/morphological endpoints used in reproduction assays, VTG induction in cellular systems or other screening assays should be used as indicators, rather than identifiers, of the potential endocrine activity of a chemical.

3.5.3.2 Cytochrome P-450 Replacement (refinement)

Cytochrome P-450 (CYP) and their associated enzymes, play a prominent role in Phase I metabolism of most xenobiotics via oxidation, reduction or hydrolysis (Goeptar *et al*, 1995; van der Oost *et al*, 2003). They are also able to facilitate the excretion of certain compounds by transformation of lipophilic xenobiotics to more water-soluble compounds (Bucheli and Fent, 1995). CYPs are part of the haem family of proteins and are characterised on the basis of their absorbance maximum at 450 nm. They are membrane bound proteins located predominantly on the endoplasmic reticulum of the liver and other tissues. The first to be identified was cytochrome P-4501A (CYP1A) and a large amount of research has since been conducted on this protein (Fent, 2001; Huuskonen *et al*, 2000).

Expression of the CYP1A gene is initiated following exposure to an agonist such as an environmental pollutant (e.g. hydrophobic aromatic compounds). Gene expression, under these circumstances, is a consequence of the agonist binding to an AHR. The AHR is a ligand-dependent transcription factor, which is translocated to the nucleus from the cytosol following binding of the agonist (Fent, 2001). According to Fent (2001), the binding potential of the ligand to the AHR is approximately proportional to the resultant gene transcription and hence the relative toxicity. Therefore, CYP1A induction can be used as a biomarker of toxicity. CYP1A protein induction can be quantified by the activity of its associated CYP1A- dependent enzyme ethoxyresorufin O-deethylase (EROD) or via direct protein content analysis (determined by immunocytochemical methods such as Western blots or ELISA) (Brüschweiler *et al*, 1995).

Sturm *et al* (2001) have compared the effects of prochloraz and nonylphenol diethoxylate on the induction of CYP1A in immortalised rainbow trout hepatocytes and induction of CYP1A in the liver of juvenile rainbow trout. The authors suggest that the results showed good qualitative correspondence between the *in vitro* and *in vivo* data, however, more *in vitro* research should be performed before good quantitative predictions can be made.

3.5.3.3 Heat shock proteins - replacement (refinement)

Stress proteins are present in all living cells and are inducible after exposure to environmental stresses such as heat, salinity or the presence of xenobiotics. The family of stress proteins include proteins such as ubiquitin, metallothionein, hemeoxygenase and the more familiar heat shock proteins (HSP). This section will look at heat shock proteins in more detail.

The exact function of heat shock proteins is poorly understood, although the general function appears to be of a protective nature against proteotoxic stresses, or to provide a regenerative function (Molina *et al*, 2002). There is also evidence to suggest that the purpose of HSP induction is to prevent apoptosis (Samali *et al*, 1999). The authors showed that cells that have been heat shocked, and have a subsequent accumulation of HSP, are resistant to apoptosis. The sequences of the HSP amino acids show good homology between most organisms and are characterised by their molecular weight (Wright and Welbourn, 2002). The protein moiety that has been most widely studied is HSP 70 since it is one of the most highly conserved proteins (Wright and Welbourn, 2002). This protein exists in unstressed cells and acts in a chaperoning function, but is also present in stressed cells after induction (Boone and Vijayan, 2002). Due to this fact, the presence of HSP70 does not necessarily prove that the organism has been exposed to environmentally stressful conditions (Sanders, 1993). More notably, Sanders (1993) points out that some steroid hormones can actually induce some heat shock proteins and also that diet can affect the response. The function of the induced form of the protein is believed to be the stabilisation, solubilisation and the removal of denatured proteins.

Kilemade and Mothersill (2001) have shown that the synthesis of HSP 70 in rainbow trout primary epidermal skin cell cultures is inducible after exposure to 2,4-dichloroaniline (2,4-DCA) and that the amount of protein synthesised is directly proportional to the exposure applied. The expression of HSP 70 has also been investigated in liver tissues of rainbow trout and chinook salmon after whole organisms were exposed to acutely toxic pulp mill effluent and sodium dodecylsulphate (SDS) (Vijayan *et al*, 1998). At sublethal concentrations, it was observed that synthesis of HSP 70 was induced suggesting that HSP70 expression could be used as an indicator of sublethal cellular responses to toxic substances (Vijayan *et al*, 1998; Kilemade and Mothersill, 2001).

The determination of heat shock protein induction could be automated through the use of transgenic fish cell lines that have the ability to express reporter genes after heat shock protein induction. This has been shown in the cell line of the nematode worm *Caenorhadbitis elegans* (Stringham and Candido, 1994) where the HSP 16 stress protein gene was fused to the *Eschericia coli lacZ* reporter gene. Subsequent β-galactosidase products formed from HSP 16 induction can then be assessed colorimetrically. Furthermore, these principles have since been applied to producing transgenic fish cell lines with the ability to express a green fluorescent protein (GFP)

following HSP induction (Molina *et al*, 2002). Molina *et al* (2002) have developed a stable transformed fish cell line derived from carp epidermal herpes virus-induced hyperplasia lesions (*Epithelioma Papulosum Cyprini* (EPC)). This cell line contains the GFP reporter gene that is under the control of the tilapia HSP 70 promoter. The clones of the cell line were selected on the basis of their ability to fluoresce after heat shock (at 37°C). Fluorescence, after heat shock, was reported to be 500 times more than those cells under control conditions. The authors continued to assess the expression of the reporter gene after exposure to different compounds and the results showed that at the concentrations tested, 56% of the chemicals induced expression of the GFP. Aït-Aïssa *et al* (2000) report that for 16 of the 18 chemicals they tested, the level of HSP induction correlated with the compound's octanol/water partition coefficient (log K_{ow}). In cases where HSP induction was not observed at a concentration that caused some evidence of cytotoxicity, it is possible that the stress response was not associated with that specific HSP promoter. Consequently, this may cause a problem if HSP induction is compound specific and it may be necessary to produce a battery of clones. Clearly, this is an area that requires further investigation. There is also the feasibility that HSP induction is not just compound specific but also specific to the cell type used to assay toxicants.

Stringham and Candido (1994) stated that HSP induction 'provides a more informative result than a simple LC_{50} because it begins to describe, at the molecular level, a pathway of events that may be involved when an animal is stressed and approaching death'. The two authors also described the effects of adding denatured β-galactosidase, which had the effect of inducing HSP. This suggests that if induction is triggered merely by protein denaturation, then HSP induction would be a beneficial tool in detecting environmentally relevant toxicants. However, Bradley (1990) suggests that heat shock proteins should be measured in the natural environment to determine whether daily or seasonal changes affect HSP induction. Furthermore, Bierkens (2000) recommends that an increase in the knowledge of the kinetics and the persistence of stress responses to complex test substances (e.g. effluents) is necessary before stress proteins can be applied in ecotoxicological evaluations. It would also be difficult to extrapolate data obtained from stress protein induction to obtain a figure that correlates with toxicity data performed *in vivo*.

3.5.4 Other alternative approaches

The two main areas of discussion in this section are largely based around physico-chemical properties and include acute predictions through chemical analysis and the use of (Q)SARs. For a comprehensive review of (Q)SARs, see ECETOC (1998 and 2003a).

3.5.4.1 Quantitative Structure Activity Relationships (QSARs)

(Q)SARs relate biological activity to the physico-chemical and structural properties of chemical compounds (Walker, 1998). Knowledge of the mode of action facilitates the choice of the most appropriate chemical parameters for predicting toxicity by (Q)SARs.

Toxicity can be related to a single parameter of uptake, metabolism or distribution (toxicokinetics), for example, the relationship between the octanol/water coefficients (K_{ow}) and fish toxicity data for compounds that have the same mode of action (Walker, 1998). (Q)SARs can aid decisions about the most appropriate toxicity test that need to be conducted thus indicating where tests on daphnids and/or algae would be more appropriate than tests on fish.

Reliable and accurate data are central to the development of any (Q)SAR, and this applies to both the biological and physico-chemical parameters. However, few databases are openly available due to confidentiality reasons and many lack proper validation and/or contain data derived from a variety of protocols (Cronin and Dearden, 1995). For a (Q)SAR to gain regulatory acceptance several principles have been suggested to enable adequate validation, these are known as the Setubal principles (Cefic, 2002). According to these principles (Q)SARs: must be associated with a defined endpoint of regulatory importance; are required to take the form of an unambiguous algorithm; and they should have a mechanistic basis. In addition, they must be accompanied by a definition of domain of applicability and it is essential that they are assessed in terms of their predictive power by using data not used in the development of the model.

The extent to which (Q)SARs can be used as alternatives to animal testing depends on the state of knowledge of mechanisms of toxicity. However, even at present, this approach can be used to make certain limited predictions of toxicity within certain groups of compounds known to share a common mode of action. For example, a *Daphnia* sp. toxicity test plus a relevant (Q)SAR might be sufficient to give an estimate of fish toxicity for an industrial chemical belonging to the same category (Walker, 1998). In the USA, some (Q)SAR estimates are already accepted at the premarketing stage of assessment, although this is not currently the case in the EU.

The following are potential uses for QSARs in the regulatory assessment of chemicals in the implementation of legislation on chemical substances and products:

1. To provide information for use in priority setting procedures, which are used to expedite the risk assessment process for substances of concern;
2. to assist in the selection of experimental test methods for the assessment of chemicals;
3. to classify chemicals on the basis of their hazardous properties;
4. to provide dose-response and environmental fate information for use in chemical risk assessment.

It should be noted, however, that mode of action-based (Q)SARs can not accurately predict aquatic toxicity for all classes of chemicals and their use requires an in depth appreciation of both toxic mechanisms, and the critical structural characteristics and properties of a chemical that govern its action by a specific mechanism. Verhaar *et al* (1992) developed a classification scheme that separates a large number of small to intermediate organic chemicals into four distinct classes that can then be assigned a mode of action. These four classes are: (1) inert chemicals (baseline toxicity), (2) less inert chemicals, (3) reactive chemicals, and (4) specifically acting chemicals:

1. Inert chemicals are chemicals that are not reactive when considering overall acute effects, and that do not interact with specific receptors in an organism. The mode of action of such compounds in acute aquatic toxicity is called (lethal) narcosis. Effect concentrations for a number of endpoints can be predicted using QSARs that were developed for these endpoints (see e.g. (EC, 2003b);
2. less inert chemicals are more toxic than those chemicals predicted by baseline toxicity estimations. These chemicals are often characterised as compounds acting by a so-called 'polar narcosis' mechanism, and can commonly be identified as possessing hydrogen bond donor acidity, e.g. phenols and anilines (Escher and Hermens, 2002);
3. reactive chemicals display an enhanced toxicity caused by the fact that they react unselectively with certain commonly found biomolecular structures, or are metabolised into more toxic species;
4. specifically acting chemicals exhibit toxicity due to (specific) interactions with certain receptor molecules (specific or receptor toxicity).

Further validation of this classification scheme showed that prediction of toxicity was only possible for class 1 type organic chemicals, however, a potential toxicity range could be predicted for the other classes of chemicals (Verhaar *et al*, 2000). A major limitation to this SAR-based classification system is that toxicity of metals, inorganics and ionisable organic chemicals cannot be predicted by the Verhaar scheme (ECETOC, 2003a).

3.5.4.2 Solid phase microextraction

Solid phase microextraction (SPME) is a method for chemical analysis where analytes partition from a sample into a polymer that is coated on a fused silica rod or stir bar. The amount of analyte that attaches to the rod is then measured by gas chromatography or high performance liquid chromatography. This method was originally used for analysis of volatile organic compounds. However more recently it has been suggested for other applications such as predicting aquatic toxicity (Parkerton *et al*, 2000), where the principle of SPME is that the polymer-coated rod (solid phase) acts as the surrogate for the organisms' lipid.

For highly lipid soluble compounds, ecotoxicity occurs in aquatic organisms when the critical body residue or burden (CBR or CBB) exceeds a critical threshold in the organisms' lipid (Parkerton *et al*, 2000). Van Wezel *et al* (1996) stated 'that once the CBR is attained within an organism, narcosis-type toxicity occurs as a result of increased membrane fluidity'. In addition, chemicals that induce narcotic-type toxicity are additive, and for hydrocarbon mixtures, ecotoxicity will occur when the amount of hydrocarbons in an organism's lipid exceeds the narcosis-based CBR. Therefore, ecotoxicity can be predicted from analytical methods by quantification of a chemical's partition to a surrogate lipid phase. If the total moles of the chemical, which partition to the surrogate phase, exceed CBR then ecotoxicity could be predicted (Parkerton *et al*, 2000). Using SPME to estimate the toxicity of complex hydrocarbons, based on the bioavailable fraction of the petroleum hydrocarbons (BPH), Parkerton *et al* (2000) related this to the acute toxicity of the BPH to rainbow trout. Using this approach and assuming a common mode of action it is thus possible to assess the toxicity of complex substances by quantifying the total moles of the hydrocarbons partitioned to an SPME fibre. One consideration when using SPME that needs to be accounted for is that the length of time a chemical takes to partition will increase with polarity and size of the molecule (see Section 4.4.3 for further details on SPME). Furthermore, especially when assessing the toxicity of mixtures, the effect of chemicals with a specific mode of action will be underestimated.

Escuder-Gilabert *et al* (2001) have suggested the use of biopartitioning micellar chromatography to predict ecotoxicity. Chromatography can also be used to evaluate physico-chemical parameters. For example, the retention of a compound on reverse phase liquid chromatography can be used to estimate log K_{ow} (Escuder-Gilabert *et al,* 2001). The retention of a compound in biopartitioning micellar chromatography depends upon the electrostatic, hydrophobic and steric nature of the chemical. The authors comment that chromatography may be used to derive quantitative retention-activity relationships (QRARs), which can elucidate the biological activity of different chemicals.

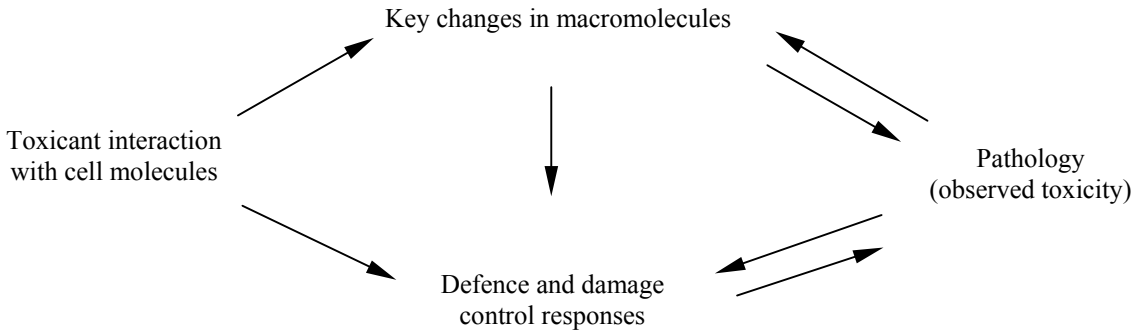
Parkerton *et al* (2000) used SPME devices to estimate the toxicity of complex hydrocarbons based on the bioavailable fraction of the petroleum hydrocarbons. Assuming a common mode of action (i.e. narcosis) and quantifying the total moles of the hydrocarbons partitioned to an SPME fibre, they demonstrated the correspondence between the BPH measurements and the toxicity of hydrocarbons to rainbow trout.

3.5.5 Toxicogenomics

Advances in molecular biology have resulted in an increase in the knowledge about the composition, architecture and function of genomes. During this time, a substantial and growing database of DNA sequence information has been assembled. Many new high throughput

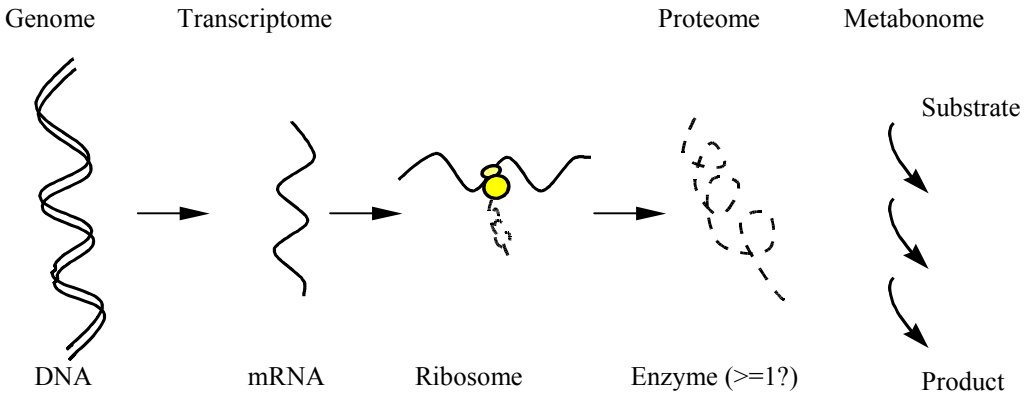
techniques for observing gene sequences, genetic variation, and gene expression have been developed. These advances have led to the development of a new sub-discipline in toxicology: 'toxicogenomics'. Aardema and MacGregor (2002) define toxicogenomics (Figure 5) as 'the study of the relationship between the structure and activity of the genome and the adverse biological effects of exogenous agents'. While such approaches are not without their challenges (particularly in data management and interpretation), they are already contributing to our understanding of basic toxicological mechanisms (Patterson, 2003; Pognan, 2004).

Figure 5: Basis of toxicogenomics (adapted from Aardema and McGregor, 2002)



The various sets of biomolecules can be broadly characterised as the genome (the organisation and sequence of DNA in chromosomes), the transcriptome (the messenger RNA from actively transcribed genes), the proteome (the entire quantity of protein in a biological sample), and the metabonome (representing the essential metabolites in a biological sample) (Figure 6). The last three are all context-specific whilst the first is more or less fixed for any species.

Figure 6: Basis of molecular life (adapted from Snape et al, 2004)



The primary aim of mechanistic toxicology is to understand how different classes of biomolecules interact in response to toxicant exposure, and the conditions under which these interactions may bring about adverse health effects. Pennie and Kimber (2002) expect that 'initial toxicogenomic evaluations of potential development compounds may streamline later mechanistic studies by providing early indicators of potential safety assessment issues; that is, to enhance our ability to predict potential toxicity issues'.

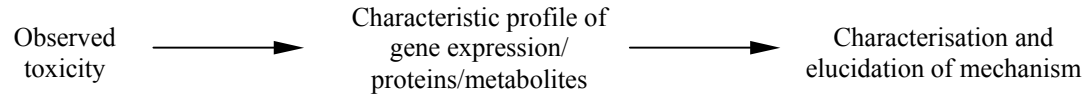
DNA microarray technology is currently receiving the greatest attention in the area of toxicogenomics. This provides a miniaturised sensor for sequence-specific and simultaneous detection of the expression of many thousands of genes. In principle it allows the entire transcriptome to be monitored simultaneously. This provides a potential method to uncover previously unrecognised responses of genes and overviews of gene complexes. Together with pattern analysis algorithms this allows the correlation of gene transcript levels with physiological responses, the generation of new hypotheses about mechanisms of action and gene responses as well as broad overviews of response profiles. This has the potential to lead to a more comprehensive characterisation of toxicity than has been previously possible.

Other techniques that may have an impact in the field of toxicology are those of proteomics (analysis of proteins and peptides), and of metabonomics (cellular metabolites). These analyses offer more information on mechanisms of action than gene transcript screening but currently are more difficult to perform. It has been recognised that 'gene expression is altered either directly or indirectly as a result of toxicant exposure in almost all cases examined' (Corton *et al,* 1999). It has also been noted that changes in gene expression associated with toxicity are often more sensitive and characteristic of the toxic response than currently employed endpoints of pathology (Nuwaysir *et al,* 1999).

Unlike other new developments in toxicology that have taken time to be adopted, genomic, proteomic and metabonomic methods are being swiftly evaluated.

Another potential advantage of toxicogenomic techniques regards 'fingerprints' of specific gene changes that provide information to design predictive toxicity screens (see Figure 7). Such pattern recognition could in turn facilitate the discovery and subsequent validation of biomarkers useful for application in higher throughput experimental systems to characterise or detect specific toxicity endpoints (Pennie and Kimber, 2002). However, for this to be a practical option, a multitude of reference data sets will need to be developed, involving the building of comprehensive databases of expression profiling.

Figure 7: Relationship between toxic effect and fingerprints of gene/protein changes (adapted from Aardema and MacGregor, 2002)



Toxicogenomics also provides the potential to predict toxic responses. Changes in the cellular molecules are thought to precede these toxic outcomes and appropriate changes may serve as early, sensitive indicators of potential toxicity (Aardema and MacGregor, 2002; Williams *et al,* 2003).Integration of these distinctive patterns may bring about the development of high through-put screening tests for the prediction of specific toxicities of chemicals and also molecular biomarkers, that indicate particular toxic mechanisms, applicable *in vivo*. The throughput requirement of such an approach linked with the limited availability of test substance will almost certainly necessitate employing *in vitro* culture systems.

The development of these new procedures may result in predictive tests that eliminate the need to conduct some of the conventional tests, or may offer the foundations for the design of an appropriate bioassay with relevant species. Because these predictive tools have the potential to provide savings in terms of time, cost, and animal use relative to traditional methods, it is expected that commercial promise will help drive the rapid development of practical assays (Aardema and MacGregor, 2002). However, it will be necessary to characterise multiple classes of agents with well defined mechanisms of action before expression profiles for new biomarkers can be used reliably in regulatory decision making (Corton and Stauber, 2000). It is therefore essential that toxicologists in industry, academia and regulatory agencies come to an agreement on the reliability and interpretation of endpoints such as these gene expression profiles before adoption into regulatory and industrial activities.

A feature of the risk assessment process is the understanding of chemical effects at low doses. Traditional toxicity studies have typically been carried out at high doses, with various extrapolation methods employed to approximate the effect at low doses. It is expected that chemical-induced changes in gene/protein expression will occur at doses lower than those observed *in vivo*. Therefore, by measuring the gene/protein/metabolite changes at low doses, and by employing pattern recognition procedures, post-genomic analyses have the potential to display biological effects at concentrations lower than would cause significant toxicity using conventional markers. This should lead to better extrapolation at low doses and improved determination of the effect of a chemical exposure to higher organisms at lower doses. This may act as an early warning trigger and even though an effect may be detected in the organism, it is necessary to understand that the multitude of coping mechanisms that many organisms have developed over time may mean that adverse effects are not manifested.

This increased understanding of gene/protein/metabolite expressions at a cellular level, coupled with knowledge of the mechanisms of action has the potential to provide a tool that can be used to compare toxic responses among species, or 'bridging biomarkers' as coined by Aardema and MacGregor (2002). Molecular genetics and genome biology have led to the recognition of conserved genes, proteins and common mechanisms between different species and phyla. The classification of key genes in the gene expression fingerprint for a given exposure to a particular toxicant in a laboratory animal in comparison to that of a higher organism should indicate that the molecular damage and response taking place in the laboratory model is similar to that occurring in the higher organism. This comprehension will also be useful for extrapolation from the *in vitro* test system to the *in vivo* condition.

One concern associated with the rapid expansion of these technologies is that many of the systems are not yet standardised. Indeed, different technical platforms are being used by different laboratories (Aardema and MacGregor, 2002) and they can give rise to divergent results. Caution in data interpretation is needed until a complete evaluation of the strengths and limitations of the different gene expression technologies through inter-laboratory studies have been undertaken.

There is also a need for refined methods of storing and analysing the vast quantities of data that will be produced, as well as standardised analysis approaches and algorithms that are required to enable sensitivity, robustness, and reproducibility comparisons to be made.

These new technologies have the potential to facilitate a more comprehensive understanding of the mechanism of toxicity as well as providing a more effective means of analysing chemicals. The extension of 'omic' technologies into environmental assessment is still at an early stage of development. Considerable research will be required before the promises of these technologies are realised and thus lead to an impact in the 3Rs in the environmental sciences.

3.6 Aquatic toxicity conclusions

In the short-term, the most promising animal alternative to the fish acute LC_{50} test is the use of the algae and daphnid acute EC_{50} tests, linked to the fish threshold (step-down) test (Hutchinson *et al*, 2003; Jeram *et al,* 2005). When it is necessary to use fish, these data with the fish acute threshold (step-down) test will lead to a reduction in the number of fish used. Further implementation of read-across and data derived from (Q)SARs in conjunction with confirmatory limit tests would also reduce the numbers of fish used. For complex substances, where it may be reasonably assumed that a common mode of action operates, SPME may also be a valuable tool that could be used.

Fish embryos may provide a longer-term alternative to the use of juvenile or adult fish in acute LC_{50} testing. However, this very much depends on the sensitivity of fish embryonic stages (e.g. embryo and/or early hatched eleutheroembryo) to different classes of chemicals. One approach that warrants further research is to evaluate removal of the protective chorion that may act as an effective barrier to many hydrophilic and ionisable chemicals.

In terms of *in vitro* tools, several of the assays discussed in this report could be used for testing of chemicals for ecotoxicity. It is probably not possible at this stage to recommend the use of individual cell lines in place of whole organisms for regulatory testing. Nevertheless, if cell lines were used in a battery approach, many of the sensitivity issues involved with the toxicity to cultured cells could be overcome. The use of *in vitro* systems (e.g. fish hepatocytes) may also offer a potentially useful research and screening tool for use in understanding the comparative pharmacology between mammals and fish in support of mode-of-action-based environmental risk assessments.

As the developments in the 'omic' technologies gather pace, it is recommended that industry keep abreast of these issues with the target for implementation of such technologies in the future. These developments, along with the capabilities of (Q)SAR applications have the potential to replace fish when they have been standardised and validated.

3.7 Proposed strategy for assessing acute aquatic toxicity using currently available techniques

The following proposed strategy is based on a tiered approach. It should be noted however, that this is a theoretical approach, which should be validated thoroughly before being applied for risk assessment purposes. The aim of this approach is where possible, to avoid using whole fish in aquatic toxicity assessment or, where there are no acceptable alternatives and fish testing is ultimately required, to significantly reduce the number of fish used. An explanation of the strategy is discussed and it is summarised in a flow diagram (see Figure 8).

3.7.1 Tier 1

The first tier relies on non-animal testing and incorporates the use of QSARs. The algal study referred to will always be the 72-h algal inhibition study (OECD 203) and the Daphnia study, the 48-h immobilisation study (OECD 202).

1. Are there valid QSARs available for algae, daphnids and fish?
    a. Consult the current regulatory systems for information on models and criteria on validity. Sources will include the ECB, OECD and the Cefic-LRI. If there are

valid QSARs for each of the three organisms, make predictions, and compare them to the available daphnia and algae effect concentrations, question 2. Note - if these are not available, they should be generated, unless the assessment process can be properly completed without this information.

b. If there are not valid QSARs for the substance being assessed, can the Verhaar scheme predict that the substance is likely to have a non-polar mode of action (type 1 inert chemicals). In such cases it is possible to predict the acute toxicity of the chemical reasonably accurately. Make predictions, and compare them to the available daphnia and algae effect concentrations, question 2.

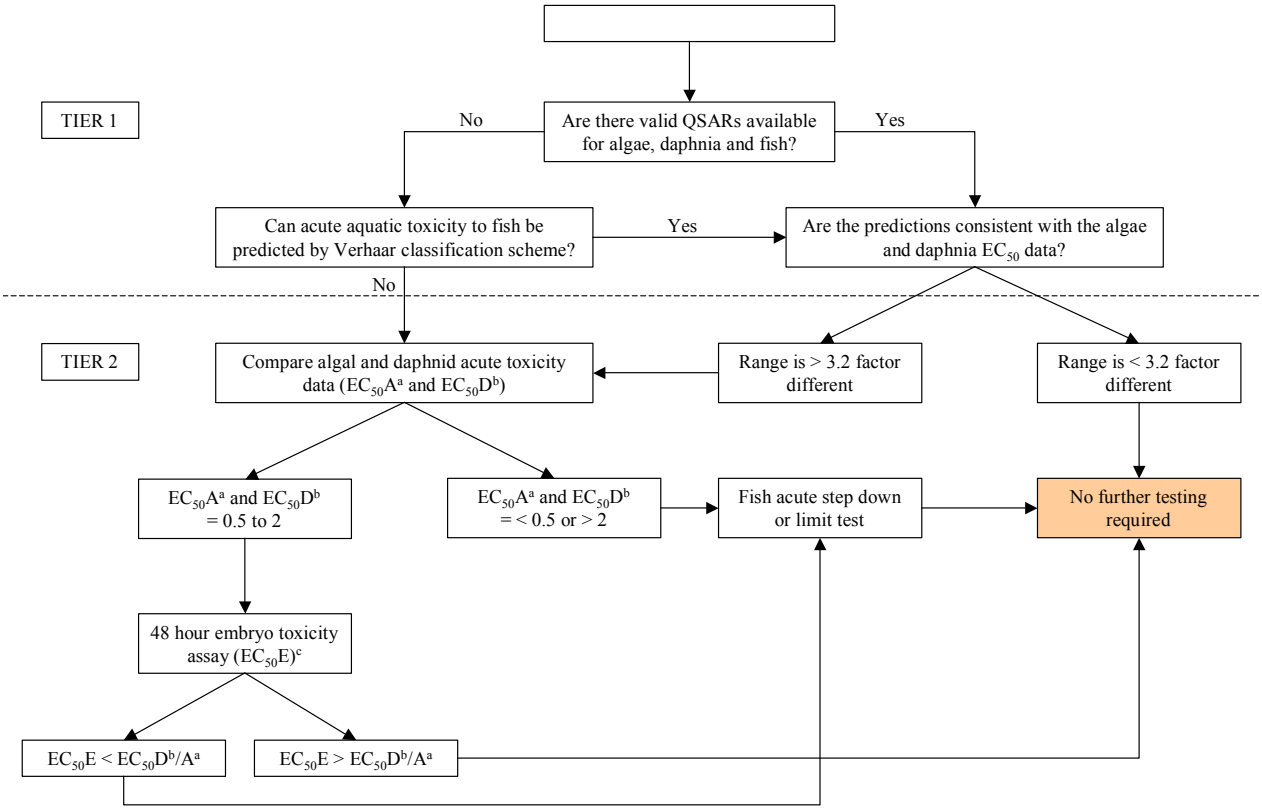
2. Are the predictions consistent with the corresponding non-vertebrate data (i.e. less than a factor of 3.2[a] different)? If so, no further testing will be required. The reason why this is satisfactory is that the chemical is a non-polar narcotic, with no specific toxicity towards the organisms assessed.

3.7.2 Tier 2

1. Compare the algal inhibition and daphnia toxicity data. If the ratio of the two EC_{50} values is < 0.5 or > 2.0 then, depending upon the data, conduct the fish acute step-down procedure or a limit test. The categories for the algae and daphnid ratios were adapted from the comparisons of data in the ECETOC EAT III database report (ECETOC, 2003b).
2. If the ratio of the two EC_{50} values is between 0.5 and 2.0 then it should be assumed that the sensitivity of the two assays is similar. In this case, a 48-h fish embryo toxicity assay (OECD, 1998) is recommended, since using fish embryos as a surrogate would give greater confidence in predicting an acute toxicity value for fish. This may change based on the recognition of other international protocols.
3. If either of the algal or daphnid EC_{50} values is more sensitive than the 48-h embryo toxicity assay, then the data from the most sensitive species will be used for the risk assessment process and no further testing will be required.
4. However, if the 48-h EC_{50} value for the embryo toxicity assay is more sensitive i.e. more sensitive by a factor of 2 compared to the most sensitive of the algal or daphnids EC_{50} values, then a fish acute step-down procedure or a limit test needs to be performed since it should be assumed that a specific mode of action might be occurring. There may be occasions where the embryo toxicity assay indicates toxicity at concentrations lower than those seen in the fish acute study. On such occasions a decision as to which endpoint should be used needs to be made on a case by case basis taking into account how the information will be used and the level of understanding of how embryos respond in these assays.

[a] The value of 3.2 is based upon a semi-logarithmic factor. Validation of this strategy will determine if this figure is over conservative.

Figure 8: Flow diagram representing the proposed strategy for assessing acute aquatic toxicity using currently available techniques (based on DIN, ISO or OECD guidelines)



[a] A: Algal test result
[b] D: Daphnia test result
[c] See Tier 2, point 2 (above)

3.8 Proposals for future research to improve the current acute aquatic toxicity strategy

The following proposals will improve the current strategy for predicting acute aquatic toxicity, however, a major consideration to these alternatives is the domain of applicability. For example, the areas of future research that have been recommended in this chapter will require appropriate validation with chemicals that have different physico-chemical properties before regulatory authorities will accept them as alternative test methods.

3.8.1 QSAR

Further research is required to confirm the Verhaar (Verhaar *et al*, 1992) approach beyond non-polar and polar narcosis and to improve the classification of substances by their mode of action.

This, together with investigation of available data sets to identify the mode of action of those chemicals for which fish were the most sensitive, will increase the potential of this approach.

3.8.2 Non-vertebrate organisms (algae, daphnids)

There is a need to determine if algae and invertebrates (various taxa) can act as a surrogate for whole fish. A number of studies have used these fish alternatives for various classes of chemicals (Mora et al, 2000; Lalah et al, 2003; Versteeg and Rawlings, 2003). Furthermore, the OSPAR guidelines for notification of offshore chemicals specifically highlights the acceptability of fish or invertebrate bioconcentration data (OSPAR, 2003; ASTM, 2002). It is recommended that comparison of algal, invertebrate and whole fish data from existing databases (e.g. EAT III database (ECETOC, 2003b); US EPA Duluth database (Russom *et al,* 1997)) should be investigated to confirm their compatibility as surrogates. It is also recommended that the step-down approach be further investigated alongside the comparisons of the existing databases.

3.8.3 Fish embryos

Further work to validate the use of fish embryos beyond whole effluent assessment on parent compounds and toxic metabolites is needed. In addition, as the majority of research on fish embryos has been conducted on zebrafish, validation will require careful assessment of toxicity on fish embryos of other OECD species, e.g. fathead minnow, medaka, etc. (recent work by Braunbeck *et al* (2005) has indicated there are limited differences; this needs confirming). Further research is also required to understand the differences in sensitivity across different embryonic life stages of different species.

3.8.4 Fish cell lines

Fish *in vitro* test systems need to be characterised better and protocols on the use of fish cells need to be standardised (Castaño *et al*, 2003). An optimised cell battery approach should be developed which can accurately predict effects *in vivo* (e.g. gill cells as a site of chemical uptake, hepatocytes for metabolic activation and an established cell line for basal cytotoxicity).

As noted previously, one of the problems with much of the work reported on fish cell lines is that they have used nominal concentrations, hence the correlation of *in vitro* to *in vivo* responses has been inconsistent. However, recent studies that have quantified bioavailable fractions or cell burdens have shown excellent correspondence with *in vivo* toxicity results (Bernhard and Dyer, 2005). Hence, if *in vitro* study results are based on nominal concentrations or total measured (i.e. not determining bioavailable fractions), then it may be expected that they show less sensitive and

or less predictable, responses. Research in this area is needed and for certain chemicals could include the use of SPME (see Section 3.5.4.2) for assessing their bioavailability.

3.8.5 Genomics

Investigations into genomic techniques are also necessary to identify mode of action. The application of genomics and proteomics may also identify more sensitive and/or specific biomarkers using diverse model systems, including fish cell lines and embryos. Some projects are already ongoing (e.g. GenDarT project) and these developments should be closely followed, especially with respect to OECD and ISO standard test organisms (Snape *et al*, 2004).

4. AQUATIC BIOCONCENTRATION

4.1 Background

4.1.1 Definition - the process

The accumulation process is generally considered as a partitioning between the water (or food) and the lipid phase of the organism. Due to accumulation, the internal concentration in an organism may reach a level causing toxic effects, even if the external concentration remains below detection limits. Consequently, the accumulation of chemicals in living organisms is of concern in environmental risk assessment. The uptake of contaminants into aquatic organisms occurs mostly by direct absorption from water, but can also be via food. The main processes of elimination (or depuration) of contaminants are diffusive transfer across gill surfaces and intestinal walls, biotransformation and excretion.

Accumulation of a chemical is the result of multiple physiological processes - absorption, distribution, metabolism, and excretion (ADME). These processes will be discussed later. Bioconcentration is defined as the net result of the uptake, distribution and elimination of substances in an organism due to water-borne exposure. The bioconcentration factor (BCF) is the ratio of the concentration in an aquatic organism (C_f) to the concentration in water (C_w), which is equivalent to the ratio of the uptake rate (k_u) and depuration rate (k_d):

$$BCF = k_u/k_d = C_f/C_w$$

This is further discussed in Section 4.2.

Bioaccumulation includes all routes of exposure (i.e. water, food, air, soil). Biomagnification may be defined as accumulation and transfer of chemicals via the food chain, resulting in an increase in the fat-adjusted internal concentration in organisms at succeeding levels in the trophic chain.

4.1.2 Data generation as part of chemical legislation

Chemical legislation, except in Japan, does not require experimental determination of bioconcentration at the basic tier, but relies on extrapolation of one of the substance's physico-chemical properties, the octanol water partition coefficient (log K_{ow}). This screening procedure assumes there is no substantial bioconcentration for compounds with a log $K_{ow} < 4$. The background for this assumption can be found in QSAR-models correlating BCF with log K_{ow} and is discussed in ECETOC (1995).

Log K_{ow} is adequate for predicting BCFs for non-polar, hydrophobic chemicals with log $K_{ow} < 6$. Above this value, non-linear relationships can be applied but in most of these cases a chemical-by-chemical evaluation is more appropriate (Nendza, 1991). Also, this log K_{ow} based QSAR approach is not reliable for all chemical classes, such as those that are poorly absorbed, e.g. organic colorants (ECETOC, 1998) or lipophilic chemicals that are biotransformed (de Wolf *et al*, 1992).

Since fish are typically secondary consumers or predators and therefore considered to represent a high trophic level, they are often the organisms of choice for assessing the bioconcentration potential of chemicals in aquatic organisms. In addition, standard methods have been developed with both fish (OECD, 1996b) and molluscs (ASTM, 1984) since they represent a potential route of exposure of chemicals to humans and wildlife predators. Furthermore, invertebrates can be used in specific situations as a representative species to assess the bioconcentration potential of chemicals, for example to assess contamination near a site of shellfish aquaculture, or native species habitat. However, the OECD fish bioconcentration test guidelines have been the most widely used experimental approach for regulatory applications.

4.1.3 Uptake/absorption

For less hydrophobic compounds (log $K_{ow} < 3$), passive diffusion of freely dissolved, bioavailable material through the cell membrane (i.e. the hydrophobic phase) is considered to be the rate limiting step for uptake. For more hydrophobic compounds diffusion is limited by the aqueous boundary layers between the fish membrane and the bulk water (Gobas and Mackay, 1987).

The uptake rate (k_u; ml/g wet fish/day) is relatively constant between log K_{ow} of 3 and 6 but varies as a function of fish weight (W; g wet fish) as follows (Sijm *et al*, 1995):

$$k_u = (520 \pm 40) \cdot W^{-0.32 \pm 0.03}$$

Where:

W = fish weight (g wet weight) at the end of uptake/start of depuration

For nonionic organic chemicals with log $K_{ow} > 6$, there is some evidence to suggest that the uptake rate may decline so the above equation would provide a conservative estimate for these chemicals.

Appendix D describes a number of rules of thumb that have been used to try to identify chemicals that will not bioaccumulate with respect to both molecular size and solubility in octanol. It is

clear from the evidence (see Appendix D) that while there may not be an absolute size cut-off, it is increasingly harder for larger molecules to diffuse through membranes than it is for smaller molecules. Recently, maximum cross-sectional diameter when applied with conformational flexibility has been shown to be effective in helping to model limited uptake of chemicals (Dimitrov *et al*, 2002a). This approach utilised within an expert system describes an experimentally derived mechanistic based model and shows promise for future development. In such an expert system, it will also help in defining the test programme of some chemicals to assess their potential to bioaccumulate.

Another good indication that bioconcentration will be limited is solubility in lipids and surrogates (octanol). This is also discussed in Appendix D where the suggested cut-off for solubility in octanol (mg/l lipid) is (0.002 × molecular weight) based on a body burden of approximately 0.001 mmol/kg wet weight, with an added precautionary factor of 10, to account for some of the uncertainty around assessing body burdens. Ionisable chemicals will need careful consideration to ensure the correct (un-ionised) proportion of the available chemical is addressed when considering bioconcentration behaviour.

An indication of uptake can be obtained from monitoring studies as presence of the chemical in biota indicates the potential for uptake. However, derivation of a BCF value from monitoring information is difficult because of uncertainty of the actual exposure concentrations and the lack of confirmation that steady state has been reached.

4.1.4 Depuration, elimination and excretion

An estimate of the rate of depuration k_d may be obtained from empirical relationships between k_d and log K_{ow}. For example, the following empirical relationship is provided in the OECD Test Guideline 305 (OECD, 1996b):

$$\log k_d = - 0.414 \cdot \log K_{ow} + 1.47$$

This relationship applies only to chemicals with log K_{ow} values between 2 and 6.5 (Hawker and Connell, 1988). An important elimination factor in bioconcentration is the possible biotransformation of substances (Sijm *et al*, 1997) which is ignored when estimating k_d via empirical relationships with K_{ow} (de Wolf *et al*, 1992). In such cases $k_d = k_e + k_m$ where, k_e represents excretion of the parent molecule and k_m elimination by biotransformation.

4.1.5 Biotransformation/metabolism

Metabolism describes the biological conversion of one chemical to another and is generally used in connection with biochemical reactions of carbohydrates, proteins, fats and other normal body constituents. Biotransformation more appropriately refers to the biological conversion of drugs and xenobiotic compounds and this meaning is used in this report. Biotransformation has been recognised in aquatic species since the late 1970s as a significant phenomenon which directly influences the fate, pharmacological and toxicological effects of xenobiotic chemicals, whereby active or inactive compounds can be converted to other inactive or active compounds (Lech and Vodicnik, 1985). Biotransformation reactions are often catalysed by enzymes present in the soluble, mitochondrial or microsomal fractions of the liver. Generally these enzymes have a low degree of substrate specificity, when compared to enzymes involved in metabolism of body constituents, and as such, tend to catalyse reactions after recognition of functional groups within a molecule. Enzymatic biotransformation reactions are divided into Phase I and Phase II reactions (see Tables 12 and 13). Products of Phase I reactions may subsequently undergo Phase II conjugation reactions. These are addition reactions where large chemical groups (e.g. sugars, amino acids) are covalently added to the xenobiotic compound and are directed towards reactions with polar functional groups (-COOH, -OH and -NH_2). The outcome of this is that the metabolic products are usually more water soluble, more rapidly excreted and less toxicologically active.

Table 12: Types of Phase I reactions

Reaction	Functional groups converted/site of action/location
Hydrolysis[a]	Esters, epoxides and amides cell-bound and extracellular.
Reduction	Halogenated organic chemicals, ketones, nitro and azo-compounds; generally occurs in the microsomal part of the liver.
Oxidation	Diverse functional groups: - by microsomal enzymes located in the membranes of smooth endoplasmic reticulum of cells[b], - by non-microsomal enzymes from mitochondrial and soluble fractions of tissues.

[a] Hydrolysis is a chemical reaction in which a molecule is split by reaction with water, often in the presence of a catalyst. Esterases belong to the group of hydrolases (carboxylester hydrolases; E.C. 3.1.1.1) that catalyse the formation or cleavage of ester bonds of water-soluble substrates. They can be extracellular or cell-bound. Epoxide hydrolase (EH) found in mammals is microsomal. In contrast to many other xenobiotic metabolising enzymes, epoxide hydrolases do not occur as a large family of isoenzymes. The majority of xenobiotic epoxides is hydrolysed by a single EH, the endoplasmic reticulum resident microsomal epoxide hydrolase (mEH). There is one additional EH in humans proficient for xenobiotic epoxide hydrolysis, the soluble epoxide hydrolase (sEH) that, in contrast to mEH, can take trans-substituted epoxides and is mainly responsible for the metabolism of fatty acid epoxides.

[b] These microsomal oxidations depend on the reducing cofactor (nicotinamide adenine dinucleotide phosphate, reduced form - NADPH) and require molecular oxygen; they may lead to increased toxicity.

Table 13: Types of Phase II reactions

Reaction	Functional groups converted
Glucuronic acid conjugation	Glucuronide transferase (microsomal enzymes in liver) catalyses interaction of UDPGA[a] with the functional group (- OH, -COOH, -NH_2).
Sulphate conjugation	Sulphotransferases (usually found in the soluble fractions of cells) catalyse the addition of PAPS to the substrate (-OH, -NH_2).
Amino acid conjugation	Addition of endogenous amino acids to aromatic or aliphatic carboxylic acids requires acetyl coenzyme A.
Acetylation	The amine (-NH_2) of the xenobiotic compound is acetylated (acetyl coenzyme A is the acyl donor).

[a] UDPGA: uridine diphosphoglucuronic acid
[b] PAPS: phosphoadenosyl phosphosulphate.

The main oxidative enzymes (monooxygenases) are comprised of a group of haemoproteins, called cytochrome P-450, because the reduced form of these haemoproteins combining with carbon monoxide have an absorption peak at 450 nm. The fact that the activity of these enzymes can be increased or decreased by specific drugs and xenobiotic chemicals has led to the use of these biochemical changes as biomarker of exposure to specific contaminants (See Section 3.5.3).

Biotransformation is strongly taxa- and species-specific (Sijm *et al*, 1997). This may be due to endogenous or exogenous factors. For instance, some species may be deficient in a specific biotransformation system, or species variations in the type of reaction may occur. In addition, there can be wide variations in the presence and activity of biotransforming enzymes due to inter-tissue variation, developmental stage or sex differences. Examples of exogenous factors are the temperature effect in poikilotherms or the nutritional status of the individual organism.

The following types of compounds can be distinguished:

- Compounds which are poorly biotransformed as a general rule;
- compounds that are poorly biotransformed by specific organisms/groups e.g. polynuclear aromatic hydrocarbon (PAH) in mussels;
- compounds which are easily biotransformed across phyla.

Negligible biotransformation higher up in the food chain implies a potential risk of biomagnification (Sijm *et al*, 1997).

4.2 In vivo bioconcentration test for BCF_{fish} measurement - OECD 305

For the experimental determination of bioconcentration factors (BCF) in fish a number of test guidelines have been documented; the most generally applied being OECD 305 (OECD, 1996b). There are two different methods to evaluate BCF. The first is to calculate it from the concentration of a chemical in fish (C_f; mg/g wet fish) divided by the concentration in water (C_w; mg/l) under steady-state conditions, (i.e. after equilibrium has been reached between fish and water for the substance). The second method uses kinetic data, i.e. uptake clearance (k_u; ml/g wet fish/day) and elimination rate constant (k_d; 1/day). This method assumes that fish can be mathematically represented as a homogenously mixed single compartment. Usually, first-order one-compartment kinetics have been found to adequately describe bioconcentration (Sijm, 1991; Kristensen and Tyle, 1991).

$$BCF = k_u/k_d = C_f/C_w$$

Experience from a ring test of the former OECD Test Guideline 305E between European laboratories showed that the variations in BCF estimates between the two methods was less than the inter-laboratory variation (Kristensen and Tyle, 1991). Agreement between kinetic and steady-state estimates of BCF for lipophilic substances can be further improved when a correction for C_w is made taking account of the bioavailable fraction in water (Schrap and Opperhuizen, 1990), thus correcting for sorption to suspended and dissolved organic materials.

Bioconcentration in aquatic organisms can be described with a simple first-order kinetic model:

$$C_f = C_w \cdot K \cdot (1-e^{-t\,kd})$$

where K is the equilibrium partition coefficient between fish and water (i.e. the BCF) and t = exposure time. In this model, k_u and k_d are independent of C_w and t, but dependent on the properties of the chemical being bioconcentrated.

OECD 305 (OECD, 1996b) is conducted in two phases: an exposure phase followed by a depuration phase. In the exposure phase, a sufficient number of fish is exposed to two sublethal concentrations of the test substance. During exposure both fish and water are sampled at regular time-intervals and the concentration of (parent) test substance measured. During the first phase the concentration of test substance in the water should be kept constant within narrow limits (± 20%). Hence the guideline recommends the use of a flow-through system. After having reached an apparent steady state (or after 28 d), the remaining fish are transferred to clean water and the depuration is followed. The BCF is expressed as a function of total wet weight of the fish and may also be expressed as a function of total lipid weight. Specific chemical analysis and radiotracer techniques may be used as analytical methods. If the latter technique is applied, a

specific chemical analysis (or a selective cleaning-up procedure) of the parent compound should be used at the end of the exposure period.

4.2.1 Number of fish used

OECD 305 (OECD, 1996b) requires three groups of fish, two exposure groups and a control group held under identical conditions. A minimum of four fish are sampled on at least five occasions during the uptake phase, and at least on four occasions during the elimination phase. (Table 14)

The guideline does not specify whether it is acceptable to reduce fish sampling in the control group, hence it has to be assumed that the sampling protocol for the control group is similar to that of the two exposure groups.

Table 14: Minimum number of fish sampled in an OECD 305 bioconcentration: flow-through test (OECD, 1996b)

	Uptake phase	Depuration phase	Total
Number of fish per sampling occasion	4	4	
Number of sampling occasions	5	4	
Subtotal	**20**	**16**	
Number of exposure and control groups	3	3	
Subtotal	**60**	**48**	**108**

4.2.2 Number of fish expected to be used under REACH for bioaccumulation

As discussed in Chapter 2 it is possible that many chemicals with a tonnage > 100 tpa will need to be tested for their potential to bioaccumulate.

X1 = number of chemicals > 100 tpa		= 5,500 (2,600 + 2,900)[a]
X2 = percentage with log K_{ow} > 2.7		= 55%[b]
Y1 = number of chemicals for BCF-testing	= X1 × X2	= 3,025
X3 = number of fish/test		= 108
Y2 = number of fish for bioconcentration testing	= Y1 × X3	= 326,700

[a] Pedersen *et al*, 2003

[b] Beek, 1991

4.3 Reduction in the use of fish to assess the bioconcentration potential of a substance

4.3.1 Reducing the number of test concentrations in OECD 305

Reducing the numbers of animals used to obtain bioconcentration information can be achieved by exposing less fish per replicate/concentration or by limiting the numbers of concentration exposures to the minimum that can be statistically justified. For example Table 15 shows that for several chemicals there was no significant difference between the low and high dose BCFs.

Table 15: Dual exposure BCF studies

Chemical	BCF values	
	High dose	Low dose
D1	1.3	0.6
D2	4.4	4.3
D3	4619	5297
D4	112	107
D5	115	120
D6	436	507
L1	562	539
L2	85	82
L3	6	11

4.3.2 Use of kinetic data in OECD 305 to reduce number of sampling points

The theoretical basis for the development of this test is that the kinetic parameters needed to estimate the BCF can be estimated from the slopes of the uptake and depuration curves. Unlike the standard OECD test, steady state does not need to be achieved. The OECD guideline calls for continuing the exposure via water until concentrations in the fish achieve a constant level and then measuring the depuration until > 95% of the material has been eliminated. The premise of the screening level assay design is that a kinetic model can accurately estimate the uptake and elimination without coming to steady state. This design results in the use of less fish because of the fewer number of sampling points required (Table 16).

This test is currently under development (Hinderleiter, 2004) and, as a screen, the benefits would include lower cost, faster execution, less waste, less chemical usage, and lower animal usage.

Table 16: Number of fish sampled in an abbreviated OECD 305 study

	Uptake phase	Depuration phase	Total
Number of fish per sampling occasion	4	4	
Number of sampling occasions	2	4	
Subtotal	**8**	**16**	
Number of exposure and control groups	2	2	
Subtotal	**16**	**32**	**48**

4.3.3 Elimination of depuration phase in OECD 305

Another approach to reducing the number of animals used in OECD 305 depends upon the purpose for which the test is being conducted. In some regulatory schemes all that it is necessary to know is whether the BCF is greater than or less than a particular trigger value. In such circumstances conducting a depuration phase may not be necessary.

4.3.4 Static exposure tests

A static exposure procedure designed by Banerjee *et al* (1984) allows for determination of uptake clearance and depuration rate constants during bioconcentration of stable substances. It requires the exposure of fish to an aqueous solution of the substance under static conditions, and measurement of loss of substance from the water as a function of time (Table 17). The rate constants are obtained from fitting the time-concentration profile to a simple mathematical model describing the exchange of substance between fish and water. The original approach assumes that no removal processes such as biotransformation, sorption or volatilisation are likely to occur.

This approach was adapted by de Wolf and Lieder (1998) to study volatile materials. This method required the exposure of fish to an aqueous solution of the substance in a fully closed system under static conditions, and measured loss of substance from the air as a function of time. The fish uptake and elimination rates were obtained from fitting the time-concentration profile to a model describing the partitioning of substance between air and water (assuming instantaneous partitioning and described by the substance's dimensionless Henry's constant) and exchange between water and fish.

A mathematical analysis of the robustness of these static exposure systems (de Wolf and Lieder, 1995) showed that reasonably accurate estimates of uptake and elimination rates are obtained when the substance concentration in fish is determined at the end of the exposure period, even in cases where (limited) loss occurs due to sorption and biotransformation. Research that compares

empirical data for metabolised substances from both static and flow-through experiments is required to fully assess the applicability of the static exposure method.

Table 17: Number of fish sampled in a static bioaccumulation study (Banerjee et al, 1984)

	Uptake phase	Depuration phase	Total
Number of fish per sampling occasion	10	0	
Number of sampling occasions	1	0	
Subtotal	**10**	**0**	
Number of exposure and control groups	2	0	
Subtotal	**20**	**0**	**20**

4.3.5 Dietary exposure

Dietary bioaccumulation tests are practically much easier to conduct for poorly water-soluble substances than the OECD 305. This is because a more constant and bioavailable exposure to the substance can be administered via the diet than via water. This test directly measures a biomagnification factor (BMF). The BAF is given by Fisk *et al* (1998):

$$BAF = C_{fish}/C_{diet} = E \cdot I/k_d = 1.44 \cdot E \cdot I \cdot T½$$

Where:

E	=	assimilation efficiency (g substance assimilated/g substance ingested)
I	=	ingestion rate (g food/g wet fish/day)
k_d	=	first-order elimination rate constant = 0.693/T½
C_{fish}	=	concentration in fish
C_{diet}	=	concentration in food
$T_{1/2}$	=	growth corrected half-life (days)

In a dietary bioaccumulation study fish are fed chemical-spiked food at a fixed concentration over a specified period of time (e.g. one to four weeks depending on the expected half-life (T½)) (Parkerton *et al*, 2001). At the end of this exposure period some fish are analysed for parent substance (time = 0 of the depuration phase). The remaining fish are transferred to clean diet and sequentially sampled and analysed over time so that a depuration curve can be established. Table 18 shows the number of fish used for such a study. From these data the half-life, dietary assimilation efficiency and BAF, defined as the steady-state ratio of the concentration in fish to that in the diet, can be readily derived. Some of the data may also be converted to a BCF value

for EU regulatory purposes. A conservative estimate of the uptake clearance k_u (see Section 4.1.3) may be divided by the depuration rate obtained in the dietary bioaccumulation study to give a reasonable overestimate of the BCF.

$$BCF = k_u \cdot T_{1/2}/0.693$$

Where:

$T_{1/2}$ = growth corrected half-life from bioaccumulation test (days)

Table 18: Number of fish sampled in a dietary bioaccumulation study (Parkerton et al, 2001)

	Uptake phase	Depuration phase	Total
Number of fish per sampling occasion	0	4	
Number of sampling occasions	0	5	
Subtotal	**0**	**20**	
Number of exposure and control groups	0	2	
Subtotal	**0**	**40**	**40**

4.4 Replacement of fish in the assessment of the bioconcentration potential of a substance

A replacement strategy can be achieved by considering information from other species, or related chemicals (read-across/chemical grouping), modelling, *in vitro* and embryos assays.

Chemical grouping/read-across, (Q)SAR, *in vitro* and other non-animal (e.g. algae or invertebrate) assays can be accepted as replacement methods as long as the models and the assays are validated for the endpoint and are fit for the regulatory purpose.

In environmental risk assessment, (Q)SARs for physico-chemical characteristics (log K_{ow} and toxicity) of certain classes of chemicals are accepted by regulators, provided that the chemical fits the applicability domain of the model (EC, 2003b; ECETOC, 2003a). Based on information provided by the Danish EPA, it has been estimated that 80% of chemicals can be covered using a combination of (Q)SARs, grouping of chemicals, and read-across techniques (Pedersen *et al,* 2003) for estimating accumulation in aquatic species. ECETOC (1995, 1998, 2003a) have extensively evaluated QSARs for predicting BCF and these reports should be read for further information.

4.4.1 Extrapolation across chemical groups

In cases where several members of a chemical class are known to have *in vivo* bioconcentration factors which are significantly lower than predicted based on log K_{ow}, and these observations are attributable to reduced absorption or significant biotransformation, then interpolation to non-tested members of the same chemical class seems warranted. Groups or categories can also be used, for example, when a definitive value is not required, but a value less than some pre-agreed limit. Such an approach, for example in the context of the EU-PBT strategy, could lead to significant reduction in the number of chemicals tested. This approach starts by identifying the chemicals within a group. This may be based on similar chemical structures or presumed bioconcentration behaviour. The purpose of the grouping would be to allow for key members of the group to be identified and tested. Interpolation within the group would then allow for BCF values to be assigned to the other group members via a 'local' QSAR.

4.4.2 QSAR

The first estimation that may be carried out is of a chemical's potential to partition between octanol and water. QSARs and experimental techniques for measuring this are described by ECETOC (1998, 2003a).

Chapter 4 of the TGD contains a section on the (Q)SARs available for bioconcentration (EC, 2003b). The model proposed by Veith *et al* (1979) is recommended for neutral chemicals that are not biotransformed and have a log K_{ow} of up to 6. For chemicals with log $K_{ow} > 6$, a parabolic equation, re-calculated from that described by Connell and Hawker (1988), is recommended. The models described are implicitly used in the EUSES system for assessment of secondary poisoning potential.

The linear (Q)SAR model described by Veith *et al* (1979):

$$\log BCF = -0.7 + 0.85 \cdot \log K_{ow}$$

n = 55; $r^2 = 0.86$; domain is mainly non-polar and polar aromatics plus some surfactants.

The original Connell and Hawker (1988) model was recalculated as a parabolic equation and presented in the TGD (EC, 2003b) for neutral substances that are not biotransformed and with log K_{ow} > 6:

$$\log BCF = -0.20 \cdot (\log K_{ow}) \cdot 2 - 2.74 \cdot (\log K_{ow}) - 4.72$$

n = 43; $r^2 = 0.78$

The model is based on experimental data (n=45) for several fish species from literature. It considers persistent chemicals, mainly chlorinated hydrocarbons with a log K_{ow} of 3.4 - 9.8.

Parkerton (2001) assessed the performance of the above methods using experimental data for 35 substances from diverse chemical classes. Many of the classes used are known or expected to be significantly biotransformed by fish and consequently BCF predictions based on this model were expected to be overly conservative. This was indeed demonstrated in the analysis.

Syracuse Research Corporation (SRC) reviewed all available BCF data in the literature and developed, in cooperation with the US-EPA, a computer program that allows the estimation of BCF-values for a wide range of chemicals. This program (BCFWIN, Meylan *et al*, 1999) estimates the BCF of an organic compound using the substance's log K_{ow}. It uses correction factors to account for the influence on bioaccumulation that certain structural and molecular characteristics have on uptake and processes such as biotransformation have on elimination. In BCFWIN, the data utilised included BCFs of chemicals that were metabolised by fish. It was reported that some of these factors could be rationalised on the basis that they were related to some degree of reactivity or known biotransformation behaviour. However, as the authors noted, this was an empirical basis and was not based on first principles.

(Q)SAR models should only be used for those chemicals that fall within the domain of the model and for which the descriptors are suitable. Surfactants are clear examples of organic materials outside the scope of (Q)SAR models that use log K_{ow}, as this is not an appropriate physico-chemical descriptor for such materials. Metals also fall outside most (Q)SAR-models because active uptake and sequestration can occur in biological systems. Finally, many models overestimate bioconcentration in cases where uptake is hindered or elimination via biotransformation is increased.

One approach that does try to address metabolism starting from first principles is that described by Dimitrov *et al* (2002a,b; 2004). In this model, BCF is first modelled as a maximum value, ignoring any mitigating factors and based only on log K_{ow} as an indicator of partitioning behaviour. Then the other factors are included, thus molecular size, maximum diameter of 1.5 nm (Dimitrov *et al*, 2002a) and metabolism (Dimitrov *et al*, 2004) are used to refine the predicted

BCF. While there is still much work to be done, this model does show considerable promise in being able to screen chemicals and reduce the number of studies necessary for addressing the potential of chemicals to bioconcentrate.

4.4.3 Biomimetic extraction

Biomimetic refers to man-made processes, substances, devices, or systems that imitate nature. Biomimetic extractions try to mimic the way organisms extract chemicals from water. Two types of biomimetic approaches will be discussed below:

- The first extraction system, described by Södergren (1987), is based on a semi-permeable membrane device (SPMD) composed of a dialysis bag filled with hexane.
- Arthur and Pawliszyn (1990) described another approach in which they constructed a solid phase micro extraction (SPME) technique using a thin polymer coating on a fused silica fibre.

Semi-permeable membrane devices

SPMDs usually consist of either a bag or a tube made of a permeable membrane within which there is an organic phase. Using these devices it has proved possible to extract a variety of organics from water (Södergren, 1987). Amongst other matrices, SPMDs have been used to assess effluents (Södergren, 1987), contaminated waters (Petty *et al*, 1998) and sediments (Booij *et al*, 1998) when the devices were used as animal replacements for assessing potentially bioaccumulative chemicals. SPMDs were then further developed based on low-density polyethylene bags that contain natural lipids or the model lipid triolein (1,2,3-tri(cis-9-octadecenoyl)glycerol). For a review see Huckins *et al* (1997).

SPMDs are relatively easy to use and will extract only freely dissolved chemicals from the water, thus they simulate the potential for aquatic organisms to bioconcentrate chemicals. The chemicals will be extracted from the water in proportion to their partitioning coefficients. However, as SPMDs are designed to mimic organisms with a high lipid content, they depend upon passive diffusion only, so the time to equilibration with the water samples can be very long. Booij *et al* (1998), for example, suggest that results from SPMDs exposed for less than 2 months should be treated with caution.

The main disadvantages of SPMD are that they are not able to simulate the ability of fish to actively transport or metabolise chemicals, thus the bioconcentration of chemicals can be over-estimated. Also as SPMDs are only capable of reaching equilibrium with freely dissolved

chemicals they are incapable of being used to address the potential for chemicals to be taken up via the gut.

Solid phase microextraction

SPME is another technique that can be used to extract only the freely dissolved (i.e. bioavailable) fraction of chemicals from water samples. When organic chemicals are extracted from a water sample with SPME fibres, the resulting concentrations in exposed fibres are proportional to the hydrophobicity of the compounds. This fibre accumulation is analogous to the bioconcentration of chemicals observed in aquatic organisms. The process is very fast, due to the high surface area to volume ratio and generally easy to set up and use (Arthur and Pawliszyn, 1990; Vaes *et al,* 1996, 1997).

Using larvae of the midge *Chironomus riparius* and disposable 15-µm poly(dimethylsiloxane) (PDMS) fibres, Leslie *et al* (2002) studied the accumulation and accumulation kinetics of a number of narcotic compounds with a range of log K_{ow} between 3 and 6. They observed that fibres, which have a larger surface area-to-volume ratio, had consistently higher uptake and elimination rate constants (k1 and k2, respectively) than midge larvae and accumulated the chemicals 5 times faster. Comparison of the relationships of the partition coefficients $K_{PDMS\text{-}water}$ and $K_{midge\text{-}water}$ (lipid-normalised) to log K_{ow} for all compounds yielded a factor of 28 for translating fibre concentrations to biota concentrations. Leslie *et al* (2002) suggested that this factor could be used to estimate internal concentrations in biota for compounds structurally similar to the compounds tested in this study.

4.4.4 *In vitro* assessment of ADME

In this section the use of *in vitro* techniques to investigate two principal mechanisms, absorption (transfer of chemicals across cell membranes) and metabolism, will be discussed.

4.4.4.1 Absorption assessment

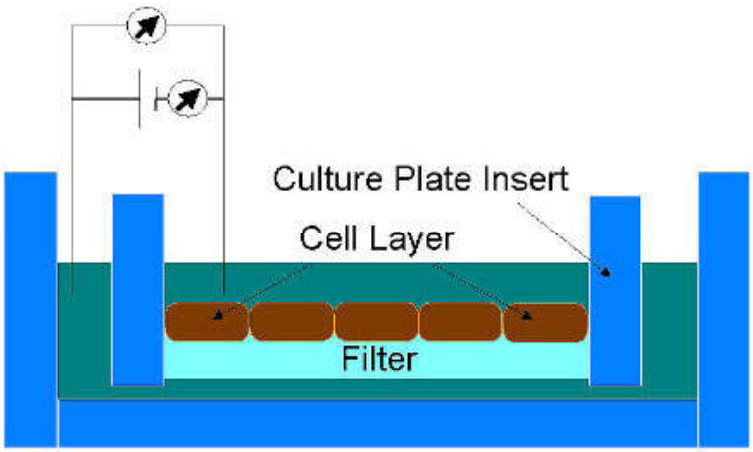
Cell culture models

Cell culture models offer many advantageous features for the analysis of chemical transport across membranes. From a basic research perspective, these systems offer the possibility for manipulating the environment or cellular properties as a means to address mechanistic questions. From a chemical uptake perspective, cell culture models can be used to expedite identification of

compounds with less favourable uptake properties, and to evaluate structure-absorption relationships.

Cultivation of epithelial cells on permeable microfilter membranes leads to a spatial separation of an upper and lower medium compartment (see Figure 9). This spatial separation results in an electrical separation of both compartments as well, which can be measured as the transepithelial electrical resistance (TEER). The epithelial cells can be seen as an electrical insulator. The TEER is an indicator for epithelial cell monolayer integrity. Epithelial cells are grown on permeable filter inserts (0.4µm). The electrical resistance (ohm) is determined with an ohmmeter. The TEER is calculated after subtraction of the control (culture plate insert without cell layer) and by multiplication with the area of the insert (ohm cm^2).

Figure 9: Method of measuring the transepithelial electrical resistance (TEER)



Wood and Pärt (1997) developed a method for the primary culture of gill epithelial cells from freshwater rainbow trout on permeable polyethylene terephthalate membranes ('filter inserts'). The cultured epithelium (approximately 8 µm thick) typically consisted of 2-4 overlapping cell layers organised as in the lamellae *in vivo*, with large intercellular spaces, multiple desmosomes and putative tight junctions. The TEER (approximately 3,500 ohm cm^2), permeability to a paracellular marker (polyethylene glycol-4000) and unidirectional flux of Na^+ and Cl^- all appeared realistic because they compared well with *in vivo* values. Cultured gill epithelia as models for the freshwater fish gill were recently reviewed by Wood *et al* (2002). They considered the cell preparation a valuable tool for characterising some of the mechanisms of active and passive ion transport in the freshwater fish gill. According to Wood *et al* (2002) an important future goal will be to develop techniques by which cultured epithelia can be stored and stockpiled for tests on demand. However, application in quantitative analysis of chemical transport across

membranes is currently limited since primary epithelial cells are used. This increases the possibility of high inter-experimental variation.

One model system that has proved useful in chemical absorption studies is the Caco-2 cell line (Hidalgo and Li, 1996). Caco-2 cells are human in origin, and can be manipulated in culture so that they exhibit many characteristics of the human small intestinal epithelium. Thus, these cells readily lend themselves to application as an *in vitro* model to study gastro-intestinal absorption in a human cell model. However, it should be noted that the cells originate from a human colo-rectal adenocarcinoma, and have been reported in some cases to retain some of the characteristics of colonic epithelium (Bailey *et al*, 1996). Caco-2 cells form monolayers of differentiated epithelial cells joined by intercellular tight junctions, which prevent the paracellular diffusion of solutes. Thus, this system provides a selective barrier for modelling structure transport relationships for both passive and carrier mediated transport. In addition, Caco-2 cells express apical efflux mechanism(s), which may play a role in restricting oral absorption. Some enzymes and transport systems are expressed to a lesser extent in Caco-2 cells compared to normal enterocytes. This difference may be due to the colonic origin of Caco-2 cells. Thus, when using the Caco-2 cell model for studying intestinal carriers, it is important to establish that transport proteins expressed by these cells are biochemically and/or functionally similar to the native transporter found in the small intestine (Hidalgo and Li, 1996). Caco-2 monolayers have been extensively used in the prediction of intestinal absorption *in vivo* (Bailey *et al*, 1996), and have been found specifically useful in identification of pharmaceuticals with potential absorption problems (Artursson *et al*, 1996).

The TEER for Caco-2 monolayers is approximately 600 ohm cm^2 (Dowty, 2004). Information on the TEER of fish intestinal monolayers is not yet available. The Caco-2 value is lower than that of fish gill epithelium (TEER of 3,500 ohm cm^2) (Wood and Pärt, 1997). A lower TEER indicates greater absorption potential. Use of Caco-2 monolayers for prediction of fish gill absorption *in vivo* will overestimate the potential gill absorption. Use of these cellular models can decrease the number of animals needed for bioconcentration studies by identifying those chemicals that have limited potential for uptake (see Section 4.5). An additional advantage of the cell culture model is that multiple studies can be performed with a relatively small amount of test chemical.

Gill perfusion model

The gills are the primary uptake site of dissolved chemicals in fish. Pärt (1990) developed a perfused gill preparation from rainbow trout (*Oncorhynchus mykiss*) as an alternative for studies *in vivo*. The perfused gill allows direct measurements of *in vivo* absorption rates of chemicals across the gill epithelium. Pärt *et al* (1992) concluded that the preparation is well suited to investigate the mechanisms behind the absorption of hydrophobic compounds across fish gills

and how the absorption rate is related to the physico-chemical properties of the chemicals, as well as to environmental factors.

Temperature dependence of uptake was studied for four hydrophobic and one less hydrophobic compound (Sijm *et al*, 1993a). The temperature dependent decrease was most pronounced for hexabromobenzene, probably due to steric effects. The rate-limiting step in uptake seemed to be diffusion, either through the aqueous diffusion layer or through the lipid membrane, and not ventilation or blood flow-rate. These latter factors do influence the uptake of hydrophobic chemicals to some extent (Sijm *et al*, 1994). Water flow will limit the uptake of hydrophobic chemicals for fish weighing > 5 g, but not in fish < 5 g, irrespective of physiological conditions and oxygen concentration. Low oxygen concentrations increase the water flow in large fish, and the uptake of hydrophobic chemicals may increase by a factor of 5 or more. Increasing the blood flow may maximally increase the uptake of hydrophobic chemicals two-fold, in small as well as large fish.

Uptake rate constants of different classes of hydrophobic organic chemicals determined in isolated perfused gills of rainbow trout (*O. mykiss*) are higher than those determined in guppy (*Poecilia reticulata) in vivo* (Sijm *et al*, 1995). Both systems show relatively high variation, however this can be significantly reduced and the uptake rate constants determined once they are normalised with a reference chemical. Subsequent extrapolation to fish of different sizes can be through use of allometric relationships (Sijm *et al*, 1995, Sijm and Hermens, 2000; Hendriks *et al*, 2001; Hendriks and Heikens, 2001).

4.4.4.2 Biotransformation and elimination assessment

In vitro tests can provide information on the types and relative abundance of possibile metabolites (see Appendix C for further details of such tests). Such biotransformation data could be useful for input into fish-specific mass balance and physiologically based pharmacokinetic (PBPK) modelling efforts. Several types of studies are available that assess the influence of biotransformation on the BCF in fish:

- Measuring the decrease of parent compound (mass balance approach). See for example, the studies of Opperhuizen (1986) on chlorinated naphthalenes;
- estimation of the *in vivo* biotransformation rates from *in vitro* assays;
- comparison of total elimination of biotransformable and non-biotransformable chemicals with a similar K_{ow} (de Wolf *et al*, 1993a).

In fish, chemicals are normally converted to more polar (more easily excreted) metabolites through the process of biotransformation (see Section 4.1.5). De Wolf *et al* (1992) demonstrated a

significant reduction in the bioconcentration of chlorinated anilines, which they attributed to biotransformation. Fish have the capabilities both for oxidative metabolism by the cytochrome P450 monooxygenase system (Phase I reactions) and for conjugation reactions (Phase II). Biotransformation activity has been measured in liver, intestine, gill, kidney and brain (Lindström-Seppä *et al*, 1981; Miller *et al,* 1989; Van Veld *et al,* 1990; Hegelund and Celander, 2003). Since biotransformation processes take place primarily in the liver, this is the organ of choice to study the metabolism of chemicals. In some circumstances other organs can play a very important role, for example Barron *et al* (1989) measured biotransformation in gill homogenates and on isolated perfused gill arches. Their conclusion was that the pre-systemic (gills) biotransformation processes were responsible for the very low bioconcentration of di-ethyl hexyl phthalate (approximately 50 times less than predicted by log K_{ow} based QSAR). Given the limited activity observed in other organs they will not be further considered in this report.

In vitro estimation of biotransformation potential

Biotransformation potential of fish has been investigated in liver slices (Schmieder *et al*, 2000), whole liver homogenates (de Wolf *et al*, 1993b), liver subfractions (Kolanczyk *et al*, 1999; Dyer *et al*, 2003; Perdu-Durand *et al,* 2004), isolated hepatocytes and cell lines (Cravedi *et al*, 2001; Segner, 1998; Segner and Cravedi, 2001; Dyer and Bernhard, 2004). Biotransformation of chemicals through enzymatic reactions can be monitored either by an increase in the activity of enzymes involved, by the decrease in the amount of substrate (parent compound), or by an increase in products.

The rate of parent disappearance in subcellular systems can be derived as well as a BCF_{cell} in hepatocytes. The xenobiotic metabolite pattern produced by fish hepatocytes *in vitro* is generally similar to that observed *in vivo* (Segner and Cravedi, 2001). For hydrophobic chemicals, the faster the rate of parent biotransformation the less likely it is that the chemical will bioaccumulate. However, because of the lack of data there are no generally accepted approaches to use the rates to alter BCFs calculated using models. This is an area for further research (see Section 4.6).

Dyer *et al* (2003) applied an approach to derive a BCF_{cell} for various surfactants. Primary hepatocytes, derived from the liver of common carp and cultured hepatocytes (PLHC-1 cells), were exposed to radiolabelled test compounds. The rates of uptake and loss of the test chemical from the cellular systems were measured using liquid scintillation and thin layer chromatography. The rates were estimated assuming first order kinetics and the BCF in the cells determined by a ratio of uptake rate to the rate of loss. The primary hepatocytes appeared to metabolise linear alkylbenzene sulphonate (LAS) more completely than the PLHC-1 cells. For LAS, the calculated BCF_{cell} was approximately 4-fold less than the BCF_{fish} measured by Tolls *et al*, (1997) in fathead

minnow. For the linear alcohol ethoxylate ($C_{13}EO_8$), the corresponding BCF_{cell} was 2 to 30-fold less, compared to the fathead minnow results generated by Tolls *et al,* 2000.

An advantage of *in vitro* methodologies for assessing biotransformation is that they are rapid and less expensive than *in vivo* tests. A compromise between conducting *in vivo* BCF testing and exposing liver systems would be to measure the same parameters in livers extracted from exposed fish. This may allow for a reduction in the number of fish used in assessing bioconcentration of a chemical; however this has not yet been investigated.

Estimation of biotransformation potential in fish embryos

The capacity of fish embryos to metabolise chemicals and to respond to known inducers of enzymes has been reported in the literature (see Table 19).

These enzymatic activities increase slowly during development and show elevations corresponding to increased stress conditions. In particular, ethoxyresorufin-O-deethylase (EROD) activity seems to significantly increase around hatching (Monod *et al*, 1996).

With regard to their metabolising capacities, fish embryonic stages could be studied for their potential capacities to predict chemical biotransformation in adult fish (Görge and Nagel, 1990). However metabolic capacities may sometimes be limited: for example, in zebrafish embryos exposed to phenol, the metabolite phenyl sulphate was detected after 24 hours and only after five days of exposure were all the other phenolic metabolites, associated with adult zebrafish, detected. This has implications for toxic metabolites that may not be synthesised within a 48-hour assay (Lange *et al,* 1995).

4.4.5 Use of invertebrates

In general, the use of invertebrates to assess bioconcentration potential of chemicals in fish is not recommended since the physiological processes that govern bioconcentration in invertebrates differ substantially from those in vertebrates. There is different compartmentalisation in the body and their biotransformation systems are less developed. However, if there were only a need to demonstrate that the BCF in fish were below a certain value, e.g. < 500 (for GHS) or 2000, in the EU PBT assessment, then it may be possible to utilise BCFs from invertebrates as a precautionary BCF. Thus if the substance had an invertebrate BCF below one of these regulatory triggers, there would be less reasonable justification to recommend a fish test. For risk assessment the BCF derived from an invertebrate could also be used as a maximal value, and if the risk assessment indicated no concern then again the use of fish to derive a fish BCF would be difficult to justify.

Table 19: Biotransformation enzyme induction in fish early life stages

Species and life stage	Test substance	Enzyme inducible	Reference
Zebrafish (*Danio rerio*), from embryonic to adult stage	These enzymatic activities increase slowly during development and show elevations corresponding to increased stress conditions.	P450 monooxygenases and glutathione S transferases activities.	Wiegand *et al*, 2000a,b,c
Rainbow trout *(Oncorhynchus mykiss)* eleutheroembryonic stage	Contaminated sediments.	Aryl hydrocarbon hydroxylase (AHH) Ethoxyresorufin-O-deethylase (EROD), aminopyrine-N-demethylase (APDM) and UDP glucuronyl transferase (UDPGT).	Vigano *et al*, 1995
Teleost fish species including killifish *(Fundulus heteroclitus)* embryos and salmonids brook trout (*Salvelinus fontinalis*) embryos	Benzo(a)pyrene (BaP) and polychlorinated biphenyls (PCBs).	Microsomal aryl hydrocarbon hydroxylase activity (AHH), aminopyrine N-demethylase-(APD) activity to increase to a lesser extent.	Binder and Stegeman, 1980, 1983, Binder *et al*, 1985; Stegeman *et al*, 1984
Rainbow trout (*Oncorhynchus mykiss*) embryo	PCB-77.	EROD (CYP1A). Glutathione S-transferase (GST).	Koponen *et al*, 1998, 2000
Turbot (*Scophthalmus maximus*) in larvae and juvenile stages	BaP and lindane.	EROD (CYP1A).	Peters and Livingstone, 1995
Rainbow trout embryos and microinjection in the yolk-sac prior to hatching	PCBs, polychlorinated naphthalenes (PCNs), polychlorinated paraffins (PCPs) and polybrominated diphenyl ethers (PBDEs).	EROD (CYP1A).	Norrgren *et al*, 1993
Rainbow trout (*Oncorhynchus mykiss*) embryos	Pregnenolone	EROD (CYP1A).	Petkam *et al*, 2003
Teleostean fishes, from embryonic to adult stage		EROD (CYP1A).	Sarasquete and Segner, 2000
Fathead minnow (*Oryzias latipes)* embryo	Tetrachlorobiphenyl (TCB)	EROD (CYP1A).	Lindström-Seppä *et al*, 1994
Zebrafish (*Danio rerio)* embryo	17β -estradiol (E2), diethylstilbestrol (DES) and bisphenol A (BPA).	Cytochrome P450 aromatase.	Kishida *et al*, 2001

4.5 Bioconcentration testing strategy

4.5.1 Introduction

The proposed bioconcentration testing strategy is shown in Figure 10. It makes use of approaches that lead to replacement and reduction in fish use as compared to the OECD 305 bioconcentration test guideline. These approaches deal with absorption, distribution, metabolism and elimination aspects of the bioconcentration process (ADME). Refinement/reduction methods are mentioned in Section 4.6, as part of the future research needs.

The testing strategy is a tiered process; Tier 1 using estimation models and Tier 2 using non-animal experimental systems. Depending upon the quality of the prediction, these tiers can lead to a replacement of animals used for assessing bioconcentration within environmental assessment. Tier 3 makes use of experimental systems but with a reduced number of animals. The full BCF test, OECD 305, is Tier 4 (the final test in the strategy). Validation of alternative approaches from Tiers 1, 2 and 3 should include a comparison of performance against results for the Tier 4 test.

4.5.2 Strategy - suitable for purpose

Central to the strategy is the question 'Is BCF suitable for purpose'. The reason for this question is to ensure that the BCF being generated is either sufficiently accurate that an assessment of indirect exposure can be conducted or that regulatory decisions can be made with sufficient confidence. Clearly the closer an estimation is to a boundary, either a specific criteria (as in classification, BCF < or > 100 or 500) or the PEC/PNEC ≥ 1, the more confidence is needed that the BCF is a reasonable estimate. In making this judgement the discussions in this section, and the variability that occurs even with OECD 305, should be considered.

4.5.2.1 Tier 1

1. The first part of the assessment addresses whether the substance has a potential for bioconcentration and utilises the approaches outlined in Appendix D. If the substance is unlikely to bioconcentrate, a surrogate or null BCF is estimated for use in an assessment. The assessor then moves to the central question re suitability of the estimate.
2. If absorption does not seem to be restricted and biotransformation seems unlikely, then the second question asked is whether log K_{ow} is an appropriate model or surrogate for describing the water-fish distribution process. For example in the case of metals and surfactants log K_{ow} is not an appropriate model and one should immediately move to point 3 below. If log K_{ow} is suitable, a measure of the octanol-water partition coefficient needs to be obtained. This can be done by model estimation (ECETOC, 2003a) or measurement methods (ECETOC, 1998).

a. To determine whether the chemical has potential to pass through biological membranes or to be absorbed, see Appendix D.
b. To determine whether the chemical has significant potential to be biotransformed, literature searches of similar substances or estimates generated by metabolism models are suggested.
c. Next is to evaluate whether there is an applicable (Q)SAR that includes the chemical in its domain. If yes, the log K_{ow} value can be used as input into the (Q)SAR to estimate the fish bioconcentration factor (see Section 4.4.2).

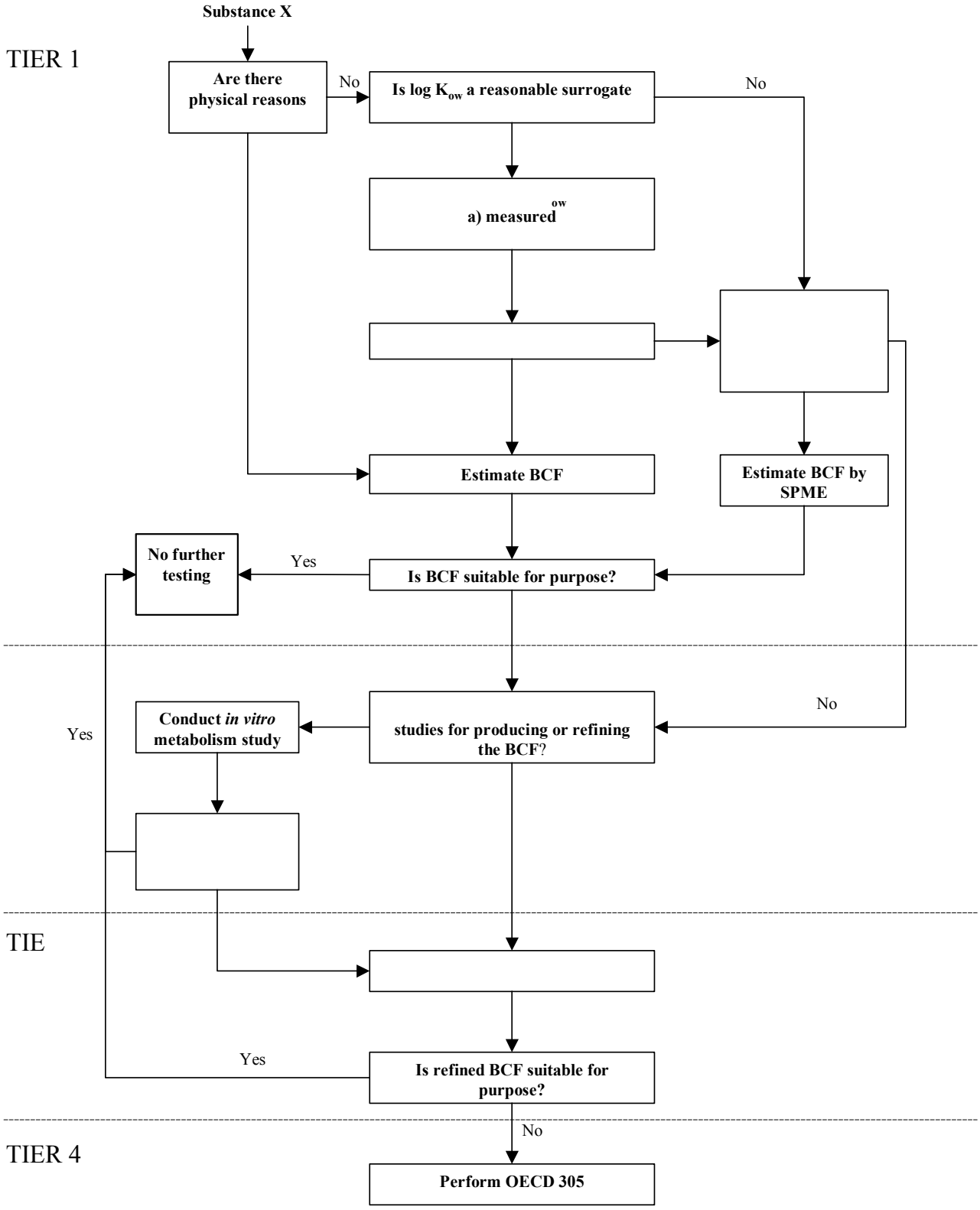
3. If log K_{ow} is not a suitable surrogate, then other approaches (e.g. SPME) can be addressed and used at this stage, if appropriate. Other options include SPMD and dialysis bags, a biotic measurement system i.e. a surrogate species not defined as a protected animal, such as mussels (discussed in Sections 4.4.3 and 4.4.5). From this measurement an estimation of a fish bioconcentration factor is obtained. The confidence in the information is again addressed via the central question. If there are no reasonable models (including SPME, etc.) move to Tier 2.

4.5.2.2 Tier 2

When a BCF has been estimated but there is significant uncertainty or insufficient precision for the assessment being conducted, then go to point 4 below. If there are no reasons for restricted uptake, no good surrogates for partitioning behaviour, then the potential for *in vitro* BCF tests should be assessed (see section 4.6.3). If this is not possible, go to Tier 3.

4. The assessment at this point addresses to what extent metabolism would impact the elimination of the substance from fish and thus reduce an estimated BCF. If absorption is not limiting and no account is taken for metabolism, K_{ow} and other approaches will predict a maximum BCF value. This can be approached by asking whether metabolism occurs in other species with potential similarity in metabolism pattern, or whether other, structurally related substances are known to be metabolised? If so, a metabolism measure could be obtained either through the use of model estimations or *in vitro* measurements (see Section 4.4.4.2). In this way a refined BCF is obtained and the suitability of the new value assessed.

Figure 10: Strategy for assessing bioconcentration



4.5.2.3 Tier 3

Testing is required when there are no reasons for restricted uptake and no appropraite surrogates for partitioning behaviour. It is suggested that a fish BCF is estimated using screening *in vivo* tests (see Section 4.3) before moving to a BCF measurement using the OECD 305 testing guideline (Tier 4 below). If the estimate from the screening assays is suitable for purpose, then exit the bioconcentration testing strategy. If not, the OECD 305 test will need to be performed before the testing strategy can be exited.

4.5.2.4 Tier 4

Conduct the OECD 305 study.

4.6 Future research

The following section identifies research needs, which are presented following examination of the decision-tree proposed in Section 4.5 as a possible bioconcentration testing strategy (Figure 10). These research needs were discussed at an ECETOC Workshop (ECETOC, 2004a) and presented at SETAC (Comber *et al*, 2004). The use of relevant existing information on biotransformation can be considered as an alternative and research into this area should be implemented. Reduction measures, while still making use of a limited number of fish, can already be applied or may need rapid development for short-term application. In the longer term, research programmes will be needed to enable the replacement tests to be implemented.

4.6.1 Immediate use of relevant information - reduction

The use of available biodegradation data and metabolism/biotransformation data from mammalian studies should be considered before conducting any fish bioconcentration test. In order to ensure that extrapolation can be done, a literature study should capture differences and similarities between different species and across the chemical class. Bacteria, invertebrates and vertebrates are capable of chemical biotransformation, but to various extents, and may incorporate different metabolic pathways. The knowledge of biotransformation patterns and extent in diverse phyla may help understand bioconcentration processes in fish.

4.6.2 Standard *in vivo* bioconcentration test and other *in vivo* experiments - reduction

The domain of application of the standard *in vivo* bioconcentration test OECD 305 should be identified. The uncertainties in the measurements obtained after conducting a standard *in vivo* bioconcentration test should be better assessed. Without this the successful validation of alternative methods to the fish bioconcentration test would be compromised. The use of only one concentration or limited uptake/depuration phases should be evaluated and implemented for relevant chemical classes.

Other *in vivo* experimental approaches, e.g. the dietary BMF protocol and abbreviated OECD 305 need to be further investigated to define their limits of applicability and eventually extend their domain. Further, the assumptions regarding rates of uptake needs confirmation and their limitations need to be understood. The use of a correction factor accounting for bioavailability needs investigation.

4.6.3 *In vitro* assays, expert systems and models - replacement

To better address the value of *in vitro* assays, and their suitability for amending predicted BCFs, additional research is needed to identify their variability and confidence limits. Research into the use of decision theory methods may also help by allowing for a better assessment of the uncertainty inherent in these techniques and with extrapolation across species.

Technical issues need to be addressed in order to better understand the use of *in vitro* methods. For the purpose of standardising protocols, recommended procedures for the isolation of fish cells, culture and exposure should be agreed and should be in compliance with the Good Cell Culture Practices. It is expected that ECVAM will publish protocols addressing these issues in 2005.

The use of suspension incubations as opposed to plated cells should be considered. The cryopreservation potential of isolated hepatocytes should be investigated so that the procedures could be made less labour intensive.

The development of *in vitro* assays, expert systems and models capable of incorporating ADME concepts should receive priority.

- Absorption: the parameters governing physical restriction of cellular absorption of chemicals should be better described and the assumed constant rate of uptake, up to log K_{ow} 6, needs to be further investigated. Furthermore, the applicability of using *in vitro* systems to assess absorption should be studied. The first step could be to evaluate whether the

mammalian intestinal cells (Caco-2 cells) are representative of fish for understanding gill absorption, uptake from food and for deriving assimilation factors. Future research is needed to further assess the impact of gill biotransformation in the absorption process. In addition, generation of information that provides more insight into the validity of extrapolation from existing approaches to fish and/or the development of fish specific absorption models is required.

- Distribution and partitioning: the applicability domain of (Q)SARs for log K_{ow}/BCF predictions should be better defined. Research into the conditions of use of SPMD/SPME, within the context of the strategy outlined above, should be performed. Their limitations and potential, for assessing poorly metabolisable chemicals and in WEA/environmental monitoring, should be explored.
- Biotransformation: the existing (Q)SARs that address biotransformation in fish need to be improved or further developed. The available *in vitro* biotransformation assays with sub/cellular fish liver systems to address metabolism should be further investigated. In order to allow the use of relevant information, the level of biotransformation potential in the different *in vitro* systems, using different fish species or classes of organisms, should be compared. The level of biotransformation potential *in vitro* should be compared to the level of biotransformation *in vivo*.

There are a number of issues in relation to the extrapolation from *in vitro* to *in vivo* for deriving a BCF. Ultimately it should be possible to relate, for example, the level of parent disappearance in microsomes with a factor that would refine the estimated BCF_{fish}, or a BCF_{cell} to BCF_{fish}. It is not yet obvious how absorption and metabolism in mammals relate to absorption and metabolism in fish. Another inherent difficulty of *in vitro* studies is the relation between responses in cellular fractions and single cells to responses/effects in whole tissues and whole organisms. This is true for toxicological responses as well as for biotransformation processes. The acceptability of *in vitro* data could be enhanced provided that parallel studies are conducted *in vivo*, for example by comparing the level of enzymatic activity in the livers of exposed fish to that in exposed liver cells. This could also be used as a refinement and reduction of the number of fish used to assess fish bioconcentration.

Measurement of changes in the genes expressing metabolic enzymes in exposed fish or specific fish cells could also be developed to assess the potential for biotransformation. Metabonomics, which is the study of low molecular weight metabolites in an organism, offers a way for tracing these changes even though they would not necessarily be reflected in gene expression profiles.

5. ENVIRONMENTAL RISK ASSESSMENT OF SEDIMENTS

5.1 Introduction

Environmental risk assessments for single substances are generally undertaken by identifying or extrapolating from the ecotoxicity data, to give concentrations at which no effects are expected and comparing this level with the estimated exposure level as recommended by the EU TGD (EC, 2003b). Although data on effects of chemicals in sediments is limited, the TGD approach allows prediction of effects of contaminants present in sediments to be extrapolated from existing aquatic toxicity data.

Using the TGD approach for the risk assessment of sediments is only appropriate if the ecotoxicological properties and environmental fate of all relevant substances are known. However, contaminants do not generally exist in isolation and not all toxic components may be identified by routine chemical analysis. Consequently, trying to predict the effects of sediments with a complex mixture of contaminants is fraught with difficulties. Because of these limitations a number of studies using fish and other vertebrates have been and continue to be undertaken to assess the hazard and risks posed by contaminated sediments.

A review of the literature indicates that fish are used to study the effects of chemicals in sediments as well as in the aquatic phase. Although there are a few specific studies to look at the hazardous effects of chemicals which may, as a consequence of their use, end up in sediments most studies are undertaken as part of environmental risk assessments to identify potential and/or actual effects of sediment bound contaminants. Sediments are often studied in environmental risk assessments because these may constitute an important repository for anthropogenic chemicals from past and present activities. Fish have been used in these assessments because they are ecologically important, often constitute a food resource for man and, as higher-level consumers, can be adversely affected by contaminants in water, sediments and other organisms. Although chemical analytical techniques can give an indication of sediment contamination, biological effects measures (e.g. toxicity) are considered to be crucial to the assessment of sediment quality as current methods for analysis and quantification of contaminant levels alone are insufficient to assess potential hazards to aquatic organisms. This is because analytical methods do not address the interaction of bioavailability and toxicity, or how this is influenced by sorptive behaviour or the sediment characteristics.

The types of sediment studies range from assessment of direct toxic effects by contact with sediments and/or via chemicals released from sediments into the overlying water (Schuytema *et al*, 1988, Vigano *et al*, 2001 and Tetreault *et al*, 2003), or indirect effects via where fish are provided a diet of benthic fauna containing toxic bioaccumulative chemicals (Mondon *et al*, 2001). This chapter provides a brief outline of the approaches where fish have been used to

improve the risk assessment of sediments by establishing the hazard profile, together with some recommendations for the potential to use alternative methods in line with the principle of the 3Rs.

5.2 Methods used to study effects of sediments on fish

5.2.1 Overview of methods

With respect to providing data for hazard assessment there are a few examples of product specific research where laboratory and/or mesocosm studies are undertaken with fish exposed to sediment to which a test chemical or waste has been added. Examples of these include studies to assess the effects of oil (Moles and Norcross, 1998) and wood preservatives like creosote (Sved *et al*, 1997). Other studies have been conducted to assess the effect of sediments on the toxicity of specific chemicals such as pesticides to support product registration and/or provide specific data to improve the environmental risk assessment to support the continued use of an individual substance. For example, Maund *et al* (1998) conducted studies to show that adsorption of the pyrethroid insecticide lambda-cyhalothrin to sediments reduces exposure and hence the apparent toxicity of the compound to fish with no adverse effects on fish seen under field conditions. The majority of the research where fish have been used to assess sediments has been conducted either:

- in the field to establish whether natural fish stocks are showing adverse effects where contaminants are present, or
- in the laboratory to
    - link to effects observed in natural populations or
    - predict potential effects in natural populations.

A variety of ecotoxicological effects are assessed including fish physiology, histology and mortality. Other measures may include biomarkers. In addition to ecotoxicological effects, fish are used to assess the influence of sediments on bioaccumulation of chemicals. For example, studies have been undertaken by Huckins *et al* (1991), Maund *et al* (1998) and McCarthy *et al* (2003) to assess how sediments affect the bioavailability of specific chemicals.

It should be recognised that the vast majority of sediment toxicity studies use invertebrate species. However, to address ecological and human health concerns fish will continue to be used because these may represent an important pathway to higher organisms such as birds and mammals including man.

5.2.2 Assessment of wild fish populations

As previously mentioned a number of studies are undertaken to try to establish links between contaminant related effects seen in the wild to specific contaminated sediment locations. Examples of these studies have been summarised in Table 20.

Biomarkers

Many of the monitoring studies use a range of biomarkers. There is debate about the role of biomarkers as to whether these are measures of effects or just exposure. They are, however, increasingly being seen as an important component of environmental monitoring surveys. Ultimately, the value of a biomarker depends upon a number of factors but they have proved their relevance in certain areas. For example, vitellogenin (a biomarker) has been used to successfully track and identify sources of fish endocrine disruptors in the UK's programme to study endocrine disruption in the marine environment (EDMAR) as summarised by Matthiessen *et al* (2001). The authors claimed that the relevance of the vitellogenin biomarker in male flounder (*Platichthys flesus*) was enhanced by the fact that induction of intersex (ovotestis) was found in 15-20% of males.

Field observations of effects

In other studies, links between contaminants in sediments and adverse biological effects have been found. For example, the work by Johnson *et al* (1998) indicates that fish from locations where sediments contained elevated concentrations of PCBs produced eggs with significantly reduced weight. I addition the overall spawning success of fish from the site with the highest level of aromatic hydrocarbons was significantly impaired. Their observations support the findings of Nagler and Cyr (1997) who observed that the hatching success of progeny of fish (*Hippoglossoides platessoides*) was impaired when they were exposed in laboratory tanks to sediments contaminated with PAHs and PCBs.

Table 20: Examples of studies in which fish have been used to assess sediments

Type of investigation	Fish used and description of study	Comments	Reference
Assessment of oil sands contaminated sediments.	Laboratory studies to assess whether oil sands contaminated sediments could induce biochemical responses (increases in 7-ethoxyresorufin-O-deethylase (EROD) activity and decreases in *in vitro* steroid production capacity) in slimy sculpin (*Cottus cognatus*). Objective was to assess whether effects seen in the laboratory tests could be linked to effects seen in fish caught in the Athabasca Oil Sands area.	EROD activity in laboratory exposed fish was comparable to that in fish native to the oil sands area. The study did not demonstrate a reduced ability of gonadal tissue of exposed fish to produce steroid hormones *in vitro*, as previously demonstrated in wild fish assessments. The laboratory bioassay was not a suitable surrogate for field studies although it appeared to have potential to identify MFO-inducing compounds in sediment using a Toxicity Identification Evaluation approach.	Tetreault *et al* (2003)
A two-part study to assess impact of aromatic hydrocarbons (AH) and polychlorinated biphenyl (PCB) contaminated sediments on ovarian development and spawning success in bottom dwelling fish.	In part 1 sexually mature rock sole *Pleuronectes bilineatus* were collected from four locations in Puget Sound, Washington during two spawning seasons. Fish were sacrificed for tissue concentration analysis for AHs and PCBs, to provide blood samples for vitellogenin analysis and histology (ovarian development).	Egg weight was negatively correlated with levels of PCBs in the liver although contaminant exposure levels were not significantly associated with gonadosomatic index, plasma estradiol concentrations, probability of entering vitellogenesis or fecundity.	Johnson *et al* (1998)
	In part 2 gravid female soles, collected from the same locations, were hormonally induced to spawn in the laboratory. Eggs were fertilised with pooled sperm from males taken from clean reference sites and the condition of eggs and overall spawning success monitored.	Fish from locations where sediments contained elevated concentrations of PCBs, produced eggs with significantly reduced weight and spawning success of fish from the site with the highest level of AH was significantly impaired.	

Table 20: Examples of studies in which fish have been used to assess sediments (cont'd)

Type of investigation	Fish used and description of study	Comments	Reference
Field studies investigating biomarker responses and sediment contamination	Similar to the studies undertaken by Johnson *et al* (1998) but using other US bottom dwelling species. Studies indicated that differences in susceptibility of fish species to sediment contaminants exist. Differences in histopathological biomarkers of response found between species.	For the starry flounder *Platichthys stellatus* and white croaker *Genyonemus lineatus* analyses confirmed that the relative risk for hydropic vacuolation of biliary epithelial cells and hepatocytes increased with the presence of aromatic and chlorinated hydrocarbons in sediment, fish bile, and fish liver. However, although hydropic vacuolation also frequently occurred in the rock sole *Lepidopsetta bilineata* the lesion showed no clear association with contaminant exposure.	Stehr *et al* (1998)
Field studies investigating biomarker responses to PAH sediment contamination	Hardhead catfish (*Arius felis*) and Atlantic croaker (*Micropogon undulatus*) were collected from locations where sediment total PAHs ranged from 68 to > 1,000 ng/g.	Studies indicated that biliary PAH metabolites and PAH-DNA adducts were sensitive indicators of exposure to PAH contamination in both species of fish but the croaker appeared to be a better species for monitoring contaminants that induce CYP1A-mediated responses.	Willett *et al* (1997)
Marine (e.g. North Sea) monitoring programmes	Wild bottom dwelling fish species such as the dab (*Limanda limanda*) were collected by trawl to assess potential sediment contamination effects. Fish were maintained on deck in clean flowing seawater until they were sacrificed for EROD, fish pathology and fish disease biomarker studies.	Monitoring programmes used to provide an environmental baseline and identify 'hot spots' which warrant further investigation. This was the case in the EDMAR programme.	CEFAS (2003)

Table 20: Examples of studies in which fish have been used to assess sediments (cont'd)

Type of investigation	Fish used and description of study	Comments	Reference
UK programme to study endocrine disruption in the marine environment (EDMAR).	Multi-disciplinary research programme to investigate the implications of observations of strongly oestrogenic effects in flounder (*Platichthys flesus*) from several UK estuaries. Looked at biomarkers such as vitellogenin (VTG) and fish histopathology.	Studies confirmed that many industrialised UK estuaries are sufficiently oestrogenic to cause strong induction of yolk protein VTG in male flounder by up to a million-fold in excess of controls. Biomarker results were backed up by the fact that induction of intersex (ovotestis) in some male individuals (15-20%) was found in some areas. At some locations VTG levels had decreased over the 3-year study period, which was linked to improvements in sewage treatment.	Matthiessen *et al* (2001)
Assessing the environmental quality of an estuary.	Fish tests included as part of a battery of water column and sediment toxicity bioassays to assess the South River, Maryland, USA. A 10-day embryo-larval survival and teratogenicity test with the sheepshead minnow (*Cyprinodon variegatus*) was used to provide toxicological risk scores for the river sediments, which were then correlated to fish diversity data.	Importance of sediment studies demonstrated because they revealed significant toxicity in stations where water column tests indicated only low-level toxic effects.	Hartwell *et al* (2000)

5.2.3 *In situ* assessments

An approach, which can be considered as an intermediate between studies on wild caught fish and laboratory studies, involves the use of *in situ* assessments. The use of organisms such as fish to improve the risk assessment of contaminants found in sediments and field situations *in situ* is currently considered to be important because these can be much more representative of actual exposure. As Chappie and Burton (2000) point out, bioassays using caged fish, bivalves, and other macroinvertebrates (*in situ* toxicity tests) supplement traditional laboratory toxicity tests and benthic invertebrate and fish population/diversity studies.

There are a number of advantages of *in situ* toxicity tests over conventional laboratory tests. These include the fact that they are more realistic because they incorporate complex site-specific conditions (e.g. suspended solids, light, dissolved oxygen, pH, salinity, and temperature), which may alter contaminant toxicity and/or bioavailability (Chappie and Burton, 2000; Fent, 2003). *In situ* studies with fish invariably rely on caged fish. An advantage of *in situ* tests over using wild caught fish species to identify contaminant effects is that wild fish frequently migrate making it difficult to establish whether the fish have been exposed to the contaminated sediments. However, it should be recognised that, although the use of caged fish avoids this problem, *in situ* studies may overestimate effects because fish are unable to avoid contaminants or periodically enter cleaner water where depuration processes could remove toxins.

Another potential limitation is that caged fish will not have access to their usual diet, which may be important. For example in the EDMAR studies reported by Matthiessen *et al* (2001) experiments with male flounder showed that they did not produce VTG when caged in oestrogen-contaminated estuaries. However, flounder experienced mild VTG induction when fed on mussels (*Mytilus edulis*) that had been held in an oestrogen-contaminated estuary (the Tees) for 3 months. The authors claimed this demonstrated that at least some oestrogenic substances have the potential for transmission through the food chain. Rice *et al* (2000) also demonstrated that feeding juvenile English sole (*Pleuronectes vetulus*) with polychaetes (*Armandia brevis*) taken from contaminated sediments could increase expression of CYP1A and formation of hepatic PAH-DNA adducts.

One of the reasons why fish have been used for *in situ* studies is that they provide sufficient material for chemical analysis to determine body burdens and provide an integrated assessment of the accumulation of contaminants. Fish can also provide sufficient material to allow periodic non-lethal sampling (e.g. blood analysis) and can be used to assess a number of biochemical (biomarker) endpoints. Historically, bivalves as well as fish have been used for *in situ* assessments. However, the reliance on these two taxonomic groups is being reduced as laboratory methods for testing a range of macroinvertebrates (e.g. cladocerans, amphipods, and chironomids) have been developed for use in the field. Chappie and Burton (2000) provide a

summary of the uses and applications of a range of species for *in situ* testing incorporating a range of techniques and endpoints. These include stressor identification, chronic bioaccumulation studies, storm water runoff assessment, evaluation of photoinduced toxicity at PAH-contaminated sites, and assessment of episodic acidification of streams.

5.2.4 Use of fish in mesocosms

A small number of fish have also been used in mesocosms to assess potential effects of sediments. Some of these have been used to assess direct effects of contaminants. In other studies the inclusion of fish was used to assess the effects of fish populations on zooplankton and phytoplankton communities rather than adverse effects on the fish themselves. For example, Beklioglu and Moss (1996) used fish in mesocosms with and without sediments to assess effects of a freshwater environment that had historically received large quantities of sewage effluent. In such studies it is unlikely that there would be any suitable alternative to the use of fish but these would still have to be considered as licenced procedures.

5.3 Ethical issues with respect to fish used in sediment tests

The severity of suffering in fish used in sediment assessment varies considerably. In most studies of wild fish, the fish are humanely killed on collection, whereas in some studies of chemicals spiked to sediments effects can be so severe as to cause mortality. In many studies where fish are collected from the field for tissue and blood analyses these fish would not have to be recorded under UK Home Office requirements. This would apply to the studies described by Stehr *et al* (1998) and by CEFAS (2003) where fish caught by trawling were held in clean flowing seawater for a few hours until they were sacrificed for necropsies.

In some instances fish use may be recorded for scientific research when it can be argued that they have not been deliberately subjected to any adverse conditions. For example, during the second phase of the studies described by Stehr *et al* (1998) the maintenance of wild caught fish was extended by transferring sexually mature fish from contaminated sites and subsequently inducing spawning in the laboratory. As such, the fish have not been deliberately exposed to chemicals or contaminated material although they have technically been subject to an experimental procedure. In terms of the UK Home Office Licence, these fish would probably be included but whether they were subject to a specific controlled procedure would depend on whether anaesthetics were used in striping gametes or hormones were added to induce spawning (as was the case in the studies reported by Johnson *et al*, 1998).

For other studies where fish have been exposed to field collected sediment (Tetreault *et al,* 2003) or spiked sediments (e.g. Muir *et al*, 1982, Schuytema *et al*, 1988 and Beklioglu and Moss,

1996), the case is more transparent. These would be included in UK Home Office statistics, as would any studies with chemicals and/or wastes incorporated into sediments. As most of these studies would be looking to assess sublethal as opposed to lethal effects the majority of the procedures should fall in the mild to moderate categories (see Section 2.1.2).

Fish used in *in situ* caged studies will come under the U.K. Home Office regulations and these would have to be covered as a special condition in the Project Licence of the scientists conducting the investigations.

Most of the fish used in such studies would not fall into the severe bands and potential to reduce suffering would be limited.

5.4 Application of the 3Rs to fish used in sediment tests

The number of fish used in the studies described above is small in comparison to those used for effluent and product assessment. However, as many of these studies are for research purposes they are more likely to be reported in the open literature.

5.4.1 Replacement

It should be recognised that the use of fish for research, to support sediment risk assessments, may be unavoidable. For example, such studies may be required when other tests and surrogates indicate that there may be risks to fish populations either directly or via exposure through the food chain. In many investigations to assess the environmental quality of fresh watercourses and the marine environment, fish tests only form part of a battery of water column and sediment toxicity bioassays. For example in the studies undertaken by Hartwell *et al* (2000) a battery of water column and sediment toxicity bioassays was used. These measured lethal and sublethal endpoints with fish, invertebrates, vascular plants and bacteria were used to assess how toxic contaminants were affecting habitat quality and living resources in Chesapeake Bay (Maryland, USA). In the UK in the National Marine Monitoring Programme (NMMP), whole sediment bioassays with invertebrate species supplement a range of flat fish biomarker assessments to monitor the status of the marine sediments (CEFAS, 2003).

5.4.2 Refinement/reduction

Although fish are likely to be used in certain assessments (e.g. for *in situ* studies) future developments in macro invertebrate tests and *in vitro* assessments could lead to a gradual reduction in their use. However, using sound reasoning and having clear objectives before

initiating risk assessment studies (i.e. to consider whether alternatives could be used), may reduce the use of fish. For example, prior to initiating their studies on the effects of sediment sorbed pesticides Clark *et al* (1989) undertook a literature review. This review demonstrated that fish were less sensitive than invertebrates and they subsequently used a combination of grass and pink shrimp in their studies.

There is also evidence that techniques to reduce animal use described elsewhere in this report can be applied to animals used to generate data for risk assessment purposes. For example, Fent (2003) provides examples where *in vitro* tests using fish cell lines have provided valuable information on the bioavailability and potential to cause adverse effects to supplement chemical analysis and improve the environmental risk assessment of contaminated sites.

In the EDMAR programme summarised by Matthiessen *et al* (2001), the yeast oestrogen screen (YES) was used in a Toxicity Identification and Evaluation (TIE) scheme to identify substances probably causing the endocrine related changes seen in the flounder. The EDMAR study indicates that the YES assay could be used to identify discharges warranting further investigation thereby reducing the numbers of fish used in future endocrine monitoring studies. It is being proposed by Matthiessen that in the future UK Endocrine Disrupter Demonstration Programme, run by the Environment Agency and the water industry, YES (or a more sensitive equivalent) will be used to identify whether sewage effluents are responding to more advanced treatment technology. Fish-based assays will then be focused on the most relevant cases (Matthiessen personal communication).

5.5 Summary

A number of techniques in which fish have been used either directly or indirectly to assess sediments have been described. Owing to the complexity of some of the uptake and exposure mechanisms it is difficult to envisage that the use of fish to assess the environmental risks posed by sediments could be entirely avoided. This is particularly true for monitoring programmes but these do not raise many ethical concerns (i.e. they are essentially the same as trawling fish). For a number of the other investigations, having clear objectives for the study and by considering a number of the alternative techniques described elsewhere in the report, it should be possible to refine studies to reduce the numbers of fish used in the risk assessment of contaminated sediments.

6. SECONDARY POISONING

6.1 Introduction

Certain hydrophobic substances, which are widely dispersed in the environment and which show a combination of resistance to degradation and biotransformation, have become prevalent in the environment and have bioaccumulated in organisms. Bioaccumulation is defined as the net result of the uptake, distribution and elimination of a substance due to all routes of exposure. Biomagnification is the increase in the internal concentration in organisms at subsequent levels in the trophic chain. Secondary poisoning is defined as the product of trophic transfer and toxicity (ECETOC, 1995).

Bioaccumulative substances can be distinguished by high persistence and toxicity, insignificant metabolism and a log K_{ow} between 5 and 8. They may present a concern when widely distributed in the environment. Therefore, where applicable, the potential of a substance to bioaccumulate in the environment should be included as an exposure related parameter in risk assessment. Bioaccumulation and secondary poisoning are not as extensive as commonly believed and have only been established for a very limited number of substances (ECETOC, 1995). However certain substances have produced unexpected toxicity (e.g. DDT, co-planar PCBs) after biomagnification. Industry and regulators therefore face a challenge to screen substances for their potential to bioaccumulate and cause toxic effects after prolonged exposure (ECETOC, 1995). Secondary poisoning has been observed in both the aquatic and terrestrial environment (e.g. by pesticides).

The assessment of risks for secondary poisoning is based on simple food chain scenarios.

Aqueous: water → aquatic organism → fish → fish-eating bird/mammal

Terrestrial: soil → earthworm → worm-eating birds/mammals

These approaches are still considered provisional due to a general lack of experience with these assessments.

6.2 Technical Guidance Document perspective on secondary poisoning

At base set level, the available physico-chemical and (eco)toxicological data can be used to determine if there is evidence for bioaccumulation potential and indirect effects. The easiest way to assess the potential of a substance to bioaccumulate in aquatic species is by experimental measurement of the BCF. A measured BCF may be available for existing substances. However, in practice such data are not generally available and the potential for bioaccumulation needs to be

assessed using physico-chemical data and data for analogous substances (see Chapter 4). Secondary poisoning should, in principle, be evaluated by comparing the measured or estimated concentrations in the tissues of the top predators with the no-effect concentrations for these predators expressed as the internal dose. Data on internal concentrations in wildlife are hardly ever available (apart from recognised persistent organic pollutants) and most no-effect levels are expressed in terms of concentrations of the food that the organisms consume (mg/kg food). The actual assessment is usually based on a comparison of the (predicted) concentration in the food of the top predator and the (predicted) no-effect concentration, which is obtained from studies with laboratory animals.

In the aquatic environment, for substances with a log $K_{ow} < 4.5$, the main uptake route is direct uptake from water. Where data on other uptake routes are unavailable, it is assumed that the direct uptake accounts for 100% of the intake. For substances with a log $K_{ow} \geq 4.5$, additional uptake routes such as intake of contaminated food or sediment may become more important.

The first step in the assessment strategy is to consider whether there is evidence for bioaccumulation potential. Subsequently, it is essential to consider whether the substance has a potential to produce toxic effects if accumulated in higher organisms. This assessment uses classifications based on mammalian toxicity data (T+, T, and Xn in combination with one or more of R48, R60, R61, R62, R63, R64, (EEC, 1967)). In addition, there are high volume chemicals that are released to the environment that may also be considered for assessment (e.g. surfactants, see Comber *et al*, 2003). The assumption is made that the available mammalian toxicity data can give an indication of the toxicity of the chemical to higher organisms in the environment. If the substance is classified, or if there are other indications (e.g. endocrine disruption), an assessment of secondary poisoning is performed.

The risk to the fish-eating predators (mammals and/or birds) is calculated as the ratio between the concentration in their food ($PEC_{oral.predator}$) and the no-effect concentration for oral intake ($PNEC_{oral.predator}$). The concentration in the fish is a result of the uptake from the aqueous phase and intake of contaminated food (aquatic organisms). Thus the $PEC_{oral.predator}$ is calculated from the bioconcentration factor and a biomagnification factor ($PEC_{oral.predator} = PEC_{water} \times BCF_{fish} \times BMF$).

For terrestrial predators the $PEC_{oral.predator}$ is equivalent to $C_{earthworm}$ (the total concentration of the substance in the worm due to bioaccumulation and adsorption to soil in the gut). This value is likely to be conservative as biotransformation in birds and mammals is generally much more extensive than in earthworms (Sijm *et al*, 1997).

The BMF is defined as the comparative concentration in a predatory animal compared to the concentration in its prey ($BMF = C_{predator}/C_{prey}$, lipid normalised). The BMF should ideally be

based on measured data. However, as the availability of such data is limited, a range of default values has been suggested (EC, 2003b). These presume that a correlation exists between the BMF, the BCF and the log K_{ow}. These BMF defaults are only considered preliminary and should be used for the identification of chemicals that require further scrutiny. These defaults have been derived from data for PCBs, toxaphene and DDT. However, the BMFs for other hydrophobic materials, volatile methylsiloxanes (log K_{ow} 4.2-7.7) were found to range between < 0.01 and 0.06 (Opperhuizen *et al*, 1987). These BMF values are much lower than the defaults (1-10) suggested in the TGD. Evidence that indicates that a substance can undergo metabolism may suggest that the potential for biomagnification is lower than the suggested default. Evidence for metabolism may include 1) data from *in vitro* metabolism studies 2) data from mammalian metabolism studies 3) evidence of metabolism from structurally similar compounds 4) a measured BCF significantly lower than predicted from the log K_{ow} (also see Chapter 4).

Table 21 gives some examples of predicted log BCF values and the corresponding values that have been measured (Meylan *et al*, 1999). It is obvious that there can be a considerable overestimation of bioconcentration. This means that the assessment of secondary poisoning which uses bioconcentration QSARs and biomagnification defaults can lead to conservative estimates, which could result in unnecessary animal testing. However, the database also contains some examples of substances that are underpredicted by the default TGD QSAR for bioconcentration.

To determine the $PNEC_{oral}$, long-term studies such as NOECs for mortality, reproduction and growth are preferred. If no adequate toxicity data for mammals or birds are available, an assessment for secondary poisoning cannot be made. For new substances (EEC, 1992) the results of mammalian repeated-dose toxicity tests (if available) are used to assess secondary poisoning effects. For existing substances and biocides, dietary studies with birds can also be used to extrapolate a $PNEC_{oral}$. Acute LD_{50} tests for rodents and birds are not acceptable, as these are not dietary tests. For the assessment of secondary poisoning the results have to be expressed as the concentration in food (i.e. NOEC rather than NOAEL) using the appropriate conversion factor. The $PNEC_{oral}$ is finally derived from the toxicity data (LC_{50} bird, $NOEC_{bird}$, $NOEC_{mammal.food.chronic}$) using the recommended application factor. The above approach has been used to show that the secondary poisoning is not a concern for HPV chemicals such as surfactants (Comber *et al*, 2003). In addition, an estimate of the BCF of concern can be derived if dietary data are available but measured bioconcentration factors are not. This can then be compared to the measured bioconcentration factor for analogous substances or BCF predictions.

Table 21: Comparison of measured and predicted BCFs

Substance	Predicted (KOWWIN) log K_{ow}	Predicted log BCF (TGD QSAR)	Measured log BCF
Substances over-predicted			
Phenothiazine	4.15	2.83	2.49
2-Ethylanthroquinone	4.37	3.01	0.94
Butylbenzyl phthalate	4.91	3.47	2.82
Pentabromotoluene	5.43	3.92	2.43
Benzo(a)pyrene	5.97	4.37	2.96
2,2-bis(4-hydroxy-3,5-dibromophenyl)propane	7.2	4.64	2.21
N-hexadecane	8.25	4.27	1.31
Tris (2-ethylhexyl)phosphate	9.49	3.27	0.82
Diisodecyl phthalate	10.36	2.20	1.16
Substances under-predicted			
2-Chlorophenol	2.15	0.2465	2.33
p-Chloronitrobenzene	2.39	0.68618	2.03
2,4-dimethylphenol	2.3	0.524	2.18
Dehydroacetic acid	0.78	-2.70448	2.11

6.3 Marine assessment

In principle, the evaluation for the marine environment uses the same methodology as for the freshwater environment. The TGD defines highly bioaccumulative substances as having a very high bioconcentration potential (log K_{ow} > 4.5 or BCF > 500) and a resistance to biotransformation in animals. Biomagnification from food is a major risk to top predators whilst direct uptake from water or sediment is of lesser importance. The principal endpoints for the secondary poisoning assessment are the predators and top predators. Biomagnification factors are especially critical in the assessment of secondary poisoning as they are applied in the calculation of the PEC_{oral} for both predator and top-predator.

The default BMF values are given in Table 22.

Table 22: TGD BMF values

Log K_{ow}	BCF_{fish}	BMF_{fish}	$BMF_{predator}$
< 4.5	< 2,000	1	1
4.5 - < 5	2,000 - 5,000	2	2
5 - 8	> 5,000	10	10
> 8 - 9	2,000 - 5,000	3	3
> 9	< 2,000	1	1

These default BMF values are considered as preliminary for use in screening and prioritisation (EC, 2003b). Studies of pelagic food webs in the Barents Sea showed that for persistent organochlorines the biomagnification factors could be quite high. For total PCB, DDT and hexachlorocyclohexane the biomagnification factors (normalised to lipid weight) between *Metridia longa* (copepod) and *Gadus morhua* (cod) were 58, 33 and 32 respectively (Klungsøyr, 2000).

However, such organochlorines have been shown to undergo little metabolism and are therefore well predicted using standard QSARS for bioconcentration. Some examples are given Table 23 (Meylan *et al,* 1999).

Table 23: Measured and predicted BCFs for organochlorines

Substance	Predicted (KOWWIN) log K_{ow}	Predicted log BCF (TGD QSAR)	Measured log BCF
O,P'-DDT	6.79	4.66	4.57
P,P'-DDE	6.51	4.64	4.71
Hexachlorobenzene	5.73	4.17	4.27
1,2,3,4,5,6-hexachlorocyclohexane	4.26	2.92	2.70
3,3',4,4'-tetrachlorobiphenyl	6.63	4.65	4.59
2,2',5,5'-tetrachlorobiphenyl	6.09	4.55	4.87

6.4 Data required to estimate secondary poisoning

From the above it can be seen that secondary poisoning estimates require, as a minimum, a BCF value and a dietary toxicity study using birds or mammals. The 28-d or 90-d oral toxicity tests in rodents are the most commonly used long-term toxicity tests. Currently there are no generally accepted methods that can be considered as a replacement for repeat-dose *in vivo* testing. It will

be an enormous scientific and technical challenge to achieve this goal (Worth and Balls, 2002). Table 24 shows the numbers of animals used in such studies. It should be noted however that, secondary poisoning assessments might be triggered for substances that have already shown significant toxicity to birds or mammals (in combination with being hydrophobic and poorly biodegradable). In this case, dietary studies are likely to be available.

The assessment for bioaccumulation in fish would be improved by dietary bioaccumulation data (especially for very hydrophobic substances) that can be used to estimate the magnitude of the BMF as well as the BCF. A direct approach for measuring the BMF is described by Parkerton *et al*, 2001, in which the chemical is spiked into fish food and fed to the fish for a period of time, usually 10-14 days. At this point the level in the fish is measured and they are transferred to a clean diet and the kinetics of loss followed. Using this approach a number of key parameters are obtained including the uptake efficiency and the growth corrected half-life for depuration. As discussed in Chapter 4, this approach also allows for an estimation of BCF.

Substances that have the potential to cause concern with respect to secondary poisoning will probably exert their effects through dietary rather than aquatic exposure (ECETOC, 1995). One source of BCF data that could also be used is that from earthworms, which as the testing does not involve vertebrate animals, (as defined in the regulations), may be considered a replacement test.

Table 24: Number of animals used to support the assessment of indirect exposure

OECD Test guideline	Species	Number of animals
Bioconcentration: flow-through test - 305	Various	Total 108
Dietary bioaccumulation study	Various	Total 40
Avian dietary toxicity test - 205	Mallard, bobwhite quail, Japanese quail, pigeon, ring-necked pheasant, red-legged partridge	Control: 20 Treatment: 50
Avian reproduction test - 206	Mallard, bobwhite quail or Japanese quail	Control: 24 - 36 Treatment: 72 - 108
Repeated dose 28-d oral toxicity study in rodents - 407	Rat (preferred)	Control: 10 - 20 Treatment: 30 - 40
Repeated dose 90-d oral toxicity study in rodents - 408	Rat (preferred)	Control: 20 - 30 Treatment: 60 - 70

6.5 Non-animal studies

For those substances that are known to be hydrophobic, persistent and toxic, sufficient data should exist (or can be predicted) to carry out a secondary poisoning assessment.

Where few data exist, the alternative methods for bioaccumulation that will be needed for a secondary poisoning assessment will be the same as those discussed in Section 4. For toxicity/effects some models are available (e.g. androgen/oestrogen receptor-binding (Q)SAR, Serafimova *et al*, 2002, Mekenyan *et al*, 2002, *in vitro* assays). In addition, useful information on the bioaccumulation and/or biotransformation may be obtained from tests using invertebrates (e.g. *Daphnia, Chironomus, Lumbriculus*) (Akkanen and Kukkonen, 2003; Artola-Garicano *et al*, 2003) or early fish embryo (pre-hatch) studies (48-h zebrafish test, e.g. Nagel, 2002, Wiegand *et al*, 2000a). Evidence for chemical biotransformation in algae or invertebrate species (e.g. arthropods) generally suggests that compared with lower vertebrates such as fish, and top predators they would be less able to biotransform chemicals. Such information needs to be considered when addressing the potential for biomagnification and hence secondary poisoning during the prioritisation of chemical testing.

6.6 Conclusions

Those substances that are prioritised for secondary poisoning assessment are likely to be large volume, poorly biodegradable, hydrophobic and toxic chemicals. Toxicity data on such substances are usually sufficient to carry out such an assessment (i.e. dietary or oral toxicity studies).

A combination of biodegradation data, expert judgement, (Q)SAR predictions, *in vitro* data and non-vertebrate bioaccumulation tests, should give a reasonable prediction of the likelihood that a chemical will pose a significant threat with regard to secondary poisoning. Hydrophobic chemicals, which are shown not to undergo biodegradation and biotransformation, should be prioritised for assessment. In the absence of existing relevant data, animal data will be required for a complete secondary poisoning assessment.

GLOSSARY

Apoptosis	Programmed cell death.
Axenically	Cultured without microbial contamination.
Bioaccumulation	The net result of multiple physiological processes (ADME: Absorption, Distribution, Metabolism and Excretion) of a substance due to all routes of exposure.
Bioconcentration	The net result of the uptake, distribution and elimination of a substance due to water-borne exposure.
Biomagnification	The accumulation and transfer of substances via the food web (e.g. algae-invertebrate-fish-mammal) due to ingestion, resulting in an increase of the internal concentration in organisms at the succeeding trophic level.
Biotransformation	The biological conversion of a substance.
Biomarker	A biomarker is defined in this report as any biochemical, physiological or histopathological indicator of exposure or response to a contaminant by individual organisms (van Gestel and Brummelen, 1996).
Caco-2 cells	Specific human colon carcinoma cells that display similar characteristics to intestinal enterocytes.
Cytochrome P450	Cytochrome P450 (CYP) and their associated enzymes. They play a prominent role in Phase I metabolism of most xenobiotics via oxidation, reduction or hydrolysis.
CHSE-sp cell line	Suspension-cultured fish cells obtained from chinook salmon (*Oncorhynchus tshawytscha*).
Chorion	Egg envelope protecting the unhatched embryo.
Crystal violet assay	Assay which evaluates the ability of cells to attach to a substrate.
DNA micro-array	Deposition of numerous oligonucleotides onto an inert substrate such as glass or silicon. These DNA chips may be probed to allow expression monitoring of many thousands of genes simultaneously.
Dedifferentiation	Process by which a differentiated cell comes back to a less differentiated stage.
Desmosome	A type of junction that attaches one cell to its neighbour. It consists of a circular region of each adjacent cell membrane, with associated intracellular microfilaments and intercellular material.
EC_{50}	Acute toxicity expressed as the concentration that induces an effect in 50% of the exposed population.
Egg	Egg refers to the unfertilised oocyte.
Embryo	Embryo refers to the fertilised un-hatched egg.
Eleutheroembryo	Embryonic phase starting at the point of hatch and ending when most or all of the yolk is absorbed and the fish starts exogenous feeding.

Embryonic period	The time from union of gametes until exogenous nutrition. This period is characterised by endogenous nutrition from the yolk of the oocyte.
Sac fry	Synonymous with eleutheroembryo: embryonic phase starting with hatching and ending when most or all of the yolk is absorbed and the fish starts exogenous feeding.
Genomics	The techniques available to identify the DNA sequence of genetic material (e.g. chromosomes) and expression of genes in response to stressors, such a drugs and toxicants.
Humane	Compassionate.
Immortalised cell lines	A cell line is a permanently established cell culture capable of continuous indefinite renewal given appropriate fresh medium and space. Lines differ from cell strains in that they have escaped from the normal limitation on growth of a finite number of division cycles (the Hayflick limit) and become immortalised. Immortalisation in culture may occur spontaneously or can be induced by mutagens or by transfection of certain oncogenes.
Inhumane	Uncompassionate, cruel, causing pain or suffering.
Insentient	Incapable of experiencing sensations or feelings.
In silico	Computer generated as opposed to *in vitro* or *in vivo*.
In vitro	In an experimental situation outside the organism. Biological work done in the test tube (*in vitro* is Latin for 'in glass') rather than in living systems.
In vivo	In a living organism.
Juvenile	The juvenile stage is the sexually immature form of the adult fish.
Larva	A larva (plural larvae) is an immature form of most invertebrates, amphibians and fish, differing markedly in form and appearance from the adult stage and must undergo some level of metamorphosis, to reach the adult stage.
LC_{50}	Acute toxicity expressed as the median lethal concentration that kills 50% of the exposed population.
LOEC	The lowest tested concentration at which the substance is observed to have a statistically significant and unequivocal effect on the test species.
Metabonomics	The techniques available to identify the presence and concentration of metabolites in a biological sample.
Micropyle	The site of sperm attachment and entry through the chorion.
Milt	Sperm of fish.
NOEC	Highest tested concentration below the LOEC where the stated effect was not observed.

Octanol-water partition coefficient	Log P (sometimes also referred to as log K_{ow}) is the ratio of the concentration of a neutral compound in aqueous phase to its concentration in an immiscible solvent. Generally, the neutral molecule exists for acids > 2 pKa units below the pKa and for bases > 2 pKa units above the pKa. Log P or log K_{ow} values for a molecule can vary according to the conditions under which it is measured (e.g. temp and pH). Log D is related to log P and the pKa of a compound, but differs from Log P in that the ionised species are considered as well as the neutral form of the molecule. Specifically, Log D (pH) = log P - log [1 + 10 (pH - pKa)] for acids and Log D (pH) = log P - log [1 + 10 (pKa - pH)] for bases. Log D is the log distribution coefficient at a particular pH. For example, 'Log D at pH 7.4' might be quoted to give an indication of the lipophilicity of a drug at the pH of blood plasma.
Proteomic	The techniques available to identify proteins.
Read-across	Information on structurally analogous chemicals: where chemicals display similar physico-chemical properties in terms of log K_{ow} and/or partition coefficients, and ecotoxicity data already exists, for example, for some members of a homologous series.
Reduction	Reduction in the numbers of animals used previously with no loss of useful information.
Refinement	Change in the experiment protocol that results in the decrease of the incidence or severity of pain, stress or distress that animals may experience.
Replacement	Methods or strategies that do not involve the use of protected animals in regulated procedures (as defined in national or international laws, such as the Animals (Scientific Procedures) Act 1986 in the UK, and Directive 86/609/EEC in the European Union.
RTG-2 cell line	Fish cell line derived from rainbow trout gonadal tissue.
Sentient	Capable of experiencing sensations or feelings.
Transcriptome	The full complement of activated genes, mRNAs, or transcripts, in a particular tissue at a particular time.

ABBREVIATIONS

ACR	Acute to chronic ratio
AH	Aromatic hydrocarbon
AHR	Aryl hydrocarbon receptor
API	Active pharmaceutical ingredient
APDM	Enzyme aminopyrine-N-demethylase
ASTM	American Society for Testing and Materials
BCF	Bioconcentration factor
BF-2 cell lines	Bluegill sunfish cell lines
BMF	Biomagnification factor
CBR	Critical body residue
CV	Crystal violet
CYP	Cytochrome P450
DNA	Deoxyribose nucleic Acid
DPH	Days post-hatch
EAT III	ECETOC Aquatic Toxicity III database
ECVAM	European Centre for the Validation of Alternative Methods
EDMAR	UK Endocrine Disruption In the Marine Environment Programme (1998)
ELISA	Enzyme linked immuno sorbent assay
ELS	Early life stages
ERA	Environmental risk assessment
EROD	Enzyme ethoxyresorufin O-deethylase
FETAX	Frog Embryo Teratogenesis Assay - *Xenopus*
FFLC	Fish full life-cycle test
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
HMSO	Her Majesty's Stationery Office
HSP	Heat shock protein
IPPC	Integrated Pollution Prevention and Control
K_{ow}	Octanol/water partition coefficient
LAS	Linear alkylbenzene sulphonate
LOEC	Lowest observed effect concentration
MAS	Metabolic activation system
MCIG	Minimum concentration to inhibit growth
MFO	Mixed function oxygenase enzymes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NIEHS	US National Institute of Environmental Health Sciences
NOEC	No observed effect concentration
NRU	Neutral red uptake
NTP	US National Toxicological Program

OECD	Organisation for Economic Co-operation and Development
OECD TG	OECD Technical Guideline
OPPTS	US Office of Prevention, Pesticides and Toxic Substances
OSPAR	Oslo-Paris convention for the protection of the marine environment of the North-East Atlantic
PAH	Polynuclear aromatic hydrocarbon
PAPS	Phosphoadenosyl phosphosulphate.
PBPK	Physiologically based pharmacokinetic
PCB	Poly-chloro-biphenyl
PEC	Predicted environmental concentration
PLHC	*Poeciliopsis lucida* hepatocellular carcinoma
PNEC	Predicted no effect concentration
PTU	6-n-propyl-2-thiouracil
QSAR	Quantitative Structure Activity Relationship
R-1 cell line	Fish fibroblastic cell line isolated from rainbow trout liver
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals.
RNA	Ribonucleic acid
3Rs	Reduction, refinement and replacement of laboratory animals, according to the definition of Russell and Burch (1959)
3Rs (other)	SOLNA principles (OECD, 1996a): these additional 3Rs state that tests for regulatory purposes need to reflect the following: biological relevance, reliability/reproducibility, regulatory acceptability.
SETAC	Society of Environmental Toxicology and Chemistry
SPMD	Semi-permeable membrane device
SPME	Solid phase micro-extraction
T3	3,3',5-triiodothyronine
T4	Thyroxin
TEER	Transepithelial electrical resistance
TGD	Technical Guidance Document
TH	Thyroid hormone
TI	Teratogenic index
vPvB	Very persistent and very bioaccumulative
VTG	Vitellogenin
WEA	Whole effluent assessment
WFD	Water Framework Directive
XEMA	*Xenopus* Metamorphosis Assay

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APPENDIX A: COMPILATION OF TOXICITY DATA WITH VARIOUS TEST SYSTEMS

	Fish, adult acute toxicity test	Fish, early life stages toxicity			Cellular toxicity		
Chemical tested	**Adult LC_{50}**	**Embryo LC_{50}**	**EC_{50} I [a]**	**EC_{50} II [a]**	**MTT_{50}**	**NRU_{50}**	**Reference**
Malathion	0.5-13.7 nm	ND [b]	10.6 nm	1.8 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
Carbaryl	7.0-139.2 nm	ND [b]	18.9 nm	13.4 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
4,6-dinitro-o-cresol	1.0 nm	ND [b]	25.7 nm	18.3 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
2,4-Dinitrophenol	3.4-124.9 nm	ND [b]	58.1 nm	25.5 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
Phenol	90.4-308.5 nm	ND [b]	386.2 nm	84.0 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
4-Nitrophenol	56.8-100.6 nm	ND [b]	322.1 nm	204.9 nm	ND [b]	ND [b]	Schulte and Nagel (1994)
Acetochlor	0.37 mg l^{-1}	0.61 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Acrylamide	59 mg l^{-1}	160 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Benzene	560 mg l^{-1}	320 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Colchine	18.5 mg l^{-1}	39 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Diethylene glycol	45,000 mg l^{-1}	30,000 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Diethyl-nitrosamine	560 mg l^{-1}	1,200 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Methanol	22,300 mg l^{-1}	290 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Triton X-100	13 mg l^{-1}	17 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Kovrižnych and Urbančíková (2001)
Alkylbenzene sulphonate mixture 54.8%	42 mg l^{-1}	75 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Cairns *et al* (1965)
Naphthenic acids	16.3 mg l^{-1}	3.5 mg l^{-1} [c]	ND [b]	ND [b]	ND [b]	ND [b]	Cairns *et al* (1965)
Potassium dichromate	180 mg l^{-1}	1,500 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Cairns *et al* (1965)

Appendix A: Compilation of toxicity data with various test systems (cont'd)

	Fish, adult acute toxicity test	Fish, early life stages toxicity			Cellular toxicity		
Chemical tested	**Adult LC_{50}**	**Embryo LC_{50}**	**EC_{50} I [a]**	**EC_{50} II [a]**	**MTT_{50}**	**NRU_{50}**	**Reference**
Potassium cyanide	0.49 mg l^{-1}	11.7 mg l^{-1}[c]	ND [b]	ND [b]	ND [b]	ND [b]	Cairns *et al* (1965)
Zinc chloride	28 mg l^{-1}	105 mg l^{-1}[c]	ND [b]	ND [b]	ND [b]	ND [b]	Cairns *et al* (1965)
Urea	250 mmol l^{-1}	ND [b]	374 mmol l^{-1}	323 mmol l^{-1}	182 mmol l^{-1}	260 mmol l^{-1}	Lange *et al* (1995)
Sodium chloride	131 mmol l^{-1}	ND [b]	205.5 mmol l^{-1}	111 mmol l^{-1}	186.5 mmol l^{-1}	86-171 mmol l^{-1}	Lange *et al* (1995)
Phenol	0.3 mmol l^{-1}	ND [b]	0.4 mmol l^{-1}	0.08 mmol l^{-1}	1.3 mmol l^{-1}	0.6 mmol l^{-1}	Lange *et al* (1995)
4-nitrophenol	0.1 mmol l^{-1}	ND [b]	0.3 mmol l^{-1}	0.2 mmol l^{-1}	8.5 mmol l^{-1}	3.5 mmol l^{-1}	Lange *et al* (1995)
2-nitroanisole	1.4 mmol l^{-1}	ND [b]	0.2 mmol l^{-1}	0.15 mmol l^{-1}	> 3.3 mmol l^{-1}	> 3.3 mmol l^{-1}	Lange *et al* (1995)
3,4-dichloroaniline	0.05 mmol l^{-1}	ND [b]	0.01 mmol l^{-1}	0.01 mmol l^{-1}	> 0.15 mmol l^{-1}	> 0.15 mmol l^{-1}	Lange *et al* (1995)
2,4-dinitrophenol	0.04 mmol l^{-1}	ND [b]	0.06 mmol l^{-1}	0.025 mmol l^{-1}	0.2 mmol l^{-1}	0.2 mmol l^{-1}	Lange *et al* (1995)
Dinitro-ortho-cresol	0.01 mmol l^{-1}	ND [b]	0.025 mmol l^{-1}	0.02 mmol l^{-1}	> 0.25 mmol l^{-1}	> 0.25 mmol l^{-1}	Lange *et al* (1995)
Carbaryl	0.03 mmol l^{-1}	ND [b]	0.02 mmol l^{-1}	0.01 mmol l^{-1}	> 0.5 mmol l^{-1}	> 0.5 mmol l^{-1}	Lange *et al* (1995)
Malathion	0.06 mmol l^{-1}	ND [b]	0.01 mmol l^{-1}	0.002 mmol l^{-1}	> 0.085 mmol l^{-1}	> 0.085 mmol l^{-1}	Lange *et al* (1995)
Pentachlorophenol	0.195 mg l^{-1}	0.38 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Ensenbach *et al* (1989)
4-chlorophenol	8.67 mg l^{-1}	37.8 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Ensenbach *et al* (1989)

Appendix A: Compilation of toxicity data with various test systems (cont'd)

	Fish, adult acute toxicity test	Fish, early life stages toxicity			Cellular toxicity		
Chemical tested	**Adult LC_{50}**	**Embryo LC_{50}**	**EC_{50} I [a]**	**EC_{50} II [a]**	**MTT_{50}**	**NRU_{50}**	**Reference**
Phenol	29.0 mg l^{-1}	49.8 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Ensenbach *et al* (1989)
4-nitrophenol	14.0 mg l^{-1}	57.2 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Ensenbach *et al* (1989)
Methanol	8.0 mg l^{-1}	22.1 mg l^{-1}	ND [b]	ND [b]	ND [b]	ND [b]	Ensenbach *et al* (1989)

[a] See Section 3.5.1.1 for EC_{50} I and EC_{50} II endpoints

[b] ND = Not determined

[c] Results expressed by Cairns *et al* (1965) were measured as TL_m (median tolerance concentration)

APPENDIX B: PROCEDURES FOR ISOLATION AND CULTURE OF FISH CELLS (E.G. HEPATOCYTES)

The procedure to isolate liver cells is reviewed in Baksi and Frazier (1990)[a] and can be summarised as follows:

Initial liver perfusion to clear off blood with buffered salt solution (1-10 min), followed by a second enzymatic (collagenase) digestion (20-45 min up to 90 min) for facilitating dispersal of the cells (preferred to EDTA perfusion). Perfusion is either via portal vein if distinct or by cannulating the arteria coeliaca or by retrograde perfusion from the heart. Perfusion is continued until the liver is soft and malleable, and shows obvious signs of digestion.

The liver is then minced with razor blades/scissors and passed through several filter screens (sterile nylon gauze, plankton net, etc).

Cells are collected in buffer, centrifuged at low speed (40-100 × g for 2-10 min depending on the species) and transferred into the final culture medium. Cells are counted on a haemocytometer and the viability of the cells assessed by trypan blue exclusion. Cell yields depend on species, strain, sex and age of donor fish and vary from 30 to 80% of the weight of the liver. There is no evidence of metabolic zonation in fish liver, as opposed to that present in mammalian liver.

Alternative procedures involve excision of (perfused) livers, cut into species and incubation in collagenase solution. Cells are dispersed by shaking in water bath or by magnetic stirring.

The conditions for the culture of hepatocytes depend primarily on the experimental design (cell suspension, or plated cells). Typically samples of cells in suspension are taken in between 1 and 4 h following initiation of cultures (but it is possible to sample up to 24 h). Temperature can range from 5-25^{o}C. Densities of 1×10^{5} to 2×10^{7} cells per ml have been reported. Alteration in media composition can affect the metabolism of fish hepatocytes.

Plate cultures of hepatocytes are used because they last significantly longer than hepatocytes in suspension (e.g. up to 20 days versus 24 h for cells in suspension in the case of catfish). Successful attachment of fish hepatocytes on plates depends on the coating of the plastic plates (collagen, horse fibronectin or extracellular matrix with or without fish serum). Attached hepatocytes flatten out and form monolayers.

[a] This perfusion method is best for fish with an encapsulated liver (e.g. rainbow trout and channel catfish) and does not work well for Cyprinids, such as carp.

APPENDIX C: CELLULAR AND SUBCELLULAR *IN VITRO* METHODOLOGIES FOR STUDYING THE BIOTRANSFORMATION POTENTIAL OF CHEMICALS

Enzyme-catalysed biotransformation reactions of xenobiotics can be studied in liver incubated with a foreign compound and appropriate cofactors. After a relatively short incubation period, the reaction mixture can be analysed. Biotransformation products can be isolated, quantified and identified. Chromatography is usually employed for qualitative and quantitative analyses of the products formed (thin-layer chromatography, gas-liquid chromatography, and high-pressure liquid chromatography). The use of radioactive (^{3}H, ^{14}C) and stable isotopes (^{13}C) adds the advantage that it is possible conveniently and reliably to account for all products formed from the parent compound. Isotopically labelled products are detected by scintillation counting. These simple, convenient methods can be applied to liver slices, homogenates and subfractions. Liver subcellular preparations (homogenates, S9 fractions, microsomes) are simple and well-defined *in vitro* methods that can be used as a rapid screen (2-h incubation) to assess biotransformation potential. The type of information generated will possibly contribute to the weight of evidence defining the significance of biotransformation in bioconcentration and environmental risk assessment (Sijm *et al*, 1997).

Description of systems

Liver slices - Precision-cut liver slices are prepared after removing the liver from the organism. Schmieder *et al* (2000) compared various slice incubation techniques. They observed that the uptake and rate of conversion of radiolabelled estradiol were faster in the liver slices submersed in medium and cultured in 12-well shaking plates than in the roller incubation flotation system (dynamic organ culture).

Cellular (primary or immortalised) - Cell cultures are incubated in sub-toxic concentrations and sampled at different time intervals to obtain rates of biotransformation in the entire sample (e.g. rate of parent loss, or rate of generation of metabolites) or cell and media fractions. Suspensions and plated cell methods have both been used to determine biotransformation rates in fish cells.

Homogenates - After tissue excision from the organism, the liver is homogenised. The resulting homogenate suspension contains both cytosolic as well as microsomal fraction of cells and thus all the Phase I and Phase II enzymes. This material presents the advantage that it can be frozen and stored at -80°C until required for further use. Centrifugation of this homogenate at different speeds produces several sub-fractions.

S9 fraction - The S9 fraction is derived from the whole-tissue homogenate by centrifugation. It removes larger debris from the tissue homogenisation process including protease-inhibiting enzymes. S9 fractions can be frozen and stored at -80°C.

Microsomes - Microsomes and cytosolic preparations are derived by ultracentrifugation of the S9 fraction (e.g. Dady *et al,* 1991). The pellet in the centrifugation tube is the microsomes, whereas the cytosolic fraction is maintained in the upper liquid layer. Microsomes are vesicles containing the CYP450 system enclosed by a mainly complete membrane system. They can be used to localise the components of the particular cells responsible for biotransformation and to identify metabolites.

Table C.1: Advantages and disadvantages of in vitro systems to assess biotransformation. Adapted from Dyer et al (2003)

		Cellular liver systems		Subcellular liver systems		
	Liver slices	**Isolated hepatocytes**	**Immortalised cell line**	**Homogenised liver**	**S9 fractions**	**Microsomes**
Description	200 µm - thick slices of liver containing multiple undisturbed cellular layers with differentiated liver tissue.	Freshly isolated cells hepatocytes from fish livers have both Phase I and II (conjugation) enzymes.	PLHC-1, *Poeciliopsis lucida* hepato-carcinoma cells) have both Phase I and II enzymes.	Homogenised whole liver. Includes both Phase I and Phase II (conjugation) enzymes.	Centrifuged supernatant of homogenised liver. Includes both Phase I (e.g. P450) and Phase II (conjugation) enzymes.	105,000 × g fraction (liver homogenates) have mainly Phase I enzymes (e.g. P450).
Advantages	Good representativeness of *in vivo* processes maintenance of intact extra-cellular matrix, cellular architecture and intercellular communication. Possibility of studying histological changes. Retention of metabolic capacity, long-term culture (72 h), ease of preparation and use when incorporating multi-well plate incubation.	Functions similar to liver *in vivo*. Standardised isolation methods. Can be cryopreserved.	High potential for standardisation.	Ease of execution Can be cryopreserved. Higher exposure concentrations may be achievable short-term (hours) assays.	Standard methods apply Can be cryopreserved. Higher exposure concentrations may be achievable short-term (hours) assays.	Standard methods apply Can be cryopreserved. Higher exposure concentrations may be achievable short-term (hours) assays.
Disadvantages	Tissue needs to be fresh and therefore cannot be frozen.	Enzymatic activity decreases through life time of cells and culture.	Full enzymatic capabilities not fully assessed.	Possible presence of interfering enzymes. Cryopreservation not fully assessed.	Preparation slightly more laborious than that of liver homogenates.	Limited to Phase I. Preparation slightly more laborious than that of liver homogenates and S9 fractions.

APPENDIX D: ASSESSING THE POTENTIAL OF CHEMICALS TO BIOCONCENTRATE

The TC NES (Technical Committee for New and Existing Substances) subgroup addressing persistent, bioaccumulative and toxic (PBT) and very persistent/very bioaccumulative (vP/vB) chemicals considered the recommendations and agreed to use them as part of the strategy of determining whether a chemical should be placed on a screening PBT/vPvB list and/or should be tested to determine whether it is B/vB.

The TC NES subgroup continue to discuss the parameters and it is recommended that prior to utilising the parameters for developing a case for a potential PBT/vPvB chemical that the strategy be discussed with a representative of the TC NES subgroup.

The criteria should not be considered as definitive, but should be considered with other information, e.g. data derived from mammalian studies. The TC-NES WG on PBTs, therefore consider the following provisional criteria in assessing chemicals.

A chemical may be considered as not B (i.e. unlikely to have a BCF > 2,000) if it has:

- a maximum cross-sectional diameter of 17.4 Å plus a molecular weight of 700 - 1,100 g/mol or
- a maximum molecular length of 43 Å or
- a measured octanol solubility (mg/l) < 0.002 × mwt (without observed toxicity or other indicators of bioaccumulation).

A chemical may be considered as not vB (i.e. unlikely to have a BCF > 5,000) if it has:

- a molecular weight in excess of 1,100 g/mol or
- a maximum cross-sectional diameter of 17.4 Å or
- a maximum molecular length of 43 Å or
- a measured octanol solubility (mg/l) < 0.002 × mwt (without observed toxicity or other indicators of bioaccumulation).

Assessing the potential of chemicals to bioconcentrate - indications for reduced or hindered uptake

The magnitude of bioconcentration (i.e. the BCF) or bioaccumulation (i.e. the BAF) of a chemical in an (aquatic) organism is estimated by a ratio of the concentration of the chemical in the body of the animal to that of the environment or food. The BCF or BAF is the result of four processes, which occur when a chemical is taken up from an animal's surrounding environment

or food. The BCF refers mainly to the process where uptake is via aqueous exposure only, the BAF takes into account multiple uptake routes. The four processes are:

- Adsorption - after the introduction of a chemical through food, water, air, sediment, or soil, its transport across a biological membrane into systemic circulation e.g. across fish gills, intestine, skin (Hodgeson and Levi, 1994).
- Distribution - after absorption, a chemical may bind to plasma proteins for circulation throughout the body, as well as to tissue components like fat or bone. The chemical may be distributed to a tissue and elicit a toxic response; other tissues may serve as permanent sinks, or as temporary depots allowing for slow release into circulation (Hodgeson and Levi, 1994).
- Metabolism - after reaching a tissue, enzymes may biotransform the chemical. During Phase I, a polar group is introduced into the molecule, which increases its water solubility and renders it a suitable substrate for Phase II reactions. In Phase II, the altered molecule combines with an endogenous substrate and is readily excreted. Metabolism is generally a detoxification mechanism, but in some cases, intermediates or final products may cause toxicity (Hodgeson and Levi, 1994).
- Excretion - a chemical with similar characteristics, primarily water solubility, to endogenous waste is eliminated by the same mechanisms. Chemicals with nutritional benefit may be broken down and ultimately exhaled as CO_2; volatile substances may also be exhaled directly through the lungs, polar molecules that are freely soluble in plasma are removed through renal filtration and passed into urine. Fat soluble chemicals may be conjugated and excreted in bile (faeces) (Hodgeson and Levi, 1994).

In addition to excretion, growth of the organism may also be relevant in reducing the chemical concentration in the organism when the rates of other elimination processes are of the same order of magnitude as the dilution due to growth rate. Elimination through the transfer of chemical to the offspring through gestation or lactation may also be important.

This section describes several chemical properties that limit the absorption and distribution of a chemical, which would sufficiently hamper the uptake, distribution or the body burden of a chemical so that the BCF can be assumed to be of no or limited concern. Metabolism, excretion processes and growth also lead to a reduction of BCF/BAF but are not discussed here.

Regulatory context

This text should be seen in the context of the European PBT and vPvB assessment of chemicals with a focus on the B or vB-assessment. Currently, if a substance has a calculated or measured BCF > 2,000 it fulfils the criterion for B. If it has a calculated or measured BCF > 5,000 it fulfils

the criterion for vB. Based on a screening criterion, a substance could be either B or vB when its (estimated) log K_{ow} is > 4.5. In this case, if a substance meets the screening criterion for B or vB and it is also shown to be or likely to be (very) persistent, it will probably undergo further bioconcentration or bioaccumulation testing to determine the experimental BCF regardless of its propensity to be metabolised or poorly absorbed.

The BCF is also relevant in other parts of the environmental risk assessment of substances, but here the focus is on the B and vB assessment.

Experimental testing to determine the BCF

The standard test to study the BCF in fish is the OECD 305 bioconcentration test (OECD, 1996b). In cases where substances meet the screening criterion for B or vB, it is probable that these substances are very hydrophobic and have a very low aqueous solubility. Due to these properties it can be very difficult to test them in aqueous exposure systems such as the OECD 305 test. Alternatively, a recently developed dietary test (Anonymous, 2004) could be used to determine bioaccumulation potential through food or to derive data to estimate a BCF. Most of the earlier studies to determine the BCF of hydrophobic substances were, however, performed following aqueous exposure. These studies did not always follow the OECD 305 test protocol and have in common that there were possible artefacts involved in the testing and in the interpretation of the BCFs from these studies. These artefacts may include:

- Difficulties in measuring the 'true' aqueous concentration due to sorption of the substances to particulate and dissolved (organic) matter;
- adsorption processes to glass walls or other materials;
- volatilisation.

It is important to realise that many of the studies that have investigated relationships between molecular dimensions and reduced uptake, i.e. based on 'lower' BCFs than expected, cannot always distinguish between the artefacts and the truly reduced uptake.

Another note is that some studies propose a reduced uptake based on experimental bioconcentration studies. The reduced uptake then usually refers to reduced uptake via the fish gills. This does not imply that there will be reduced or no uptake possible via the gut uptake, i.e. from food, where other uptake mechanisms may play a role. The extent to which those additional uptake mechanisms play a role in bioaccumulation, however, is inadequately quantified for fish and aquatic invertebrates.

Mechanisms of absorption

The route a chemical follows from the point of initial exposure to the site of action or storage involves passage through a number of tissues and every step involves the translocation of the chemical across multiple membranous barriers (e.g. mucosa, capillary wall, cell membrane), each containing distinct lipid types and proteins. Four primary mechanisms operate to absorb a compound into the body from the environment (Hodgeson and Levi, 1994):

Passive transport - molecules diffuse across cell membranes into a cell and can pass between cells.

Active transport - like passive transport, works in both directions to absorb and exsorb a wide range of chemicals. This special protein, or carrier-mediated, transport is important for gastrointestinal absorption of essential nutrients. In rare instances, toxicants can be actively transported into the cell. Efflux proteins, such a P-glycoprotein, shunt molecules out of the cell. Because of the specificity of this mechanism, it cannot be generally modelled.

Filtration - small molecules can fit through channels, but molecules with molecular weights (mwt) greater than 100 g/mol are excluded. Most compounds have limited access through these pores; filtration is considered more important for elimination than absorption.

Endocytosis - the cell membrane flows around the toxicant to engulf it and transfer it across the membrane. This mechanism is rare except in isolated instances for toxicants, such as for carrageenans with mwt around 40,000 g/mol.

This appendix focuses on passive transport as the significant mechanism of absorption for most toxicants. This mechanism is the only one that can be modelled due to recent work to determine the physico-chemical parameters affecting simple diffusion across a membrane.

Molecular properties

Lipinski *et al* (1997) first identified five physico-chemical characteristics that influence solubility and absorption across the intestinal lumen using more than 2,200 drug development tests. These characteristics have been rigorously reviewed (Wenlock *et al*, 2003; Proudfoot, 2005), used to develop commercial models to estimate absorption in mammals, and are commonly used by the human and veterinary pharmaceutical industry. Although less research in absorption, distribution, metabolism and excretion (ADME) processes has been conducted in fish, data indicate significant similarity among all vertebrates, as described below.

'Lipinksi's Rule of 5' allows the prediction of poor solubility, and poor absorption or permeation from chemical structure. A chemical is not likely to cross a biological membrane in quantities sufficient to exert a pharmacological or toxic response when it has more than 5 Hydrogen (H)-bond donors, 10 H-bond acceptors, molecular weight > 500, and has a log K_{ow} value > 5 (Lipinksi *et al*, 1997). Wenlock *et al,* (2003) studied about 600 additional chemicals and found that 90% of the absorbed compounds had < 4 H-bond donors, < 7 H-bond acceptors, molecular weight < 473, and had a log D value < 4.3. Recent work by Vieth *et al* (2004) and Proudfoot (2005) supports the lower numbers. Molecular charge and the number of rotational bonds will also affect absorption by passive diffusion across a membrane or diffusion between cells.

Although these studies on almost 6,000 substances focussed on absorption, generally of perorally dosed drugs across the intestinal wall, the similarity in tissue structures of mammals and fish imply the equations and concepts can be reapplied to estimate absorption in fish. The 'leakiness' of a tissue, or its ability to allow a chemical to passively diffuse through it, can be measured using trans-epithelial electrical resistance (TEER) and can be used to compare tissue capabilities. A low TEER value indicates the tissue has greater absorption potential. Data indicate that fish and mammalian intestines are equally 'leaky' and that fish gills are more restrictive, similar to the mammalian blood brain barrier (Table D.1). The table also shows whether P-glycoprotein has been detected and could be a functional efflux protein active in the tissue.

Table D.1 Tissue absorption potentials

Tissue	P-glycoprotein efflux?	TEER ohm cm^2	References
Fish intestine	Yes	25-50	Trischitta *et al* (1999)
Mammal intestine	Yes	20-100	Okada *et al* (1977); Sinko *et al* (1999)
Blood-brain barrier	Yes	400-2000	Borchardt *et al* (1996)
Fish gill	Yes	3500	Wood and Pärt (1997)
Human skin	No	20,000	Potts and Guy (1997)

Molecular weight

A number of values have been suggested for the molecular weight cut-off for absorption across fish tissues. The EU TGD (EC, 2003b) indicates that molecules with a mwt greater than 700 g/mol are less likely to be absorbed and bioconcentrate. The US EPA, exempts chemicals with a molecular weight of above 1,100 g/mol in the PBT assessment conducted under the Toxic Substances Control Act (US EPA, 1999). Anliker *et al* (1988) suggested that a pigment could be excluded from needing a fish bioaccumulation test if it has both a molecular weight of greater

than 450 and a cross section of over 1.05 nm[a] (as the second smallest van der Waals diameter or C_{eff}). Rekker and Mannhold (1992) suggested that a calculated log K_{ow} of > 8 can be used on its own, or in combination with a molecular weight of > 700-1,000 to conclude (with confidence) that the compound is unlikely to bioaccumulate. While there has been limited experimental evidence for a molecular weight cut-off, Burreau *et al* (2004) did demonstrate reduced bioconcentration and no biomagnification for high molecular weight polybrominated diphenyl ethers, with six or more bromines, molecular weight 644-959.

Conclusion

Evidence from both mammalian and fish studies indicate that molecular weights have been used to estimate a chemical's limited bioaccumulation potential. Considering that molecular size and shape vary versus molecular weight, molecular weight alone is insufficient. However, it does suggest that once the molecular weight is in the region of 700 - 1,100, depending on other factors, a reduced BCF may be expected.

While recognising the uncertainties in the interpretation of experimental results, it is recommended that to demonstrate a reduced BCF a substance should have either:

- a molecular weight in excess of 1,100 g/mol, or
- a molecular weight of 700 – 1,100 g/mol with other indicators (see later discussion).

Molecular size

Molecular size may be considered as a more refined approach, taking into account molecular shape and flexibility explicitly rather than molecular weight alone. Opperhuizen *et al* (1985) found a limiting molecular size for gill membrane permeation of 0.95 nm, following aqueous exposure. In their study on polychlorinated naphthalenes (PCNs), bioconcentration increased with increasing hydrophobicity, i.e. the degree of chlorination, with uptake and elimination rate constants comparable to those of chlorinated benzenes and biphenyls. For the PCN-congeners studied, BCFs increased with increasing hydrophobicity up to higher log K_{ow} values (>10^5). No further increase was observed at higher K_{ow} values. For the hepta- and the octachloronaphthalenes no detectable concentrations were found in fish. It was suggested that the absence of increasing bioconcentration was due to the inability of the hepta- and octachloronaphthalenes to permeate the gill lipid membrane, due to the molecular size of these compounds, brought about by the steric hindrance of the additional chlorine atoms. A cut-off of

[a] 1 nm = 10 Å

9.5 Å (0.95 nm) was proposed as the cross-sectional diameter which limited the ability of a molecule to cross the biological (lipid) membrane.

Anliker and Moser (1987) studied the limits of bioconcentration of azo pigments in fish and their relation to the partition coefficient and the solubility in water and octanol. A tetrachloroisoindolinone type and a phenyl azo-2-hydroxy-naphthoicacid type, both had low solubility in octanol, < 1 and < 0.1 mg/l, respectively. Their cross-sectional diameters were 0.97 and 1.68 nm, respectively. Despite the high log K_{ow} calculated for these chemicals, the experimentally determined log BCFs were 0.48 and 0.70, respectively. The explanation for this apparent inconsistency of high log K_{ow} and low BCF is the very limited absorption and fat (lipid) storage potential of these pigments, indicated by their low solubility in n-octanol (see next section) and their large molecular size.

Anliker *et al* (1988) assessed 23 disperse dyestuffs, two organic pigments and a fluorescent whitening agent, for which the experimental BCFs in fish were known. Sixteen halogenated aromatic hydrocarbons were included for comparison. Two characteristics were chosen to parameterise the size of the molecules: the molecular weight and the second largest van der Waals diameter of the molecules, measured on conformations optimised by force field calculations (Opperhuizen *et al,* 1985). None of the disperse dyestuffs, even the highly lipophilic ones with log K_{ow} > 3, accumulated significantly in fish. Their large molecular size was suggested to prevent their effective permeation through biological membranes and thus limit their uptake during the time of exposure. Anliker *et al* (1988) proposed that a second largest cross section of over 1.05 nm with molecular weight of greater than 450 would suggest a lack of bioconcentration for organic colorants.

Although the lack of bioconcentration of some chemicals with a cross section of > 0.95 nm has been explained by limited membrane permeability, a number of other studies have demonstrated the uptake of pollutants with large cross sections (e.g. some relevant dioxin and PBDE congeners) by fish and other species. Therefore a simple parameter may not be sufficient to explain when reduced BCF/BAF occurs. Dimitrov *et al* (2002a**)** have tried to develop a more mechanistic approach to address this concept, using molecular weight, size, and flexibility in their BCF estimates.

Dimitrov *et al* (2002b) found that for compounds with a log K_{ow} > 5.0, a threshold value of 1.5 nm (15 Å) for the maximum cross-sectional diameter (i.e. molecular length) could discriminate chemicals with log BCF > 3.3 from those with log BCF < 3.3. This critical value was found to be comparable with the architecture of the cell membrane, i.e. half the thickness of the lipid bilayer of a cell membrane. This is consistent with a possible switch in uptake mechanism from passive diffusion through the bilayer to facilitated diffusion or active transport. In a later paper, Dimitrov *et al* (2003) used this parameter to assess experimental data on a wide

range of chemicals. Their conclusion was that a chemical with maximum cross-sectional diameter larger than 15 Å would not have a BCF > 5,000, i.e. would not meet the EU PBT criteria for vB chemicals. More recently, in unpublished work, following further assessment of their data set, they have changed this value to 17.4 Å. This number will probably change as more chemicals are added to the training set.

Earlier Opperhuizen *et al* (1987) proposed that a substance greater than 43 Å would not pass membranes at all, either in the gills or in the gut based on a series of bioaccumulation and bioconcentration studies with linear and cyclic polydimethylsiloxanes (silicones) varying in chain length. To allow such large substances to pass is very unlikely since it would mean that the entire interior of the lipid membrane would be disturbed. Molecular weight did not explain reduced uptake, since one of the substances with a molecular weight of 1,050 was found in fish. The cross-sectional diameter of these substances could in itself also not explain the reduced uptake since they were smaller or equal to those of PCBs that did bioaccumulate strongly.

Opperhuizen *et al* (1987) also referred to a study by Hardy *et al* (1974) where uptake of long chain alkanes was disturbed for alkanes longer than $C_{27}H_{56}$ in codling. This chain length corresponds to a molecular dimension, i.e. molecular length, of 43 Å, equal to the length of the PDMS congener where reduced uptake was observed.

Loonen *et al* (1994) studied the bioconcentration of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans and found that the laterally substituted (2,3,7,8 substituted) were bioconcentrated while the non-laterally substituted were not. The main reason for this was attributed to metabolism (previously reported by Opperhuizen and Sijm, 1990, and Sijm *et al*, 1993b), however, lower lipid solubility and lower membrane permeability were also considered to have played a role in the reduced BCFs observed. The non-accumulating structures would all have exceeded the effective cross-sectional diameter of 0.95 nm.

Conclusion

Again there would appear to be no clear cut-off. While recognising the uncertainties in the interpretation of experimental results, it is recommended that:

- A maximum molecular length of 43 Å indicates no uptake and indicates a chemical is not B or vB;
- a maximum cross-sectional diameter of 17.4 Å indicates a chemical is not vB;
- a maximum cross-sectional diameter of 17.4 Å plus a molecular weight of 700 - 1,100 would suggest a chemical is not B.

Solubility in octanol

The concept of having a value relating a chemical's solubility in octanol to reduced BCF/BAF is derived from two considerations: firstly, that octanol is a reasonable surrogate for fish lipids, and secondly, that, if a substance has a reduced solubility in octanol (and therefore by extrapolation in lipid) this may result in a reduced BCF/BAF. The former is reasonably well understood and indeed forms the basis of the majority of models for predicting BCF using log K_{ow}. Further, octanol solubility (or better, the ratio of n-octanol/water solubilities) can characterise the transport of some small molecular sized, neutral compounds through biological membranes (Józan and Takács-Novák, 1997).

When a substance has a low solubility in octanol (S_{oct}) as well as a low solubility in water (S_w), the resulting ratio S_{oct}/S_w could range from very low to very high, with no clear idea on how this would affect the magnitude of the BCF/BAF. Still, it could be argued that a very low solubility in octanol could be used as an indication that only low body burdens can be built up in an aquatic organism.

Chessells *et al* (1992) looked at the influence of lipid solubility on the bioconcentration of hydrophobic compounds and demonstrated a decrease in lipid solubility with increasing K_{ow} values for superhydrophobic compounds (log $K_{ow} > 6$). It was suggested that this led to reduced BCFs. Banerjee and Baughman (1991) demonstrated that by introducing a term for lowered octanol/lipid solubility into the log K_{ow} BCF relationship, they could significantly improve the prediction of bioconcentration for highly hydrophobic chemicals.

Body burdens

The meaningful implication of bioaccumulation that needs to be addressed for PBT chemicals, e.g. as in the EU TGD (EC, 2003b), is to identify the maximum concentration(s) in organisms that would give rise to concern. The concept of critical body burdens (CBB) for acute effects is reasonably well established (McCarty and Mackay, 1993; McCarty, 1986) especially for chemicals that act via a narcosis mode of action. Recently there have been a number of reviews of this concept, Barron *et al* (1997, 2002), Sijm and Hermens (2000) and Thompson and Stewart (2003). These reviews are summarised as follows:

- There are very few data available, especially for specifically acting chemicals and for chronic effects, upon which to make decisions relating to generic CBBs;
- much of the variability in CBBs can probably be explained by species sensitivities, biotransformation, lipid content, the measurement of organ versus whole body measurements and whether the chemical is correctly assigned to a mode of action category;

- not withstanding this, it is possible to identify ranges of CBB values for specific modes of action. This is easier for narcosis type mode of actions, and becomes increasingly prone to error moving towards more specifically acting chemicals.

Table D.2 summarises three sources of information:

1. Sijm (2004) - an expert judgement view to arrive at an approximate single value based on three references, McCarty and Mackay (1993), Van Wezel and Opperhuizen (1995) and Sijm and Hermens (2000).
2. Thompson and Stewart (2003) - based on a literature review, the data range beyond the narcosis mode of actions has been drawn from their report.
3. Barron *et al* (2002) - based on Figure 10 of Barron *et al* (2002).

When comparing the expert judgement of Sijm to the ranges indicated and to the figures in the respective publications, it is clear that the values chosen are in the approximate mid-point of the ranges/data. However, there is a lot of variability and therefore uncertainty in deciding on the actual CBB value to use. Choosing the value of 0.001 mmol/kg ww (mid-point for respiratory inhibitors) allows for approximate protection for all the modes of action with the exception of the most toxic chemicals. The rationale for this would be that chemicals that act by the lowest and most specific mode of action would probably be toxic (T) and hence sufficiently bioaccumulative to be of immediate concern. The choice is therefore pragmatic but protective.

Table D.2 Summary of various ranges of CBB - lethality (mmol/kg ww)

Mode of action and source	Narcosis	AChE inhibitors	Respiratory inhibitors
Sijm (2004)	2	0.01	0.001
Thompson and Stewart (2003)	2 - 8	0.000001 - 10	0.000001 - 10
Barron *et al* (2002)	0.03 - 450	0.00004 - 29	0.00002 - 1.1 (CNS seizure agents)
McCarty and Mackay (1993)	1.7 - 8	0.05 - 2.7	0.00005 - 0.02 (CNS seizure agents)

Lipid normalising the chosen CBB of 0.001 mmol/kg ww, and assuming a lipid content of 5%, gives a lipid normalised CBB of 0.02 mmol/kg lipid or 0.02 × molecular weight mg/l lipid. However, given the uncertainty involved in deciding on the CBB that should be used, it is suggested that an application factor of 10, to account for species differences and organ versus body differences be applied to this solubility in lipid/octanol, giving an octanol solubility (mg/l lipid) of 0.002 × molecular weight. This would mean octanol solubilities of 1 and 2 mg/l lipid, respectively, for substances with molecular weights of 500 and 1,000.

Conclusion: it is proposed that where a chemical has a solubility of less than (0.002 × molecular weight) mg/l in octanol it should be assumed that the compound has only a limited potential to establish high body burdens and to bioaccumulate. If it does bioaccumulate, it would be unlikely to give rise to levels in biota that would cause significant effects. However, if the substance demonstrates high toxicity or has been shown to be bioaccumulative in organisms, then this factor must be treated with caution. It is unlikely in such cases that the TC-NES WG would use the lack of octanol solubility as indicating limited potential for bioconcentration.

Conclusion

The following indicators either alone or in combination are therefore recommended as indicating that the chemicals will not bioconcentrate to a level of concern, recognising the uncertainties in the interpretation of experimental results:

- A molecular weight of > 1,100 g/mol would suggest a chemical is not vB (i.e. BCF < 5,000);
- a maximum molecular length of 43 Å indicates no uptake and indicates a chemical is not B or vB;
- a maximum cross-sectional diameter of 17.4 Å indicates a chemical is not vB (i.e. BCF < 5,000);
- a maximum cross-sectional diameter of 17.4 Å plus a molecular weight of 700 - 1,100 g/mol would suggest a chemical is not B (i.e. BCF < 2,000);
- a measured octanol solubility (mg/l) < 0.002 × mwt (without observed toxicity or other indicators of bioaccumulation).

Other indicators for further consideration

The two indicators, molecular size and lipid solubility, are the most frequently cited physical limitations for low bioconcentration. However, there are other indicators that could also be used for indicating whether the bioconcentration of a chemical is limited or reduced despite having a log K_{ow} > 4.5. These include:

- Biotransformation - discussed in Chapter 4 (de Wolf *et al*, 1992, 1993a; Dyer *et al*, 2003) and clearly needs development to improve how such information may be used;
- other indicators for low uptake, these could for example include lack of observed skin permeability, low or reduced uptake in mammalian studies e.g. OECD 420, 423, 425, 435 (OECD, 2001a,b,c; OECD, 2004d).

Both these approaches would benefit from further research and investigation of their potential to indicate limited or reduced bioconcentration. While it is not possible, based on the current level of information, to use these indicators alone to predict low bioconcentration, they can act as supporting information to other indicators in arriving at this conclusion.

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