Intelligent Testing Strategies in Ecotoxicology: Mode of Action Approach for Specifically Acting Chemicals

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CONTENTS

SUMMARY

There is widespread regulatory and scientific interest in developing intelligent testing strategies (ITS) for the environmental risk assessment (ERA) of chemicals that may enter aquatic ecosystems. This is especially relevant to the bioaccumulation and ecotoxicity aspects of aquatic risk assessment, with significant benefits likely to be gained from an ITS in terms of animal welfare and efficient use of finite laboratory and economic resources. The Task Force supports the current use of predictive methods (e.g. structure activity relationships, toxicity threshold concentrations and *in vitro* screening) to prioritise *in vivo* testing needs for chemicals with non-specific modes of action. The Task Force has also developed a 'protein target ITS' approach for *in vivo* testing of chemicals that are specifically intended to interact with target sites in living organisms (e.g. certain agrochemicals which target the nervous system of insects).

This report provides an in-depth assessment of the current science underlining the use of mode of action (MOA) information for specifically acting chemicals; see glossary for list of all abbreviations used in this report. The MOA describes the understanding of selected key events that lead to toxic effects, whereas the mechanism of action refers to a comprehensive understanding of the entire sequence of events that result in toxicity (ECETOC, 2006a). After a review of different MOA classification schemes, the Verhaar *et al* (1992) approach was adopted as a starting point for this exercise. Briefly, the Verhaar *et al* scheme is based on acute effects information and comprises MOA1 (inert chemicals with narcotic properties), MOA2 (less inert chemicals showing polar narcosis), MOA3 (reactive chemicals which reactive unselectively with biomolecules) and MOA4 (specifically acting chemicals with selective biomolecular interactions). To date, the MOA4 class of chemicals has proved most difficult to fit into a robust ITS approach and, to our knowledge, has not been utilised to date for chronic effects assessment. Considering both acute and chronic effects, the Task Force has therefore extended the MOA4 class into four pharmacological sub-classes based on: (a) protein receptors, (b) enzymes, (c) ion channels and (d) transporters (after Rang *et al,* 2003). While there are a minority of specifically acting chemicals that do not fit into the Rang *et al,* scheme (e.g. certain genotoxic chemicals which bind to DNA), the Task Force view is that this provides a consistent basis to review the MOA for both therapeutic (intended) effects and also for toxicity (e.g. MOAs for neurotoxicity and tumour induction by some carbamates). Importantly, recent molecular insights to modern phylogeny provide useful guidance on the evolution of many proteins across species; topical examples include the characterisation of oestrogen receptors across the Deuterosomes (including vertebrates and echinoderms) or moulting hormones across the Ecdyzoans (including arthropods and nematodes). Conceptually, such information provides a valuable and growing knowledge base, with significant potential to contribute to an ITS for *in vivo* chronic testing of MOA4 chemicals and ERA.

Expanding upon published ITS schemes (Figure 2.1), the Task Force has developed a simple five step flow-chart (Figure 2.2) which starts with data gathering on physical-chemical data, SAR predictions, *in vitro* tests, and data from mammalian studies for the compound of interest and for related chemicals. Evaluation of these data provides information on exposure to, and possible MOA of, the substance. Such insight may come from efficacy or therapeutic data, or from read-across within chemical classes. Concerning effects, valuable guidance may be obtained from molecular, biochemical or cellular toxic responses measured in both *in vivo* and *in vitro* studies. For example, fadrozole is an anti-cancer drug which is designed to inhibit aromatase activity in breast tissue (*in vivo* and *in vitro*) and which also decreases vitellogenin levels in female fish (and in fish hepatocytes *in vitro*). Short-term changes in vitellogenin responses can be used to improve the design of the longer-term fish reproduction test and life-cycle studies (see Chapter 5 for details). This culminates with a pragmatic, stepwise prioritisation for the assessment of chronic effects in regulatory aquatic test species, providing guidance on the selection from microbial, plant, invertebrate or vertebrates. The report is focussed on publications and examples from aquatic organisms; however, the principles are also relevant to terrestrial ecotoxicology. The application of the 'specific MOA flow-chart' (Figure 2.2) is illustrated through five case studies, summarised below (full details in Chapter 6).

(i) Ion channel mediated effect case study: cypermethrin. Synthetic pyrethroids are neurotoxic to target invertebrates as a consequence of their rapid absorption and subsequent interaction with the sodium ion channel. The target protein (neuronal sodium channels) is found throughout the animal kingdom (but not in microorganisms or plants) and cypermethrin is slowly metabolised by invertebrates and fish. Examples of aquatic test species which include the target protein are crustaceans, fish, insects and molluscs. In terms of an ITS approach for regulatory testing, non-mammalian ADME information (and log K_{ow} = 6.6) suggests that aquatic arthropods will likely be among the most sensitive aquatic organisms (existing toxicity ranking, arthropods > fish > plants = microorganisms). The available acute data support this logic, whereby the median acute toxicity HC_{50} values are 0.095, 2.1 and 44000 µg/L for aquatic arthropods, fish and algae, respectively. Thus, the acute 'base set' interspecies sensitivity factor is nearly 463,000, a value much higher than typical for MOA1-3 chemicals.

(ii) Receptor mediated effect case study: 17α-ethinylestradiol (EE2). A large amount of *in vitro* and *in vivo* mammalian data on this pharmaceutical clearly demonstrates that it has a specific mode of action via oestrogen receptors (MOA4). EE2 is also designed to be resistant to animal metabolism. Oestrogens are key hormones throughout the Deuterostomes (from sea squirts to mammals) but they appear to be absent from Ecdyzoans (e.g. crustaceans and insects). Oestrogen receptors (the target protein) have been shown in amphibians, fish and molluscs and recent molecular studies suggest their occurrence in echinoderms and related marine invertebrates. In an ITS context, knowledge of ADME (log K_{ow} = 3.67) and oestrogen receptor biology aquatic chronic toxicity gives ranking of fish > arthropods > plants = microorganisms. The regulatory

base set (algal-crustacean-fish) interspecies sensitivity ratio is almost 6.8 (which is not very informative since it lies within the range seen for MOA1-3 chemicals). More importantly, the MOA focus on mammalian reproductive systems suggests that an assessment of fish developmental and reproductive toxicity will add significant value to the ERA for this type of chemical.

(iii) Transporter protein mediated effects case study: fluoxetine. Fluoxetine's therapeutic value is based upon its ability to inhibit serotonin (5-HT) reuptake, thereby prolonging its availability at neuronal synapses. Information on aquatic organisms indicates that serotonin is an important neurotransmitter in amphibians, arthropods, fish and molluscs (but has no known role in plants or microorganisms). In terms of regulatory testing implications, acute toxicity to daphnids and fish is 940 and 1570 μg/L and the 14 d algal EC_{50} is 1.1 μg/L (as fluoxetine free base), giving an interspecies sensitivity ratio of 58 (which is higher than is typical for MOA1-3 chemicals). The possible mechanisms behind the greater sensitivity of algae to fluoxetine are unclear but such a hydrophilic compound (log K_{ow} of 1.8 at pH 7) would be expected to be less toxic to fish and daphnids over an acute exposure period.

(iv) Enzyme mediated effect case study: ketoconazole. This lipophilic compound (log K_{ow} of 4.35 at pH 7) is used as an antimycotic for human medication and is a member of the azoles, which limit ergosterol synthesis (the predominant component of fungal cell membranes) as a result of cytochrome P450 (CYP) inhibition. Comparing taxa, CYP enzymes are found in many taxa, with high activity in amphibians, crustaceans, fish and insects but generally lower activity in annelids, molluscs and plants. In an ITS context, many studies show that high doses of ketoconazole may inhibit mammalian and fish CYPs and steroid hormone biosynthesis. The acute toxicity to algae, daphnids and fish are 1800, 400 and 3900 μg/L, respectively (interspecies sensitivity ratio of 9.8). These data indicate that the chronic toxicity ranking may be fish = daphnids > plants = microorganisms (no chronic data available).

(v) Microbial enzyme inhibitor case study: triclosan. Triclosan is a broad spectrum antimicrobial agent intended for use in consumer products. It has been shown to inhibit Fab I, enoyl-acyl carrier protein reductase in bacteria but the MOA in other organisms is unknown. In terms of regulatory testing implications, triclosan (log K_{ow} 4.80) has an acute ecotoxicity profile of 1.4 μg/L (algae), 390 μg/L (daphnids) and 602 μg/L (medaka), giving an interspecies sensitivity ratio of nearly 430. A 21 d chronic medaka study gave a ^{reproduction}NOEC of 200 μg/L and ^{vitellogenin}LOEC of 20 μg/L, suggesting that triclosan (or a metabolite) may be weakly oestrogenic with the potential to induce vitellogenin but with no adverse effect on reproductive success and offspring development.

Conclusions and recommendations. The use of the protein target ITS approach, as observed in previous ECETOC work on toxicological MOAs in human risk assessment, is a rapidly evolving area of science. Depending on the exposure conditions, some chemicals may induce biological

effects that suggest more than one MOA (e.g. carbamates) and caution is needed when using the protein target ITS approach to guide test protocol design (but not predict precise chronic toxicity values). Therefore, the Task Force recommends an ITS approach to aquatic ecotoxicity testing that includes the following key elements:

- Gather MOA information on the primary pharmacological/toxicological activity (and any additional toxicity MOAs) for the chemical of interest for the target species as well as mammalian data, also considering structurally-related chemicals, to be used in a weight-of-evidence type of approach;
- make use of non-traditional sources of biological information, especially the growing biomedical and 'omics' electronic databases on zebrafish, marine invertebrates and other non-mammalian species;
- if there is evidence for the main MOA being via a protein target (e.g. enzyme or hormone receptor), use this insight to guide the efficient selection of regulatory test methods;
- measure biomarker responses (e.g. vitellogenin) if desired for read-across purposes or setting test concentrations, however, focus on population relevant endpoints (survival, development growth and reproduction) for generating NOEC (or EC_x) values or calculation of PNEC values for application in environmental risk assessment;
- be cautious of using acute interspecies sensitivity ratios (ISRs) for algae, crustaceans or fish, since the available data suggests they are of limited value for ITS application, presumably since acute high levels exposure induces different MOAs compared to chronic low level exposures (note: available data suggest that MOA4 chemicals can have ISRs from <10 to >500, compared with ISRs for MOA1 (<10), MOA2 (8.6 to 343) and MOA3 (<5 to >25).

Finally, the Task Force has identified key knowledge gaps around regulatory test species which create major uncertainty in developing the ITS approach for many MOA4 chemicals. Therefore, the Task Force has identified five research needs that, if addressed, will help reduce the scale of this problem in the ITS context. Taking a pragmatic approach to present and prospective internationally accepted *in vivo* test methods (e.g. ISO and OECD), these recommendations are:

- To critically review data (especially chronic studies) from a wider range of chemicals in the context of the proposed MOA and ITS framework, including chemicals where the mammalian MOA is less specific than for agrochemicals and pharmaceuticals;
- to develop aquatic plant ADME models with special regard to understanding key biotransformation enzymes;
- to strengthen the use of small invertebrate models by investing in a hierarchy of biological understanding (including genomics, proteomics and population responses) in the commonly used freshwater and marine invertebrate species (including both arthropods and non-arthropods);

- for animal welfare reasons, to minimise the need for *in vivo* fish bioconcentration testing by developing *in vitro* fish protocols for chemical metabolism and also develop a small-scale invertebrate bioconcentration test method;
- to support risk assessments of endocrine disrupters, develop a database of the normal (baseline) range for developmental and reproductive endpoints in aquatic organisms measured across different laboratories;
- to capitalise on the learning from zebrafish biomedical and 'omic' research, there would be value in the establishment of a publicly available database on zebrafish ADME and toxicity information;
- to support continuing research into SAR validation by providing 'training data sets' based on high quality *in vivo* chronic tests with plants, invertebrates and fish.

1. INTRODUCTION

Scientific and regulatory activity in environmental (ecological) risk assessment (ERA) continues to progress globally, taking into account the diversity of issues around single chemicals and whole effluent assessment in different countries. In Europe, for instance, the REACH programme (EC, 2006a) and new requirements for environmental assessment of human pharmaceuticals (EMEA, 2006; Länge *et al*, 2007) represent globally significant developments in regulatory environmental risk assessment procedures for industry. Moreover, the science underpinning environmental risk assessment also links directly into related areas of international significance, such as supporting Environmental Quality Criteria and related activities in Europe (Killeen, 1997; Bloch, 1999; Barth and Fawell, 2001), North America (US EPA, 1991; Caux *et al*, 1998) and the Asia-Pacific region (Tsvetnenko, 1998; Yin *et al*, 2003). Therefore, the growing scope and complexity of data being generated to support ERA simultaneously underscores the importance of finding efficient scientific approaches to experimental design and data interpretation. As pointed out by Bradbury *et al* (2004) " … scientists and regulators are faced with three significant challenges: streamlining the risk-assessment process, quantifying risks in a spatially explicit manner, and acquiring the correct kind of environmental data to enable regulatory programs to effectively focus on future environmental protection activities".

ERA comprises two elements: exposure assessment and effects (or hazard) assessment. Environmental risk assessment includes the evaluation of potential for persistence, bioaccumulation and (eco)toxicity (ECETOC, 2005a). This technical report focuses on the ecotoxicity element of ERA in an aquatic context (including sediment ecotoxicology). In regulatory aquatic ecotoxicology, the most commonly used suite of acute lethality tests (e.g. OECD fish and invertebrate test guidelines) was developed in response to the historical concern of over diagnosing and preventing gross pollution problems (Sprague, 1969a,b, 1971). Over the past decade, success in dealing with many acute environmental pollution issues in developed countries has generally been superseded by concerns over more subtle, chronic contaminant impacts. For example, an important international development in the past decade has been the on-going development of OECD *in vitro* and *in vivo* mammalian and wildlife test guidelines for assessing endocrine disruption related to developmental and reproductive effects assessment (Vos *et al*, 2000).

It is also important that ecotoxicologists continue to pursue actively the principles of replacement, reduction and refinement (Russell and Burch, 1959) in the context of regulatory environmental assessments. In terms of acute ecotoxicity testing with fish, there are a number of proposals now under consideration which could lead to a reduction of up to 70% in fish acute lethality testing if adopted by regulatory bodies (Hutchinson *et al*, 2003; Jeram *et al*, 2005). In the fish chronic testing arena, it may be possible to minimise the future use of fish for bioconcentration testing through the use of structure-activity relationships (SARs), *in vitro* methods (e.g. fish hepatocyte

assays) or smaller scale and more-efficient invertebrate tests (ECETOC, 2005b; de Wolf *et al,* 2007). To maximise this potential, there is clearly a need to develop a framework of understanding based on the 'mode of action' that can help decide if and when a chronic invertebrate test could be a protective surrogate for a fish chronic test and aquatic animals in general (Livingstone, 1998; Esher and Hermens, 2002). This important theme is addressed in this report using a range of case studies, which seek to identify the scientific strengths and weaknesses of the MOA approach at the current time.

In recent years, many mammalian toxicologists have defined the 'mode of action' (MOA) as a category or class of toxic mechanisms for which selected key events are sufficiently understood. In contrast, the 'mechanism of action' for a chemical is a complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome (Clewell, 2005; Boelsterli, 2007). Knowledge about the mechanism of action of a chemical is often very rare and a chemical's main MOA is much easier to determine than the precise mechanism. In turn, human risk assessments that are based on MOA data, rather than default assumptions which are scientifically more reliable. This report seeks to address the principles of how the MOA approach may be usefully extended beyond mammals to aquatic organisms. Based on current science, this report also recommends that efforts to gather MOA biomarker information be properly balanced with a focus on apical toxicity endpoints (principally survival, development, growth and reproduction) to inform environmental risk assessments that are protective of wildlife populations (Bradbury *et al*, 2004).

A widely used MOA approach initiated in the early 1990s is the Verhaar categorization system for predicting acutely toxic effect concentrations of organic environmental pollutants to fish (Verhaar *et al,* 1992). This scheme separates organic chemicals into four distinct classes that can be assigned a mode of action (MOA). These four classes are as follows: MOA1 - inert chemicals (baseline toxicity); MOA2 - less inert chemicals; MOA3 - reactive chemicals; and MOA4 - specifically acting chemicals. 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 MOA of such compounds in acute aquatic toxicity is called (lethal) narcosis. Less inert chemicals (MOA2) are slightly more toxic than predicted by baseline toxicity estimations. These chemicals often are characterised as compounds acting by a so called 'polar narcosis' mechanism and commonly can be identified as possessing hydrogen-bond donor acidity, such as phenols and anilines (Escher and Hermens (2002), suggested that MOA1 and MOA2 are probably the same based on the lethal membrane concentration). Reactive chemicals (MOA3) display an enhanced toxicity that is related to the phenomenon that these chemicals can react unselectively with certain chemical structures commonly found in biomolecules or can be metabolized into more toxic species. Specifically acting chemicals (MOA4) exhibit pharmacological and toxicological effects because of (specific) interactions with certain receptor molecules. For example, agrochemicals and pharmaceuticals typically have one of the following protein targets:

(a) receptors, (b) ion channels, (c) enzymes, (d) transporters (Seiler, 2002; Rang *et al,* 2003). The Verhaar categorisation scheme does not include metals or other inorganic substances and ionisable organic substances.

Another MOA scheme is that described by Russom *et al* (1997) which classifies substances into one of seven groups: narcotics (three distinct groups), oxidative phosphorylation uncouplers, respiratory inhibitors, electrophiles/proelectrophiles, acetylcholinesterase inhibitors, or central nervous system seizure agents. Overall, the Task Force chose to follow the Verhaar *et al* (1992) approach, since this could be clearly augmented by modern pharmacological and toxicological perspectives (ECETOC, 2006a; Rang *et al*, 2003) and therefore cover a wider range of substances than appears to be the case for the Russom *et al* (1997) approach.

In aquatic ERA, the environmental threshold of no toxicological concern (ETNC) is now being developed as a tool to aid in decision making for MOA1-3 chemicals (de Wolf *et al*, 2005). Moreover, accurate (eco)toxicity prediction of such a safety threshold for MOA4 chemicals remains a major scientific challenge (Roex *et al*, 2000). In the short-term, therefore the assessment of chronic impacts of MOA4 chemicals is likely to require generation of experimental data (Roex *et al,* 2000; Brietholz *et al*, 2006). However, experiences with chemicals like environmental oestrogens suggest that consideration of the MOA can be used to guide species selection (Hutchinson, 2002; Escher *et al*, 2005; Sumpter and Johnson, 2005) and even help in the efficiency of test design (Hutchinson *et al*, 2006). Preliminary estimates suggest where this approach can be used to avoid unnecessary fish chronic testing, generating cost savings of approximately 50% for an individual ERA and with the concomitant animal welfare benefits. In conclusion, there is regulatory interest to the MOA approach across the agrochemical, industrial chemicals and pharmaceutical sectors, as a tool to improve the focus and efficiency of ecotoxicity testing of specifically active acting chemicals.

ECETOC established a Task Force with the following Terms of Reference:

- Identify available sources of data on pharmacological and toxicological modes of action in a regulatory context;
- review the state of the art in interspecies biology as applied to common biological targets for chemicals and their relevance to regulatory ecotoxicity test species;
- describe case studies where mode of action approaches have been pursued, addressing both strengths and weaknesses;
- identify research needs to develop the application of the MOA approach to regulatory ecotoxicity testing for fate and effects.

Importantly, this report also seeks to manage expectations about different MOA approaches to chemicals assessment, based upon current scientific data but also acknowledging some major

knowledge gaps. In this sense, the primary goal of the report is to give guidance on the prudent application of MOA principles as applied to regulatory test species selection and efficient chronic test design. However, the report does not seek to advocate a rigid process that must be applied to all classes of synthetic chemicals, recognising the different regulatory risk assessment characteristics of different industrial sectors (e.g. agrochemicals, biocides, industrial chemicals or pharmaceuticals).

2. STRATEGY FOR EFFICIENT CHRONIC ECOTOXICITY TESTING

2.1 The need for efficient risk assessment

Risk assessment stakeholders in academia, government and industry collectively recognise the need for greater efficiency in the way the correct data are gathered to support ERA. The challenge of stream-lining the risk assessment process reflects the need to gather scientifically robust data on exposure and effects, while also optimising on use of finite experimental and economic resources and minimising the use of vertebrates (Bradbury *et al*, 2004).

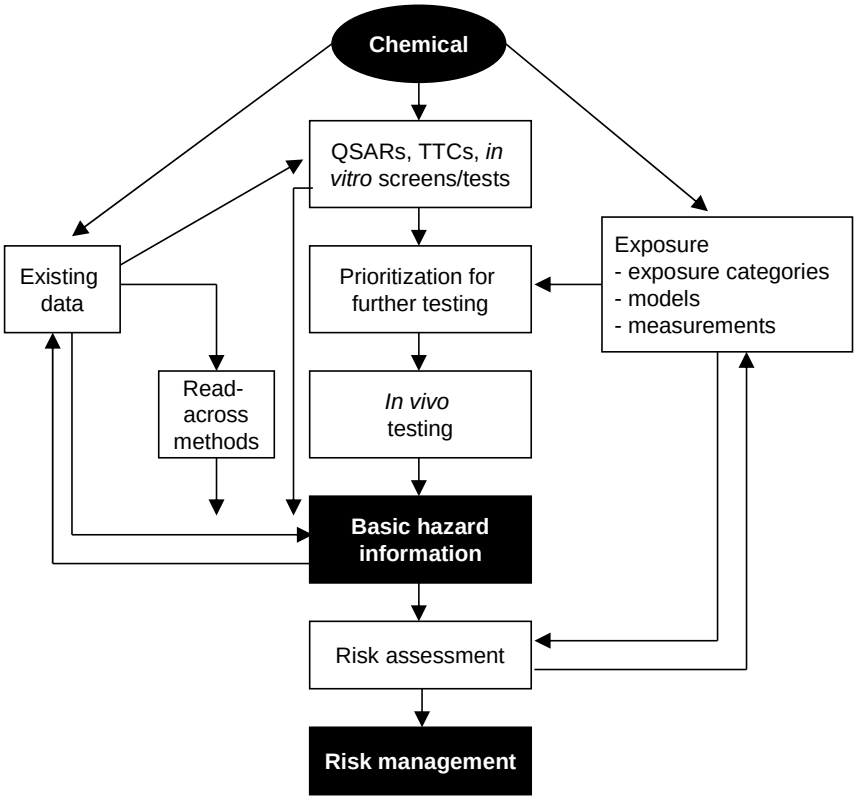
This report focuses on the aquatic effects assessment component of this debate (although the same principles apply to terrestrial effects assessment), an important subset of the wider challenge for chemicals that lack toxicological and exposure data. As stated by Bradbury *et al*, (2004) "… the researcher's challenge is to create ways to efficiently and credibly predict toxic potency and exposure levels. These predictions would help assessors make reasonable decisions about whether empirical studies are required to further refine a risk assessment. In the context of regulatory programs, where data generation is required to make regulatory decisions, the challenge is to move in a scientifically credible and transparent manner from a paradigm that requires extensive hazard testing to one in which a hypothesis-and risk-driven approach can be used to identify the most relevant *in vivo* information". The way forward is clearly with a philosophy of improving the efficiency of environmental risk assessment, as shown in Figure 2.1. This 'intelligent testing strategy' includes the use of exposure information, thresholds of toxicological concern (TTCs), validated structure-activity relationships, read-across methods, *in vitro* test protocols and prioritisation of non-vertebrate ecotoxicity tests where scientifically appropriate.

The approach is based on information obtained from quantitative structure-activity relationships (QSARs), read-across methods, thresholds of toxicological concern (TTCs) and *in vitro* tests prior to *in vivo* testing as a more rapid and efficient way to perform risk assessment of chemicals (after Bradbury *et al*, 2004).

This report focuses on the central boxes in Figure 2.1 that address the prioritisation for further testing and the *in vivo* testing needs. Within this context, there is a need to recognise that based on currently available data there appears to be a limited number of chemical substances that have specific MOA such that they warrant further analysis. To put this into context, de Wolf *et al* (2005) recently addressed the concept of environmental thresholds of no toxicological concern for freshwater systems ($ETNC_{aq}$) for organic chemicals. They analysed environmental toxicological databases (acute and chronic endpoints) and substance hazard assessments. Lowest numbers and 95th-percentile values were derived using data stratification based on

mode of action (MOA 1 = inert chemicals; 2 = less inert chemicals; 3 = reactive chemicals; 4 = specifically acting chemicals).

Figure 2.1: Principles of efficient environmental (ecological) risk assessment



Their preliminary analysis with complete MOA stratification of the databases shows that in the case of MOA1 or MOA2, the $ETNC_{aq}$ value could be even higher than 0.1 mg/L. A significantly lower $ETNC_{aq}$, MOA4 value was observed based on the long-term toxicity information in the most recent ECETOC database (ECETOC, 2003). De Wolf *et al* (2005) concluded that "When the chemical producer has access to acute toxicity information, that producer can apply an appropriate assessment factor (e.g. 100 or 1,000) to derive a PNEC value and, subsequently, to compare this with the $ETNC_{aq}$, MOA1 to 3. When the $ETNC_{aq}$, MOA1-3 is higher, the chemical should be examined in greater depth, because a special situation with regards to the chemical's toxicity might apply". In other words, there is a need to think further about specifically acting chemicals based on currently frameworks of understanding.

2.2 Aquatic toxicity testing - MOA as a guide for species prioritisation

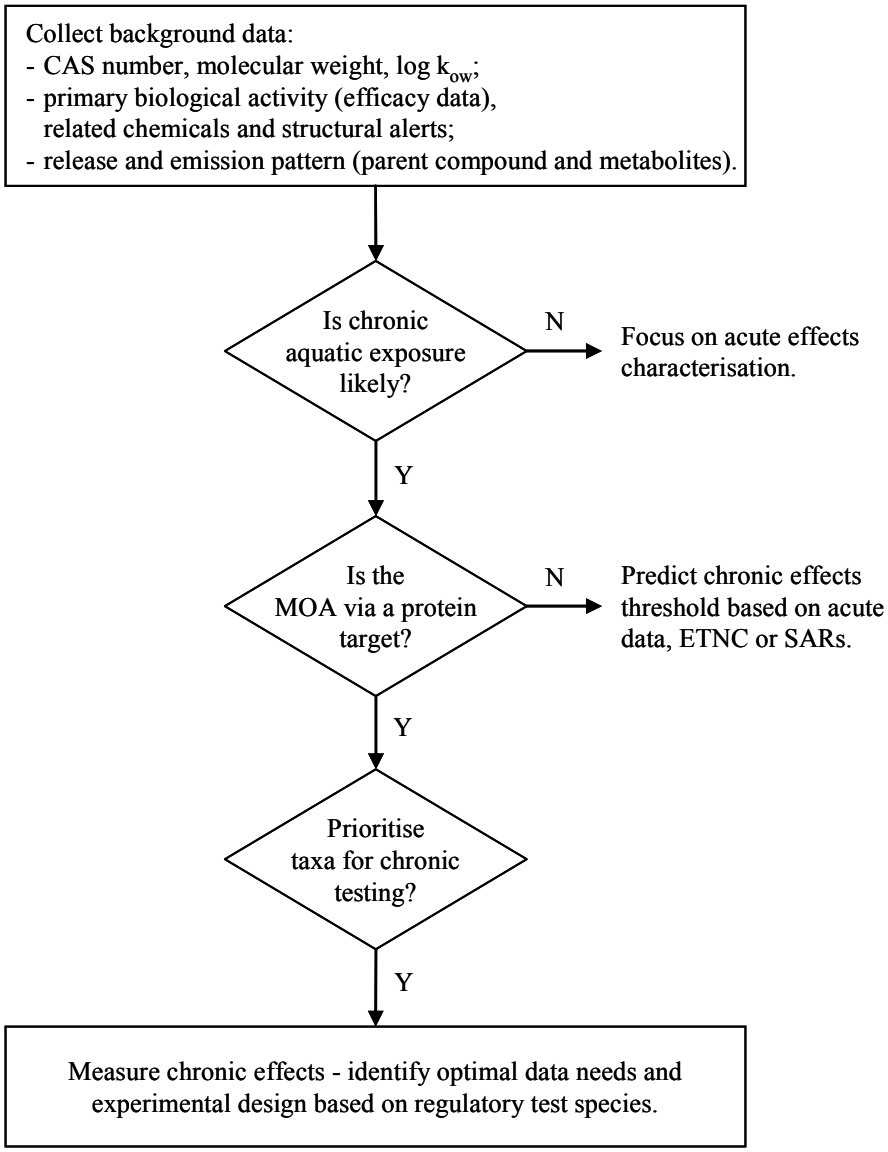
This report builds upon the efficient risk assessment scheme proposed by Bradbury *et al* (2004), taking in some of the wider lessons learned from MOA research over the past decade. For example, Escher and Hermens (2002) argue that knowledge of the mode of toxic action is indispensable for developing descriptive and predictive models in ecotoxicology, emphasising the benefits of information on biological target sites, interactions with the these target site(s), and mechanisms. In terms of assigning sublethal impacts on populations, the past 10 years have also witnessed a major step forward in knowledge regarding reproductive (eco)toxicology and endocrine disruption (Sumpter and Johnson, 2005; Hinton *et al*, 2006; Hutchinson *et al*, 2006). The area of endocrine disruption and aquatic organisms shows, for example, that not all invertebrates respond in the same way as do vertebrates to endocrine-disrupting chemicals, and equally unlikely that all invertebrates respond in the same way. For example, steroidal oestrogens such as 17β-oestradiol and 17α-ethinylestradiol are extremely potent oestrogens in fish, but have little, if any, endocrine effect on arthropods invertebrates. However, these compounds have reported to have reproductive effects on aquatic molluscs at concentrations similar to those that cause feminisation in fish (Sumpter and Johnson, 2005). Initial data on beta-blocker pharmaceuticals similarly points towards major interspecies differences for aquatic organisms (Huggett *et al*, 2002).

A further growing source of MOA information relates to the increasing use of non-mammalian species, for example zebrafish (*Danio rerio*) in pharmaceutical research and development, providing a valuable learning resource for ecotoxicologists (Goldsmith, 2004; Zon and Peterson, 2005). The basic principle of this approach is that where information is available, the MOA of a chemical can serve as pragmatic guide for deciding whether it is necessary to either measure aquatic chronic effects or whether it is justifiable to instead predict chronic aquatic toxicity based on measured or predicted acute ecotoxicity data (Figure 2.2). As a cautionary note, however, it should be borne in mind that MOA4 chemicals may produce diverse exposure-dependent effects that suggest one MOA for biological activity (efficacy/therapeutic effect), together with a MOA for toxicity measured in other tissues. For example, acute exposure to carbamates rapidly kills insects through cholinesterase inhibition and also produces neurotoxicity (and lethality) in mammals. However, tumour formation and other non-neoplastic lesions observed in long-term exposure studies are not readily explained by cholinesterase inhibition and these lesions are considered to be formed due to another MOA (Sternberg, 1979; Karalliedde *et al*, 2000; Pope *et al*, 2005).

The approach shown in Figure 2.2 is based on exposure and MOA (based on the pharmacology of the four key target proteins described by Rang *et al*, 2003). In Figure 2.2, it is important to emphasise the need to gather comprehensive background information before moving to a decision on whether or not to predict the acute and chronic toxicity (Bradbury *et al*, 2004). In the case of

agrochemicals, for example, valuable MOA data may be available from the efficacy screens that today cover a wide range of microbial, plant and invertebrate systems (Stenersen, 2004). With human pharmaceuticals, there will instead be opportunities to leverage the MOA information generated during pre-clinical safety assessment (Huggett *et al*, 2005).

Figure 2.2: Stepwise prioritisation of prediction or measurement of chronic effects in regulatory test species



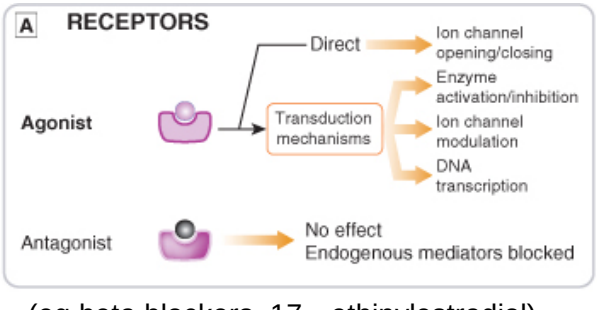
For industrial chemicals, opportunities may exist for read across from related classes of chemicals, supplemented by use of validated SARs and possibly *in vitro* data from mammalian, fish or invertebrate cell lines (Crespi *et al*, 1997; Fent, 2001; Castaño *et al*, 2003;

Dinan *et al*, 2001; Bernhard and Dyer, 2005). Furthermore, if chronic aquatic exposure is unlikely, then it is most sensible to focus on addressing the potential for acute effects in aquatic organisms, where possible using the types of predictive tools advocated by Bradbury *et al* (2004). Importantly, there may be a need to also consider continuous versus time varying exposures in some risk assessment scenarios.

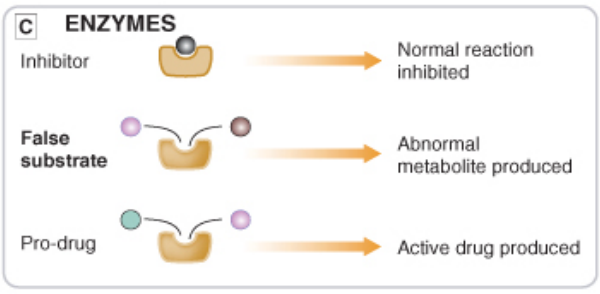
For specifically acting chemicals (termed 'MOA4' by Verhaar *et al*, 1992), these can be usefully classified into one of four major protein targets, as is the case in modern pharmacological nomenclature (Rang *et al*, 2003). As represented in Figure 2.3, this modern classification approach covers pharmaceuticals and agrochemicals which are designed to primarily target either: (a) receptors; (b) ion channels; (c) enzymes and (d) transporters. This approach appears equally applicable to agrochemicals, as well as human and veterinary pharmaceuticals (Table 2.1).

The compounds cited in Table 2.1 and Figure 2.3 are only examples, while it should be noted that there are a small number of anti-neoplastic drugs (e.g. bleomycin, cyclophosphamide and mitomycin C) that target DNA or other intracellular systems to prevent cell division (Rang *et al*, 2003).

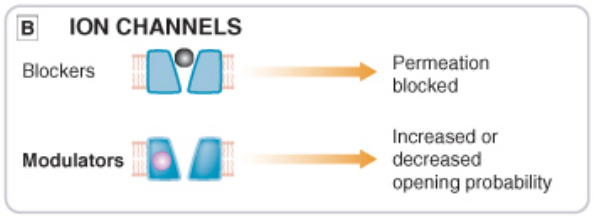
Figure 2.3: Four protein groups as the major pharmaceutical targets (after Rang et al, 2003)



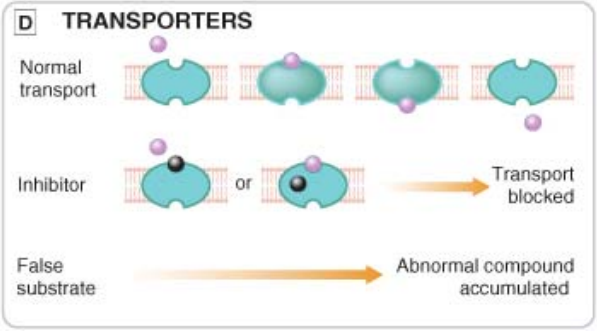
(eg beta blockers, 17α-ethinylestradiol)



(eg aspirin, ketoconazole)



(eg local anaesthetics, cypermethrin)



(eg fluoxetine, omeprazole)

Note – a few drugs target DNA rather than proteins (eg mitomycin C).

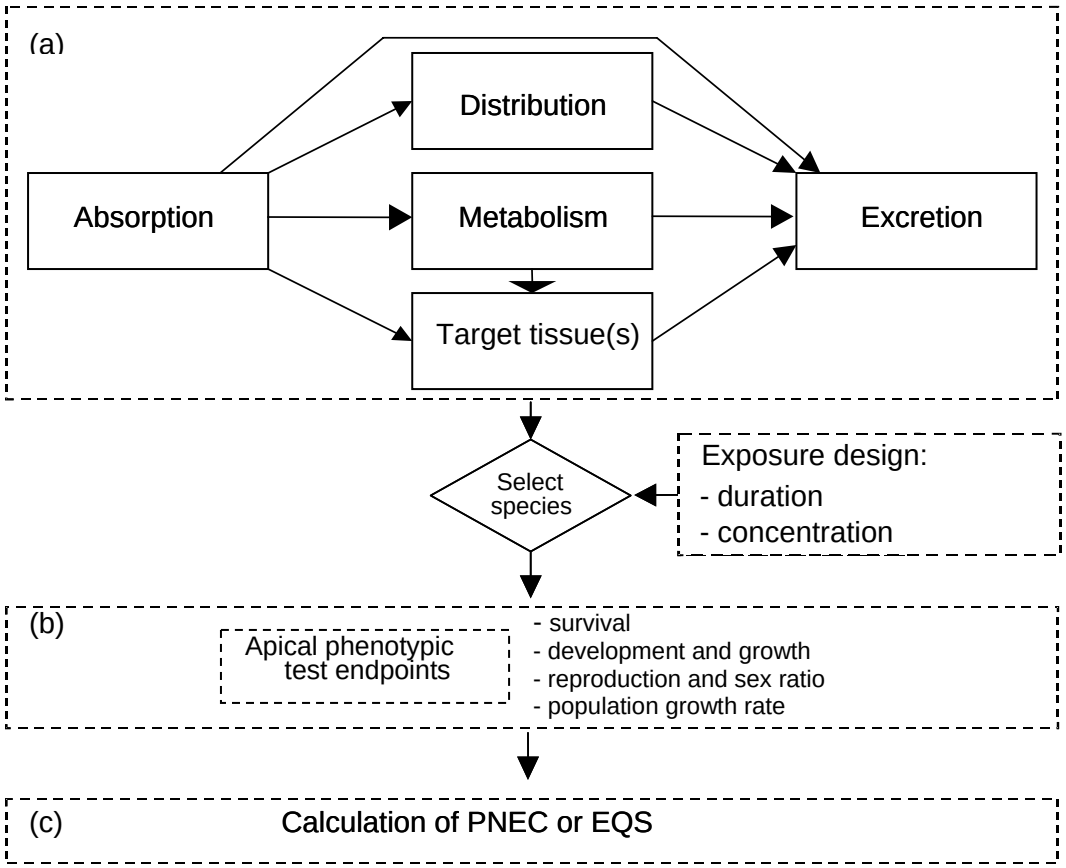
Table 2.1: Examples of compounds which are designed to primarily for major target proteins

Protein target	Examples	Reference
(a) Receptors	Oestrogen receptors (e.g. 17α-ethinylestradiol)	Rang *et al* (2003)
	Adreno-receptors (e.g. atenolol)	Toda (2003)
(b) Ion channels	Voltage-gated calcium channels (e.g. cypermethrin)	Burr and Ray (2004)
	Voltage-gated potassium channels (e.g. 4-aminopyridine)	Rang *et al* (2003)
(c) Enzymes	Cyclooxygenase (e.g. aspirin)	Rang *et al* (2003)
	Cytochrome P450s (e.g. hexaconazole)	Ghannoum and Rice (1999)
(d) Transporters	Proton pump inhibitors (e.g. omeprazole)	Rang *et al* (2003)
	Selective serotonin reuptake inhibitors (e.g. fluoxetine)	Rang *et al* (2003)

In human toxicology, substances which mainly work via these four protein targets need to be studied in the context of individual tissues and organs of individuals (Haber *et al,* 2001; ECETOC, 2006a). In contrast, ecotoxicology ultimately addresses population impacts and hence changes in biological responses measured in selected tissues may be useful but is not normally regarded as an adverse ecotoxicological endpoint. In ecotoxicology, the measurement of such tissue specific (e.g. molecular or enzyme) response to a chemical exposure, for instance, is often termed a biomarker. For the purposes of this report, biomarkers are defined as a change in a biological response (ranging from molecular through cellular and physiological responses) that can be related to exposure to, or toxic effects of, environmental chemicals (after Huggett *et al*, 1992). In principle, biomarkers may be usefully applied to both *in vitro* and *in vivo* tests (for example for measuring hormonal responses such as aromatase induction (Tyler *et al*, 1998; Hutchinson *et al*, 2006) and hence form one 'tool in the toolbox' for read across between chemicals (e.g. aromatase inhibiting drugs) or provide a link between field and laboratory studies. Taking endocrine disrupters as an example, however, it is the adverse effect endpoints (e.g. altered development, growth, and/or reproduction) from efficiently designed chronic tests that are most valuable for calculating ^{adverse}NOEC (No Observed Effect Concentration) or $^{adverse}EC_{10}$ (effective concentration for a 10% response) and subsequently deriving predicted no effect concentrations (PNECs). With current uncertainties, ^{biomarker}NOEC or $^{biomarker}EC_{10}$ data should not be used in isolation to derive PNECs (Hutchinson *et al*, 2006). More broadly, in the context of evaluating background data mentioned in Figure 2.4, it is useful to consider an Adsorption-Distribution-Metabolism-Excretion (ADME) framework for analysing biomarker-type data measured in either *in vitro* or *in vivo* mammalian and non-mammalian tests (Ekins *et al*, 2005). For clarity, this ADME approach can be seen as the first step in a two-phase approach for using the MOA to select a regulatory test species and then design an optimal chronic test. This ADME-linked population assessment approach has recently been described in the context of selecting invertebrate species and endpoints (e.g. biomarkers or population relevant parameters) for assessing endocrine disrupters (Hutchinson, 2007) and is summarised in Figure 2.4.

The summary of the ADME parameters shown in Figure 2.4 should not be interpreted as a recommendation to conduct such routine measurements to support risk assessment. Rather the intention is to develop a framework to use ADME biomarker data where it may arise. Relevant biomarker data may have been generated during *in vitro* or *in vivo* efficacy studies (e.g. information on the neuronal ion channels in insect pests for an insecticide) or during mammalian pharmacology and toxicology studies (e.g. reproductive endocrine effects of a pharmaceutical).

Figure 2.4: Via efficient selection of a regulatory test species, ADME can be usefully linked to population assessment (after Hutchinson, 2007)



Finally, biotransformation of natural and synthetic chemicals (xenobiotics) has been studied extensively in mammals but far less often in many aquatic organisms. This is mainly due to problems associated with isolating adequate quantities of tissues for *in vitro* assays that are used to characterise metabolic activity, resulting from the small size of many of these organisms and also the often low enzyme activities (Chambers and Carr, 1995; Livingstone, 1998; Brown, 2005). Table 2.2 summarises examples of this field, using traditional terminology but also a recent alternative suggestion.

Table 2.2: Traditional and alternative terminology of xenobiotic transformation

Usage	Description
Traditional	Phase I processes – the enzymes of Phase I metabolism (including cytochrome P450 monooxygenase or mixed-function oxygenase (MFO) system, flavoprotein monooxygenase system, epoxide hydratase and flavoprotein reductases) introduce a functional group (-OH, -COOH, - NO_2, etc) into the xenobiotic compound for subsequent Phase II conjugation
Traditional	Phase II processes – the Phase II enzymes (glutathione *S*-transferase (GST), UDP-glucuronyl transferase, UDP-glucosyltransferase, sulphotransferase, and amino acid conjugases) attach a large polar moiety to the intermediate metabolite from Phase I, thereby rendering the final metabolite water soluble and amenable to excretion
Alternative	Josephy *et al* (2005) argue that the Phase I and II biotransformation dichotomy is misleading and instead propose four key biotransformation reactions (oxidations; reductions; conjugations; and nucleophilic trapping processes) that 'overlap' in terms of their roles to first introduce a functional group and subsequently attach a polar moiety to the group prior to excretion

3. FISH AND AMPHIBIANS

Fish and amphibians form an important and visible part of many freshwater and saltwater communities and are susceptible to exposure to chemicals in surface waters. Environmental hazard and risk assessors (EC, 2003; EMEA, 2006; US EPA, 1992) have recognised the need to understand the potential for effects on fish, and to a lesser extent amphibians, and methods have been promulgated allowing standardised testing of these organisms (OECD, US EPA test method references). Typically, effects testing may include acute or chronic test designs requiring approximately 100 to 300 organisms, respectively. As discussed above, there is the need to conserve testing resources and reduce the number of organisms used in toxicity tests. Hence, it is appropriate to use all methods to understand the potential for adverse effects on aquatic organisms. Multiple opportunities exist to apply existing data to understand effects on aquatic organisms resulting in the most appropriate test designs being employed. In many cases, these optimised test designs will reduce the number of organisms used in testing, however, it must also be accepted that for a limited number of chemicals with greater uncertainty in the mode and site of toxic action and with risk ratios approaching 1.0, optimisation of the test design may call for additional species to be tested or test designs requiring additional organisms. Information, which can be used to optimise the test design on aquatic organisms, include: structure activity relationships (SARs), *in vitro* test systems, measured data on mammals, data on related chemicals (close structural analogues and chemicals with similar physical and chemical properties).

3.1 Uptake

The first step in the ultimate manifestation/expression of toxicity in fish or amphibians is the exposure to the chemical and uptake of the chemical by the organism. Uptake typically occurs at the gills or lung, skin and intestinal tract with uptake across the skin and gill/lung the major uptake routes for compounds with log K_{ow} values less than 5 (Nichols *et al*, 1990; Cowan *et al*, 1995; Randall *et al*, 1998). However, not all compounds which are presented to these organ systems are available for uptake due to sorption to organic carbon or particulate material in the water (bioavailability, Di Toro *et al*, 1991), poor lipid solubility, and a molecular size which precludes uptake across a biological membrane (Hayton and Barron, 1990). Chemical uptake across the skin is controlled by hydrophobicity, hydrogen binding, and molecular weight or molecular volume among other parameters (Moss *et al*, 2002). Molecular size restricts uptake due to essentially a physical exclusion effect. For dermal exposure of humans, compounds larger than approximately 500 Daltons do not readily cross the skin, and compounds of 1300 Daltons do not readily pass the mucosa (Bros and Meinardi, 2000). Yamamoto *et al*, (2001) compared uptake of five compounds (350-9100 Daltons) in the lungs and intestine of male rats. For all compounds uptake across the lungs was substantially greater than uptake in the small intestine and other tissues and uptake dropped as molecular weight increased. While there are techniques

to move larger compounds across the surface of the skin (ultrasound, uptake 'enhancers'), these mechanisms are not relevant to the environment. The relative importance of the gill/lung, skin, and intestine in overall exposure is related to the physiology of the organism and the physical and chemical properties of the compound in question. Given their small size, uptake of oxygen, and by analogy substances, across the skin can be significant in larval fish (Saarikoski *et al*, 1986). In fact, Rombough (2002) has shown that larval zebrafish can survive up to day 3 without gill function. By day 7, the gill was needed to provide ionoregulation but not oxygen uptake and by day 14 the gill was needed to provide both ionoregulation and oxygen. In larval Atlantic salmon, cutaneous oxygen uptake is more important than branchial uptake representing 60 to 80% of total oxygen uptake in fish less than 0.1 grams body weight (Wells and Pinder, 1996). The highly vascularised yolk sac contributes to this uptake which declines as the gills develop, the yolk is resorbed and the skin thickens with age. Overall uptake across the skin differs with species and individual size with the gills take on increasing importance in the uptake of chemicals and oxygen with skin responsible for approximately 1 - 8% of total uptake in mature fish (Namdari and Law, 1996; Nichols *et al*, 1996). For tetrachlorobiphenyl, branchial and skin uptakes were shown to be similar in mature medaka and fathead minnows (Lien and McKim, 1993).

Continuous flow of water over the gill with a counter current blood-water exchange interface allows blood oxygen levels to rapidly approach that of the inspired water. The lung uses tidal gas flow into and out of cul-de-sac arrangement of alveoli (Hahn and Farmery, 2003). So, while the function of the gills and lung and the distance between the media and blood are similar, physiological differences lead to differences in oxygen uptake rates and extents and, as a result, will probably lead to differences in the uptake rates and efficiencies of xenobiotics. These differences will likely prevent direct extrapolation of uptake rates and extents between air breathing animals and fish, but relative uptake rates for fish and mammals may be similar. Also, the rate of oxygen consumption has been shown to be directly related to the toxicant uptake rate in several species and sizes of fish (Yang *et al*, 2000).

3.2 Metabolism

Metabolism in vertebrates is generally divided into two types of transformations, phase I transformations which involve oxidation, reduction, and hydrolysis, and phase II reactions which involve conjugation (Lech and Vodicnik, 1985). Many diverse cell and organ systems are capable of metabolising chemicals but the liver, intestine, kidney, and gill/lung play the largest role in fish. Organisms in the environment ranging from fish and frogs to mice, rats, mink, and deer are constantly exposed to trace levels of xenobiotics, both man-made and natural chemicals. Increased hydrophobicity leads to increased uptake rate and storage. Fortunately, biotransformation or metabolism of compounds typically reduce hydrophobicity and increase elimination of the product relative to the parent (Parkinson, 1996). The biotransformation or

metabolism of chemicals in mammalian species has been extensively studied and computer-based programs to predict metabolism based on known metabolic pathways have been developed (Klopman *et al*, 1994; Testa *et al*, 2004). While studies in aquatic vertebrates are not as prevalent, understanding in this area has grown rapidly in the past 15 to 20 years. There are considerable similarities in anatomy and physiology between aquatic vertebrates and mammals, and it is relevant to consider the similarities in metabolic capabilities. While it has been said that metabolism in fish is, in general, not as extensive as in mammals (Parkinson, 1996), certain metabolic pathways function in similar ways and to a similar extent in fish and in mammals (e.g. see PAH metabolism below). Nabb *et al* (2006) compared the metabolic rate in freshly isolated hepatocytes for seven different processes in fish (*Oncorhynchus mykiss*) and rats. Enzyme reactions included O-dealkylation, aromatic ring hydroxylation, aliphatic chain hydroxylation, and a conjugation reaction mediated by glutathione S-transferase. Reaction rates in fish were slower than in rats with rates 3.4 to 18.4 times slower. While reaction types and rates for many reactions are comparable between fish and mammals, there can be significant differences in metabolism. For example, while PCB metabolism is measurable *in vitro* and *in vivo* in mammals, PCB metabolism in fish is slow (White *et al*, 1997) to nonexistent (Murk *et al*, 1994).

The metabolism of PAHs have been well studied in a variety of fish and mammal species due in large part to the observation that benzo[a]pyrene (B[a]P), which requires metabolic activation to bind to DNA, has been observed bound to fish DNA at greater levels than in similarly dosed rodents (Varanishi *et al*, 1986). Overall, it appears the nature and extent of metabolism of two to five ring PAHs are similar in the fish and mammal species which have been studied (Pangrekar *et al*, 2003). As with other reactions, the rate of PAH metabolism in fish is generally slow than in rodents. For example, in addition, the quantitative formation of metabolites in fish and rodents differ. For example, Varanishi *et al* (1986) showed that while similar levels of the 9,10 diol was formed as a percent of the initial oral dose, fish (English sole and Starry flounder) formed greater amounts (as a percent of initial dose) of the 7,8 diol and the 1, and 3, hydroxy metabolites of B[a]P than did rats. Rats tended to form higher concentrations of the 4,5 diol and quinones. This is supported by the work of Pangrekar *et al*, (2003) and Yuan *et al*, (1999). Pangrekar *et al*, (2003) studied the metabolism of chrysene (a four-ring PAH) by brown bullheads noting greater formation of the chrysene 1,2-diol, the proximate carcinogen, in fish than in rat liver microsomes. Yuan *et al* (1999) compared the metabolism of dibenzo[a,l]pyrene (a six ring PAH) by rainbow trout and rats observing the formation of the DB[a,l]P-11,12-diol, the proximate carcinogen, at greater rates in fish than in the rat. Rats formed the K-region diol, the DB[a,l]P-8, 9-diol to a greater extent.

3.3 Sites of pharmacological and toxicological action

Taxonomically, fish and 'higher' vertebrates separated millions of years ago and have evolved independently since that time. Despite this separation, mammal and fish currently share significant biological (genetic, physiological, structural, etc.) and toxicological similarities that enable fish to be used as models for mammalian disease, drug, and toxicological research (and *vice versa*). While many differences in the basic biology of mammals and fish exist, their similarities suggest that mammalian toxicity data can be used to begin to understand responses in fish. This similarity has been clearly show by comparing the relative sensitivities of fish and mouse cell lines *in vitro* (Gülden *et al*, 2005). Fish and mammals have:

- Advanced immune systems which are compromised by environmental toxicants (Weeks and Warinner, 1986);
- endocrine systems which react similarly to a variety of endocrine active compounds (Wester *et al*, 2004);
- neurological (Scalzo and Levin, 2004), cardiac (Heideman *et al*, 2005), developmental (Teraoka *et al*, 2003), and genetic systems which serve as models in mammalian biology.

In many ways, fish are not that physiologically different from mammals. Aquatic vertebrates appear to have very similar enzyme and receptor systems as mammals (Table 3.1) (Evans *et al*, 1993). A large number of receptors in fish have been identified through cloning and sequencing techniques in both the central and peripheral organs of fish (Table 3.1). Overall sequence homologies for receptors and enzymes in fish range from 31 to 88 %, with homologies within the functional regions being much higher. For example, β-adrenergic receptors, peroxisome proliferation activated receptors (PPAR γ), and serotonin receptors (5-HT) have been identified in representative fish species with sequence homologies of 63, 47 and 72 %, respectively (Nickerson *et al*, 2001; Yamaguchi and Brenner, 1997; Andersen *et al*, 2000). Since fish have many similar enzyme and receptor systems, this potentially makes them susceptible to similar biochemical and physiological mechanisms of activation/inactivation. Because of the conservation of enzyme and receptor systems between mammals and these fish, chronic and target organ toxicity identified in mammalian safety assessments is likely to be useful in predicting the need for additional toxicity evaluation in teleosts.

Based on the above analysis, it would seem that most pharmacological and toxicological targets that are present in mammals are also present in fish. If the target has not already been identified in environmental species, then isolation of the DNA for Southern blot analysis using an array of organisms may be a reliable first step. By performing Southern blots one can quickly determine the presence or absence for the gene of a therapeutic target. If the target is not present then, the likelihood that a chronic effect is manifested is low, when compared to a target that is expressed.

Table 3.1: Summary of select receptor and enzyme expression in teleost species (adapted from Huggett et al, 2003)

System	Species	Homology [1]	Reference
Receptor [2]			
PPARγ	*S. salar*	47	Andersen *et al*, 2000
Erα / ERβ	*D. rerio*	47 / 47	Menuet *et al*, 2002
AR	*P. major*	45	Touhata *et al*, 1999
5-HT_{1A} / 5-HT_{1D}	*F. rubripes*	72 / 71	Yamaguchi and Brenner, 1997
β_2-adrenoceptor	*O. mykiss*	63	Nickerson *et al*, 2001
A_{1A}-adrenoceptor	*O. latipes*	61	Yasuoka *et al*, 1996
NMDA (NR1 subunit)	*A. leptorhychus*	88	Dunn *et al*, 1999
GlyR (α subunit)	*D. rerio*	77	David-Watine *et al*, 1999
GluR (R3 subunit)	*Oreochromis*	87	Chang *et al*, 1998
IGF	*D. rerio*	63	Maures *et al*, 2002
IR	*Salmon*	83	Chan *et al*, 1997
AH	*D. rerio*	43	Andreasen *et al*, 2002
OR_1	*D. rerio*	66	Rodriguez *et al*, 2000
NPY	*Cod*	50	Sharma *et al*, 1999
BK_2	*D. rerio*	35	Duner *et al*, 2002
IL_1	*S. salar*	31	Subramaniam *et al*, 2002
GnRH	*O. mykiss*	45	Madigou *et al*, 2000
FSH	*I. punctatus*	53	Kumar *et al*, 2001a
LH	*I. punctatus*	47	Kumar *et al*, 2001b
TSH	*M. saxatilis*	57	Kumar *et al*, 2000
GH	*C. auratus*	42	Lee *et al*, 2001
RARα	*F. rubripes*	58	Wentworth *et al*, 1999
SST	*C. auratus*	62	Lin *et al*, 2000
Enzyme			
Lipoprotein lipase	*O. mykiss*	56	Lindberg and Olivecrona, 2002
P450 1beta-hydroxylase	*O. mykiss*	33	Kusakabe *et al*, 2002
COX	*D. rerio*	67	Grosser *et al*, 2002
P450 aromatase	*D. labrax*	50	Dalla Valle *et al*, 2002
Creatine kinase	*D. rerio*	86	Dickmeis *et al*, 2001.
Caspase-3	*D. rerio*	60	Yabu *et al*, 2001
Stearoyl-CoA desaturase	*C. chanos*	63	Hsieh *et al*, 2001
ADH	*D. rerio*	81	Dasmahapatra *et al*, 2001
AChE	*D. rerio*	62	Bertrand *et al*, 2001

[1] Percent homology to mammalian receptor/enzyme

[2] PPAR = peroxisome proliferator activated receptor, ER = estrogen receptor, AR = androgen receptor, 5-HT = serotonin receptor, B-AR= beta adrenoceptor, A-AR = alpha adrenoceptor, NMDA = N-methyl-D-aspartate receptors, GlyR = glycine receptor, GluR = glutamate receptor, IGF = insulin like growth factor, IR = insulin receptor, AH = aryl hydrocarbon, OR = opiate receptor, NPY = neuropeptide Y, BK = bradykinin, IL = interleukin, GnRH = gonadotropin releasing hormone, FSH = follicle stimulating hormone, LSH = lutinizing hormone, TSH = thyroid stimulating hormone, GH = growth hormone, RAR = retinoic acid receptor, SST = somatostatin , COX = cyclooxygenase, ADH = alcohol dehydrogenase, iNOS = inducible nitric oxide synthase, AChE = acetyl cholinesterase

Knowing that a target receptor with some identity is present in environmental species will trigger further studies aimed at determining how a given compound interacts with this receptor. Traditional receptor binding studies with tissue homogenates or transfected cell lines will determine the degree to which binding will occur, as well as measures of density and affinity. These data can then be compared to the mammalian data and known agonist/antagonists to determine next steps. For instance, researchers determined that the mammalian anti-androgen vinclozalin did not bind to the androgen receptor in the fathead minnow (Makynen *et al*, 2000).

Further evidence in the Atlantic croaker indicates that most anti-androgens bind with low affinity to fish androgen receptors (Sperry and Thomas, 2000). Partial life-cycle studies with these same anti-androgens further validate that these weakly binding anti-androgens elicit endocrine/reproductive responses at concentrations above those expected in the environment (Panter *et al*, 2004).

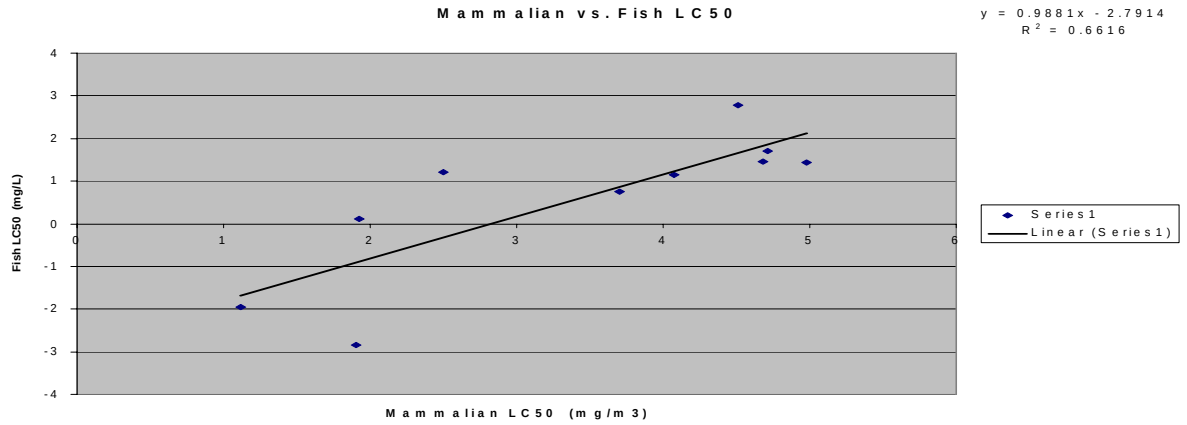
Other methods for determining receptor dynamics include reverse transcriptase-polymerase chain reaction (RT-PCR) to semi-quantitatively/quantitatively determine whether a receptor/enzyme is up-regulated or down-regulated after drug treatment. If the genetic sequence is known, this could be a fairly straight-forward study. Quite often the genetic sequence in various environmental species is not known, therefore degenerate primers would need to be developed which provides additional complexity. Assays utilising antibodies (i.e. ELISA and Western blots), aimed at measuring protein level receptor/enzyme dynamics can also be used. These antibody-based assays can be extremely important if evidence exists that the pharmacological target is not translated into a protein in all species. Antibodies for human targets are available during the drug and often times the methodology for western blots and ELISAs are available. Developing genomic and proteomic technologies may further aid in the determination of potential response pathways. For instance, Wintz *et al* (2006) illustrated that 2,4-dinitrotoluene exposure in fathead minnows results in genomic changes that correlated well with the known mammalian toxicity data.

Several researchers have compared fish and mammal acute toxicity data focusing on the use of acute LC_{50} (mg/L in exposure aquaria) with LD_{50} (mg/kg via an oral exposure) (Kenaga 1981, Rosenkranz *et al*, 1993). Statistically significant relationships suggest utility of data on mammals in predicting fish responses, and visa versa. However, we would expect that extrapolation of a toxic oral dose in mice or rats to a toxic concentration at the respiratory surface in fish would not be as successful as a relationship based on inhalation data in mammals and gill exposure in fish. To evaluate the potential for such a relation, the TF used the ECETOC EAT 3 database (ECETOC, 2003) and the ECETOC report "Guidelines for Setting Occupational Exposure Limits: Emphasis on Data-Poor Substances" (ECETOC, 2006b). Mammalian endpoints (oral LD_{50} (mg/kg), MTD (maximum tolerated dose) (mg/kd day), OEL (occupational exposure level) (mg/m^3), and the inhalation LC_{50} (mg/m^3)), against fish LC_{50} values (mg/L) (see Table 3.2).

Mean fish acute toxicity values for all compounds in the EAT 3 database were obtained and regressed against each mammalian endpoint. All values were log transformed. The equations are shown below:

log (LC_{50} fish) = 0.624 (log LD_{50} mammal) - 0.9715	R^2 = 0.2159	n=63
log (LC_{50} fish) = 0.5668 (log MTD mammal) - 0.3	R^2 = 0.2462	n=63
log (LC_{50} fish) = 0.7566 (log LC_{50} OEL) - 0.0654	R^2 = 0.3899	n=12
log (LC_{50} fish) = 0.9881* (log LC_{50} mammal) - 2.7914	R^2 = 0.6616	n=12

Figure 3.1: Relationship between mammalian and fish lethality concentrations



Despite only having data on 12 chemicals, a statistically significant correlation coefficient was derived (Figure 3.1). Similarly, Delistraty (2000) showed a correlation of 0.68 for rat inhalation LC_{50} and rainbow trout LC_{50} values for 231 chemicals. While additional research is needed to better understand this relationship, its uncertainty and limitations, this effort suggests that mammalian acute inhalation data can be used to provide initial estimates on fish acute toxicity values. With additional data, one might be able to understand the role of other physical and chemical parameters on this acute toxicity relationship, providing potentially useful guidance for fish acute toxicity testing.

In addition to extrapolation of LC/LD_{50} values from mammals to fish, researchers have also begun to determine the applicability of extrapolating effective plasma concentrations (Cmax or AUC) across species (Huggett *et al*, 2003, 2005). In many instances, mammalian studies determine the plasma concentration at which a pharmacological or toxicological response is observed. When considering acute fish toxicity experiments (i.e. 48-96 h LC_{50}), it may be possible to use effective mammalian plasma concentrations as a preliminary surrogate. Reviewing the available data for human pharmaceuticals, there was no instance where the acute

LC_{50} value was less than the mammalian Cmax value (Table 3.3). This suggests that acute testing may be greatly reduced if mammalian plasma values are known.

For chronic studies, focusing on effective plasma concentrations, as opposed to dose concentrations, processes such as absorption, distribution and elimination may be better accounted for across exposures. Moen *et al*, (2004) demonstrated that for 17-α ethinylestradiol (EE2), vitellogenin in rainbow trout was induced when the EE2 plasma concentrations were at or above the human therapeutic plasma concentration. In addition, rainbow trout growth was decreased when plasma concentrations exceeded the human therapeutic plasma concentration for propranolol (Owen *et al*, 2007). While more studies are needed to determine the applicability of this approach, data to date suggest that it may provide a useful screening tool for fish testing.

Table 3.2: Mammalian and fish toxicity data used to generate toxicity extrapolation equations. Mammalian toxicity data taken from ECETOC (2006b) and fish acute toxicity data from ECETOC (2003)

CAS number	Chemical name	log K_{ow}	Mammal LD_{50}	Observed MTD	OEL	Inhalation LC_{50}	Fish acute toxicity
52-68-6	Trichlorfon	0.51	150	50	-	-	16.42
56-23-5	Carbon tetrachloride	2.83	2,920	50	6.41	51270	50
56-38-2	Parathion	3.83	13	2.83	0.1	84	1.29
57-06-7	Allylisothiocyanate	2.15	148	25	-	-	0.077
58-89-9	γ-HCH or γ-BHC	3.72	88	21.24			0.092
60-57-1	Dieldrin	5.4	69	2.25	0.25	13	0.011
67-66-3	Chloroform	1.97	1,186	80	9.9	47702	28
67-72-1	Hexachloroethane	4.14	6,000	20	-	-	1.24
71-43-2	Benzene	2.13	4,894	200	-	-	19.64
71-55-6	1,1,1-Trichloroethane	2.49	12,300	750	556	94492	26.71
72-20-8	Endrin	5.2	17	0.22	-	-	0.0017
72-43-5	Methoxychlor	5.08	5,000	38.03	-	-	0.049
75-09-2	Dichloromethane	1.25	2,136	1,000	-	-	231.8
75-25-2	Bromoform	2.4	1,147	200	-	-	7.1
76-01-7	Pentachloroethane	3.22	4,000	150	-	-	7.3
76-44-8	Heptachlor	6.1	100	3.51	-	-	0.00875
78-59-1	3,5,5-Trimethyl-cyclohex-2-enone	1.7	2,330	500	-	-	140
78-87-5	1,2-Dichloropropane	1.98	2,196	125	-	-	61

Table 3.2: Mammalian and fish toxicity data used to generate toxicity extrapolation equations. Mammalian toxicity data taken from ECETOC (2006b) and fish acute toxicity data from ECETOC (2003) *(cont'd)*

CAS number	Chemical name	log K_{ow}	Mammal LD_{50}	Observed MTD	OEL	Inhalation LC_{50}	Fish acute toxicity
79-00-5	1,1,2-Trichloroethane	1.89	835	92	-	-	52.35
79-01-6	Trichloroethylene	2.42	7,159	1,000	-	-	33.05
79-34-5	1,1,2,2-Tetra-chloroethane	2.39	250	108	-	-	19.39
80-05-7	4,4'-Isopropyl-idenediphenol	3.32	4,040	90	-	-	7.31
85-68-7	Butylbenzyl phtalate	4.73	2,330	240	-	-	1.16
85-68-7	Benzylbutyl phthalate	4.73	2,330	540	-	-	1.16
86-50-0	Azinphos-methyl	2.75	26	7.02	-	-	0.018
86-57-7	1-Nitronaphthalene	3.19	120	81	-	-	9
87-86-5	Pentachlorophenol	5.12	27	10	-	-	0.708
88-06-2	2,4,6-Trichlorophenol	3.69	820	450	-	-	5.23
88-72-2	*o*-Nitrotoluene	2.3	891	12	-	-	30.1
91-23-6	*o*-Nitroanisole	1.73	740	30	-	-	216.1
91-23-6	2-Nitroanisole	1.73	740	90	-	-	216.1
95-50-1	1,2-Dichlorobenzene	3.43	500	120	-	-	11.95
95-80-7	4-Methyl-*m*-phenylene-diamine	0.14	260	7.92	-	-	912
100-42-5	Styrene	2.95	5,000	2,000	107	11800	14.2
100-52-7	Benzaldehyde	1.48	1,300	400			8.77
106-46-7	1,4-Dichlorobenzene	3.44	500	300	122	5000	5.75
106-47-8	4-Chloroaniline	1.83	310	22.5	-	-	24.35
106-93-4	1,2-Dibromoethane	1.96	125	4	-	-	32.1
107-05-1	3-Chloropropene	1.93	700	77	-	-	10
107-06-2	1,2-Dichloroethane	1.48	770	95	-	-	119
108-46-3	Resorcinol	0.8	301	112	-	-	54.75
108-46-3	Resorcinol	0.8	301	225	-	-	54.75
108-90-7	Chlorobenzene	2.84	2,910	120	-	-	5.11
108-94-1	Cyclohexanone	0.81	1,535	315	40.9	32720	595.3
108-95-2	Phenol	1.46	480	225	8	316	15.95
110-80-5	2-Ethoxyethanol	–0.32	3,000	2,000	-	-	26400

Table 3.2: Mammalian and fish toxicity data used to generate toxicity extrapolation equations. Mammalian toxicity data taken from ECETOC (2006b) and fish acute toxicity data from ECETOC (2003) *(cont'd)*

115-29-7	Endosulfan	3.83	43	42.84	0.1	80	0.00144
115-32-2	Dicofol	5.02	1,100	42.39	-	-	0.325
116-06-3	Aldicarb	1.13	1	0.27	-	-	3.81
120-83-2	2,4-Dichlorophenol	3.06	580	225	-	-	6.086
121-75-5	Malathion	2.36	1,375	180	10		0.176
123-31-9	Hydroquinone	0.59	320	50	-	-	0.64
127-18-4	Tetrachloroethylene	3.4	12,982	750	-	-	14.04
140-11-4	Benzylacetate	1.96	2,490	500	-	-	4
140-11-4	Benzylacetate	1.96	2,490	500	-	-	4
298-00-0	Methyl parathion	2.86	14	1.8	-	-	6.15
333-41-5	Diazinon	3.81	250	36	0.1	-	4.90
563-47-3	3-Chloro-2-methylpropene	2.48	580	150	-	-	14
1582-09-8	Trifluralin	5.34	1,400	360	-	-	0.153
1746-01-6	2,3,7,8-Tetra-chlorodibenzo-*p*-dioxin	6.8	0.02	0.00001	-	-	0.009
7487-94-7	Mercuric chloride	–0.22	1	1	-	-	0.074
7632-00-0	Sodium nitrite	–2.37	180	70	-	-	2.91
26628-22-8	Sodium azide	0.16	27	5	-	-	23.92

Table 3.3: Comparison of mammalian effective plasma concentrations (i.e. Cmax) and fish acute LC_{50} values for selected human pharmaceuticals

Compound	Mammalian Cmax (μg/ml)[1]	Fish LC_{50} (μg/ml)[2]
Propranolol	0.05	> 24
17-α ethinylestradiol	0.0001	1.6
Fluoxetine	0.1	0.705
Naproxen	94	560
Caffeine	7.0	151
Nadolol	0.062	>100
Metoprolol	0.045	>100
Ibuprofen	30	173
Paroxetine	0.1	2
Metformin	5	>982

[1] From RxList.com or Physicians Desk Reference Online;
[2] From Huggett *et al*, 2003; Brooks *et al*, 2003; Webb, 2001.

3.4 Excretion

The elimination of chemicals from fish was studied extensively, especially using rainbow trout as an experimental model. For example, McKim *et al*, (1986) showed how trout excreted approximately 50% of ^{14}C-pentachlorophenol over the gills, 30% in faeces and bile, and 20% in the urine. Subsequent studies have demonstrated the importance of excretion via the gills (Nichols *et al*, 1990; Cravedi *et al*, 1993; Randall *et al*, 1998; Fitzsimmons *et al*, 2001), kidney (McKim *et al*, 1999) and biliary system (McKim *et al*, 1986; Lindholst *et al*, 2001). Similar studies have also been reported in selected amphibian species (Noshiro and Omura 1984; Longo *et al*, 2004).

3.5 Summary

This chapter focuses on the use of mammalian data in a weight-of-evidence type of approach to inform the toxicologist on the potential for exposure and effects in fish. The focus is on fish because of to the amount of information available on this taxa but the rationale and approaches can be extended to amphibians. We have discussed the application of mammalian uptake, metabolism and excretion data to help make decisions about the need for similar studies in fish. These specific biological processes were selected for inclusion due to their importance to risk assessment and the general applicability of their function across chemicals. Other biological processes and toxicological endpoint such as endocrine modulation (Gray *et al*, 2002),

carcinogenicity (Chengelis, 1990), immunotoxicology (Sweet and Zelikoff, 2001), and genotoxicity testing (Rao *et al*, 1995), to name but a few, could have been discussed and should be included in a weight-of-evidence approach to defining the appropriate test approach for fish and amphibians, or in eliminating aquatic vertebrate testing altogether (see Tables 3.4 and 3.5 for examples of current and prospective draft regulatory test methods).

The Task Force proposes a flow-chart for an intelligent test strategy that applies the weight-of-evidence gathered from non-fish animal studies to stimulate debate in this important area. Currently, we see the developing ITS approach as continuing to evolve in light of emerging scientific knowledge and regulatory initiatives around the MOA philosophy for mammals and other species (Haber *et al*, 2001; Clewell, 2005; Ekins *et al*, 2005). As new tools are critically evaluated and validated, additional opportunities to minimise or avoid acute and chronic testing of fish and amphibians will become clearer (Huggett *et al*, 2003; Hutchinson *et al*, 2003; Jeram *et al*, 2005; Owen *et al,* 2007).

Table 3.4: Examples of test guidelines for sublethal effects assessment in fish

Identification	Description	Species	Endpoints	Reference
ASTM E 1241-88	Standard guide for conducting early life-stage toxicity tests with fishes	Fathead minnow Medaka Rainbow trout Sheepshead minnow Zebrafish	Embryo hatching, larval development and growth	ASTM, 1988
ISO 15088-1 (draft)	Water quality - determination of the acute toxicity of waste water to zebrafish eggs	Zebrafish	Embryo hatching and development	ISO, 2005
OECD TG210	Fish, early-life stage toxicity test (adopted: July 1992)	Fathead minnow Medaka Rainbow trout Zebrafish	Embryo hatching, larval development and growth	OECD, 1992a
OECD TG215	Fish, juvenile growth test (adopted: January 2000)	Medaka Rainbow trout Zebrafish	Growth	OECD, 2000a
OECD new TG (draft)	Fish 21 d endocrine screening test guideline (validation in progress)	Fathead minnow Medaka Three-spined stickleback Zebrafish	Secondary sexual characteristics and vitellogenin	OECD, 2006a

***Table 3.4: Examples of test guidelines for sublethal effects assessment in fish** (cont'd)*

Identification	Description	Species	Endpoints	Reference
OECD new TG (draft)	Fish sexual development test guideline (validation in progress)	Fathead minnow Medaka Zebrafish	Gonad histology, somatic growth, secondary sexual characteristics and vitellogenin	Holbech *et al*, 2006
US EPA OPPTS 850.1400	Fish, early-life stage toxicity test (adopted: April 1996)	Fathead minnow Medaka Rainbow trout Zebrafish	Embryo hatching, larval development and growth	US EPA, 1996a
US EPA new (draft)	Fish short-term reproduction test (validation in progress)	Fathead minnow	Fecundity, secondary sexual characteristics and vitellogenin	Ankley *et al*, 2001
US EPA OPPTS 850.1500	Fish life-cycle toxicity **(a**dopted: April 1996)	Fathead minnow Sheepshead minnow	F_0 embryo hatching, larval development and growth and fecundity; F_1 embryo hatching, larval development and growth	US EPA, 1996b

Table 3.5: Examples of test guidelines for sublethal effects assessment in amphibians

Identification	Description	Species	Endpoints	Reference
ASTM E-1439-98	Standard guide for conducting the FETAX assay (revised 1998)	*Xenopus laevis* *Xenopus tropicalis*	Embryo development	ASTM, 1998a
ISO FDIS 21427-1 (draft)	Water quality - evaluation of genotoxicity in amphibians	*Xenopus laevis and Pleurodeles waltl*	Micronuclei in peripheral erythrocytes	ISO, 2006

4. INVERTEBRATES

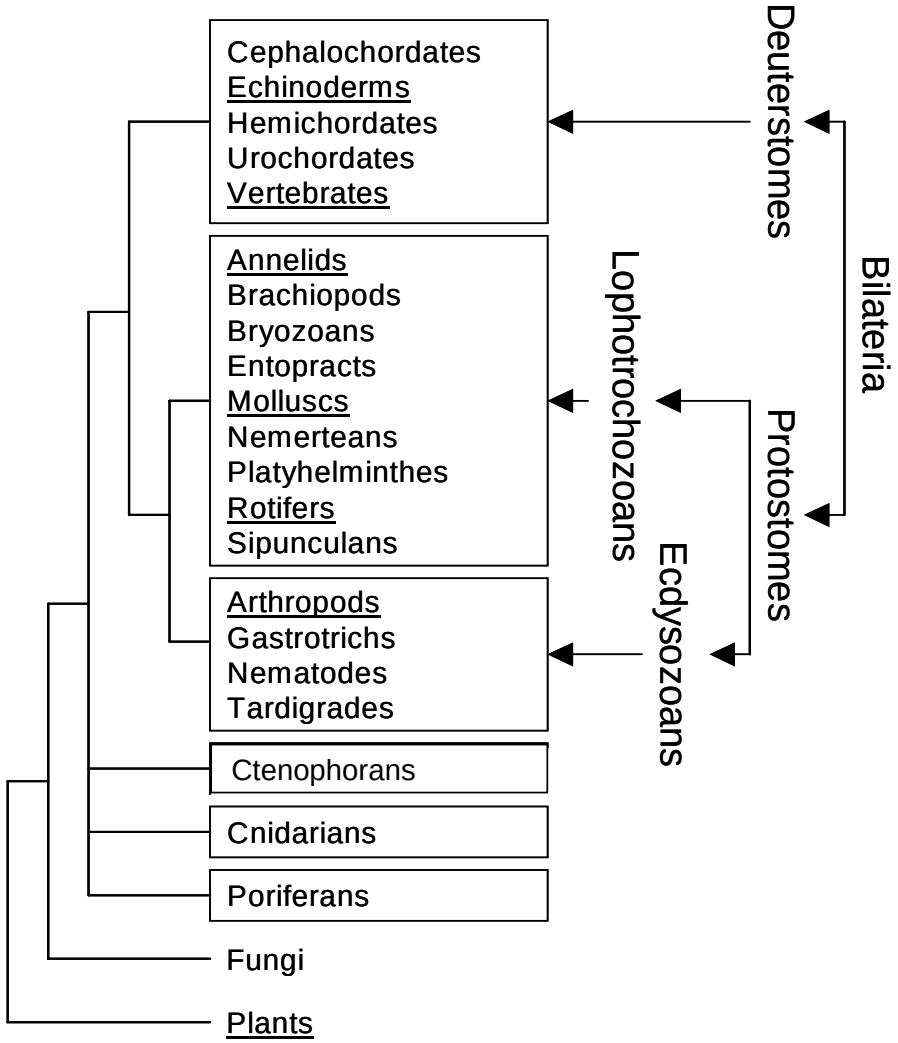
A number of regulatory test protocols have been developed using aquatic invertebrates, reflecting their ecological importance and their economic value in aquaculture. Invertebrates comprise roughly 95% of all animal species (Wilson, 1999) and a number of invertebrate taxa are often used when conducting ERAs and for Whole Effluent Assessment (WEA). Knowledge from invertebrates is also integral to the derivation of Environmental Quality Standards (Zabel and Cole, 1999) and in the wider context of the European Water Framework Directive (Allan *et al*, 2006; Hering *et al*, 2006). Despite this rich complexity of morphological species differences, the evolutionary biology (phylogeny) of invertebrates shows increasingly well-understood molecular themes across different taxa (Adoutte *et al*, 2000; Philippe and Telford, 2006; Schubert *et al*, 2006). For example, the Ecdyzoa brings together the arthropods (crustaceans and insects), nematodes and other invertebrate phyla frequently used in ecotoxicology (Figure 4.1). These phyla all share the presence of a molting cuticle (albeit of very different composition, chitin in arthropods, collagen in nematodes), hence the name 'Ecdysozoa'. Arthropods represent the largest invertebrate taxa and are widely used in freshwater (e.g. chironomids and daphnids) and marine (e.g. copepods and mysids) regulatory risk assessments for single substances and effluents. In the context of this report, these often share some important physiological features that may affect their response to contaminants (e.g. development and reproduction controlled by ecdysteroid and juvenile hormones). Other important aquatic phyla that have been well studied and are periodically used in ecotoxicology include annelids, ascidians, echinoderms, molluscs, rotifers and tunicates (Hutchinson, 2007). From the perspective of defining sublethal endpoints (e.g. growth, development and reproduction – see ADME Figure 1.4) for use in ERA, several of these non-arthropod phyla share some common physiological elements (e.g. reproductive endocrinology based on androgens, oestrogens and related steroids) (deFur *et al*, 1999). This chapter is not intended to be an exhaustive review of invertebrate zoology but focuses on the ADME principles that have potential value as a guide in MOA ecotoxicology. The chapter distinguishes between arthropods and non-arthropod invertebrates given the often marked differences in their sensitivities to chemicals (Maltby *et al*, 2005).

4.1 Uptake

Aquatic invertebrates may be exposed to chemicals via the surrounding water, the diet or in some cases via sediments (e.g. ingestion by annelids). The life stage of animals may be a significant factor affecting the potential for chemical exposure (e.g. bivalve molluscs are usually filter feeders throughout their lives whereas aquatic insects have distinct larval versus adult life stages) (Barnes *et al,* 2001). Also, soft-bodied invertebrates are characterised by integumental nutrient transport processes. These processes are qualitatively similar to the Na-dependent transporters of intestinal epithelia, have kinetic and energetic characteristics that make them particularly well

suited for accumulating organic compounds and providing a major energy source from the extremely low substrate concentrations found in surface waters (Wright and Manahan, 1989). For practical reasons, the majority of published data relate to macroinvertebrates, especially with respect to the need to consider contamination risks to shellfish and subsequent human and wildlife exposure.

Figure 4.1: Modern animal phylogeny based on recent molecular data, representing an important aspect of MOA ecotoxicology (abbreviated version based on Adoutte et al, 2000) with taxa used in regulatory ecotoxicology underlined)



However, the conservation of gills as both respiratory and feeding organs across diverse invertebrate taxa (e.g. echinoderms and molluscs) hypothetically suggests that xenobiotic uptake patterns might be consistent across these invertebrate taxa. In a mechanistic sense, this will fundamentally depend on the following xenobiotic uptake mechanisms: (1) diffusion through the lipid; (2) diffusion via aquaporins ('protein pores') that traverse the membrane; (3) combination with transmembrane carrier proteins; or (4) pinocytosis (after Rang *et al*, 2003; Shitara *et al*, 2006).

4.1.1 Uptake mechanisms in arthropods

Depending upon the life stage and species, uptake of organic chemicals into crustaceans and aquatic insect larvae can occur via the water column (over the gills or cuticle), diet or theoretically via ingested sediment. More broadly, lipophilic insecticides (e.g. organochlorines and synthetic pyrethroids) are effective in terrestrial insects as they are able to quickly cross the moulting cuticle. Several aquatic invertebrates have been shown to have high total lipids (with crustaceans up to almost 12% and mussels almost 8% whereas values for fish may be rather lower; summarised in Table 4.1). Arthropod dietary uptake studies are rarely conducted, although Gomes *et al* (2004) showed it to be a minor factor in oestrone bioconcentration studies with *Daphnia magna*. For feeding studies, gut passage time in *Daphnia magna* and crustaceans also need to be considered (Gillis *et al*, 2005). In a regulatory context, however, chemicals that are potentially capable of depositing on, or sorbing to, sediments to a significant extent are often assessed for toxicity to sediment-dwelling invertebrates (e.g. freshwater chironomids or marine amphipods). In general, substances with a K_{oc} < 500 - 1000 L/kg are not likely sorbed to sediment. To avoid extensive testing of chemicals a log K_{oc} or log K_{ow} of ≥ 3 is used as a trigger value for sediment effects assessment (section 3.5.2, EU Technical Guidance Document) (EC, 2003). Theoretically, the uptake of chemical-spiked sediments could be a 'triple mechanism' including sediment particle ingestion, gills and cuticle via the interstitial 'pore' water.

From a theoretical point of view, uptake of chemicals across the body surface of invertebrates is likely to be influenced by a number of factors. Baird and van den Brink (2007) looked for patterns of sensitivity that correlated with a variety of organism traits in the Arthropoda, concluding that 71% of a species' sensitivity to chemicals was due to four traits (morphology, life-history, physiology and feeding ecology). Recently, Buchwalter *et al,* (2002) used a combination of tritiated water (as a quantitative surrogate for exposed cellular surface area) and [^{14}C]chlorpyrifos to show that respiratory strategy is a major determinant of organophosphate uptake in aquatic insects. Air-breathing aquatic insect taxa were significantly less permeable to both $^{3}H_20$ and [^{14}C] chlorpyrifos. The degree of permeability of the exoskeleton may also be of relevance.

Table 4.1: Total lipid content in aquatic invertebrates compared to fish

Organism	Tissue	Total lipids (mean % of dry weight)	Reference
Amphipod	Whole animal	18.3 - 45 23 - 30	Gardner *et al,* 1985 Lehtonen, 1996
Annelids	Whole animal	2.6 - 6.5	Barnes and Blackstock, 1973
Barnacles (2 species)	Whole animal	6.5 - 11.8	Barnes and Blackstock, 1973
Copepods	Whole animal	58	Barnes and Blackstock, 1973
Crab	Hepatopancreas	15.4	Barnes and Blackstock, 1973
Crab	Muscle	4.0	Barnes and Blackstock, 1973
Echinoderms	Soft tissues	11.1 - 14.4	Barnes and Blackstock, 1973
Fish (cod)	Eggs Larvae	13.5 10.2 - 21.2	Petersen and Kristensen, 1998
Fish (herring)	Eggs Larvae	11.4 15.2 - 19.4	Petersen and Kristensen, 1998
Fish (turbot)	Larvae	18.2 - 26.0	Petersen and Kristensen, 1998
Fish (zebrafish)	Eggs Larvae Juveniles	10.3 19.6 11.2	Petersen and Kristensen, 1998
Fish (herbivorous)	Whole animal	0.72	Covaci *et al,* 2006
Fish (omnivorous)	Whole animal	1.29	Covaci *et al,* 2006
Fish (piscivorous)	Whole animal	0.44	Covaci *et al,* 2006
Gammarids	Whole animal	3.2	Barnes and Blackstock, 1973
Mussel	Soft tissues	8.1	Barnes and Blackstock, 1973

This has been investigated for some aquatic insects, where extent of uptake of chlorpyrifos was shown to be related to be water permeability of the exoskeleton (Buchwalter *et al*, 2003). In theory, permeability will be determined by the degree of chitinisation in the cuticle of arthropods and may be influenced by whether the organism 'breathes by diffusion' across its body surface (e.g. mollusc larvae) or uses gills (e.g. dragonfly larvae); the latter being likely to enhance uptake (see Van der Ohe and Liess, 2004; Sherratt *et al*, 1999; Stark, 2005, for a wider discussion of interspecies sensitivity traits). Landrum *et al* (2001) showed that for organic chemicals with a log K_{ow} of 6 of more, water-only uptake was inversely proportional to the size of the amphipod. It has also been observed that smaller crustaceans have much higher rates of uptake from sediment than large or medium sized amphipods (Landrum *et al,* 2001). No information could be found on organic chemicals to distinguish between surface adsorption versus body absorption but this has

been studied on metals (Vijver *et al*, 2005). Hare (1992) and Rainbow (2002) have thoroughly reviewed work on trace metal bioaccumulation in aquatic invertebrates.

4.1.2 Uptake mechanisms in non-arthropods

In an ADME context, compared to fish and mammals, there are relatively few mechanistic studies published on uptake mechanisms in non-arthropod invertebrates. Annelids, echinoderms and molluscs are noteworthy examples given the frequent use of these organisms in OECD, ISO and other regulatory test guidelines. A number of bioconcentration studies with annelids (e.g. the freshwater oligochaete *Tubifex tubifex*) have been applied to metals (Ingersoll *et al*, 1995; Redeker *et al*, 2004), organochlorines (Egeler *et al*, 1997), aromatic hydrocarbons (Leppänen and Kukkonen, 2000) and nitrotoluenes (Conder *et al*, 2004) and phthalate esters (Wofford *et al*, 1981). For marine annelids, polychaetes have been extensively used for laboratory studies of chemicals uptake from food, water or sediments (Ahrens *et al*, 2001; Timmermann and Andersen, 2003; Ruus *et al*, 2005). The age, size and diets of annelids are clearly important issues in studies of xenobiotic uptake. For example, Selck and Forbes (2004) stress the importance of worm surface area for metal uptake from the dissolved phase (polychaetes absorb diet-bound cadmium and that absorption does not relate to the bulk sediment concentration alone but rather to the amount of contaminated food or sediment passing through the gut). Ahrens *et al* (2001) concluded that for hydrocarbons, gut chemistry is likely to set upper and lower limits on absorption efficiency of food and sediment-bound in marine polychaetes, while body size, and in the case of food uptake, differences in gut passage time probably account for most of the variance within this range (the gut passage time in polychaetes was nearly 5 to 10 times shorter in juveniles than in adults).

For the echinoderms phyla, sea urchins (echinoids) have been used for bioaccumulation studies of metals (Warnau *et al*, 1996) and organochlorines (Zeng *et al*, 2003: Danis *et al*, 2005), however, no tissue specific uptake rates were reported. No studies of xenobiotic uptake could be found for marine starfish (crinoids). In terms of natural substances, Schneider and Whitten (1987) described a unique nucleoside uptake and transport system in sea urchin embryos that is quite different from those previously described in mammalian cells. The implications of these observations for environmental chemicals have not been studied.

Freshwater bivalve molluscs (e.g. *Dreissena polymorpha*) are periodically used for bioconcentration studies (Voets *et al*, 2006), while there are few studies published for freshwater prosobranchs such as *Potamopyrgus antipodarum* (Jensen *et al*, 2001). Marine mussels and oysters have been extensively used for field monitoring and for experimental purposes to support ERA (e.g. Gardinali *et al*, 2004; ASTM, 2001). It is also known that the embryo-larval stages of bivalve molluscs are able to take up free amino acids, peptides and other organic compounds

across the body surface, prior to the maturation of the gills system (Wright and Manahan, 1989; Preston, 1993). The implications for this route for xenobiotic exposure have not been published in the available invertebrate literature.

4.2 Metabolism

Biotransformation of natural and synthetic chemicals has been studied extensively in mammals and fishes, but far less often in many aquatic invertebrates. This is mainly due to problems associated with isolating adequate quantities of tissues for *in vitro* assays that are used to characterise metabolic activity, resulting from the small size of many of these organisms and somewhat lower enzyme activities compared with mammals (Chambers and Carr, 1995; Livingstone, 1998; Brown, 2005). Traditionally and more recent approaches to biotransformation have been defined as:

- Traditional Phase I processes – these enzymes of Phase I metabolism (including cytochrome P450 monooxygenase or mixed-function oxygenase (MFO) system, flavoprotein monooxygenase system, epoxide hydratase and flavoprotein reductases) introduce a functional group (–OH, –COOH, $–NO_2$, etc) into the xenobiotic compound for subsequent Phase II conjugation (see Table 4.2).
- Traditional Phase II processes – the Phase II enzymes (glutathione *S*-transferase (GST), UDP-glucuronyl transferase, UDP-glucosyltransferase, sulphotransferase, and amino acid conjugases) attach a large polar moiety to the intermediate metabolite from Phase I, thereby rendering the final metabolite water soluble and amenable to excretion (see Table 4.2).
- Modern terminology from Josephy *et al*, (2005) – with four key biotransformation reactions (oxidations; reductions; conjugations; and nucleophilic trapping processes) that 'overlap' in terms of their roles to first introduce a functional group and subsequently attach a polar moiety to the group prior to excretion.

4.2.1 Metabolism in arthropods

Many arthropod species can actively metabolise xenobiotics. Research into pesticide resistance has meant the metabolic processes have been intensively studied in insects (Scott, 1999) and some of this biochemical data can be used as a guide for crustaceans. For oxidases, Nelson (1998) observed that there are currently 37 cytochrome P450 families identified in the animals. Of these only 16 are found in mammals. The other 21 families are exclusively from insects (six families), molluscs (two families) and the nematode *C. elegans* (13 families). Evidence for cytochrome P450 activity has been observed in amphipods (Ankley and Collyard, 1995), chironomids (Ankley and Collyard, 1995; Sturm and Hansen, 1999) and daphnids (Barata *et al*,

2001; Akkanen and Kukkonen, 2003; Kashian, 2004; El-Merhibi *et al*, 2004; Ikenaka *et al*, 2006) (Table 4.2).

Table 4.2: Examples of biotransformation reactions measured in selected aquatic invertebrates compared with fish (after Livingstone 1998)

Enzyme amount or activity (mg^{-1} protein)	Fish	Echinoderm	Crustacean	Mollusc
Total cytochrome P450 content (pmol mg^{-1})	322 ± 35 (38)	63 ± 12 (2)	328 ± 70 (14)	73 ± 10 (9)
BPH activity[a] (pmol $min^{-1}.mg^{-1}$)	307 ± 208 (9)	8.4 ± 5.5 (2)	40 ± 31 (10)	21 ± 3.4 (5)
FMO activity[b] (nmol $min^{-1}.mg^{-1}$)	0.35 ± 0.21 (3)	ND	ND	0.63 ± 0.13 (2)
UDPGT activity[c] (pmol $min^{-1}.mg^{-1}$)	394 ± 105 (10)	ND	10 (1)	3800 (1)
GST activity[d] (nmol $min^{-1}.mg^{-1}$)	776 ± 139 (12)	11 to 52 (5)	1202 ± 842 (6)	4230 ± 2184 (14)

Note: Activities g-1 wet weight are for liver (fish), pyloric caeca (echinoderm), hepatopancreas (crustacean) and digestive gland (mollusc) and measured in cytosolic (GST) or microsomal fractions. Values are mean ± SEM or range (n = number of species). ND = no data, a = B[a]P hydroxylase, b = Flavoprotein monooxygenase (substrate: N,N-dimethylaniline, methimazole, others), c = UDP-glucuronyl transferase (substrate: p-nitrophenol) and d = GST (substrate: 1-chloro-2,4-dinitrobenzene).

Cytochrome P450-mediated metabolism may lead to either detoxification or activation of xenobiotics (James, 1989; Ankley and Collyard, 1995; Drenth *et al*, 1998). For example, malathion and diazinon require P450-mediated metabolic activation to exert toxicity (Baldwin and LeBlanc, 1994a; Ankley and Collyard, 1995), while many insecticides such as the pyrethrins are detoxified by P450 through the same mechanism (Ankley and Collyard, 1995). More recently, a number of biochemical studies have been undertaken in *Daphnia magna* in order to characterise antioxidant activity (Borgeraas and Hessen, 2000), acetylcholinesterase (Fisher *et al*, 2000), multiple steroid hydroxylase (Baldwin and LeBlanc, 1994b) and the biotransformation of testosterone by phase I and II detoxication enzyme activities (Baldwin and LeBlanc, 1994a). Typically, total P450 protein and associated enzymatic activities in invertebrates are found to be 10-fold lower than in mammals (Livingstone, 1998). In addition, several studies have failed to measure P450-catalysed activities *in vitro* in crustaceans (Singer *et al*, 1980; James and Little, 1984; Baldwin and LeBlanc, 1994a) possibly because of the presence of endogenous inhibitors in microsomal preparations (James, 1989). Therefore, the *in vitro* detection of both aromatase and hydroxylation activities in crustaceans are also expected to be affected by similar problems. Overall, studies of pesticide resistance and other research suggest overall that arthropods can actively metabolise many diverse xenobiotics.

4.2.2 Metabolism in non-arthropods

Compared to crustaceans and insects, there is a paucity of data on metabolic processes in freshwater non-arthropods, although a number of marine species have been studied. In terms of annelids, Ankley and Collyard (1995) did not detect evidence of cytochrome P450 activity in freshwater oligochaetes (*Lumbriculus variegatus*) (see also other examples in Table 4.2). However, evidence has been gathered for metabolic activity in marine polychaete adults (Lee *et al*, 1979; Livingstone, 1991; Forbes *et al*, 1996; Giessing and Lund, 2002; Rewitz *et al*, 2006) and polychaete embryos (e.g. Jha *et al*, 1996).

Echinoderms have also been shown to have active steroid metabolism and cytochrome P450 monooxygenase system (Den Besten *et al,* 1991). A major theme of work has been to understand the metabolism of steroids (androgens, progestogens and oestrogens), as demonstrated by several high quality studies (Voogt *et al*, 1986 and 1992; Hines *et al*, 1992; Wasson and Watts, 2000).

Molluscan metabolic processes have also been investigated in terms of cytochrome P450 in adults and embryos (Dixon *et al*, 1985; Livingstone *et al*, 1989), essentially showing levels at a maximum of 10-times the mammalian tissue levels. Again, a major theme of work has been to understand the metabolism of steroids and the impacts of alkyltins and other contaminants on these processes (De Longcamp *et al*, 1974; Morcillo *et al*, 1999; Gooding and LeBlanc, 2001; Janer and Porte, 2006).

4.3 Sites of pharmacological and toxicological action

As the purpose of this report is to illustrate the principles of MOA ecotoxicology, this section gives examples of known sites of action for the four principle protein targets shown in Figure 2.3. Through invertebrate genomics and proteomics, this area of knowledge is expanding exponentially at the current time, fuelled by interest in evolutionary biology (Adoutte *et al*, 2000; Hervé and Telford, 2006; Schubert *et al*, 2006; Wilson *et al*, 2006) and biomedical applications (Dengg and van Meel, 2004). Recently published molecular information suggests a striking difference between the Ecdyzoa (including crustaceans, insects and nematodes) versus the chordates (including mammals), echinoderms and molluscs. As for other groups of organisms, the current weight-of-evidence suggests that specifically active chemicals may affect invertebrate systems via the following pharmacological MOA groups:

- Receptor targets – for example, steroidal androgens, oestrogens and thyroid hormones all operate via nuclear hormone receptors which are also found in fish, amphibians and other vertebrates and typically show high acute: chronic ratios (i.e. ACR > 1000) in terms of chronic developmental and reproductive effects (Ankley *et al*, 2005). Currently, early

evidence suggests that these hormones and their mimics may be active in echinoderms and molluscs (Thornton *et al*, 2003; Janer and Porte, 2006) but are inactive in Ecdyzoan tissue *in vitro* assays (see Oberdörster *et al* (1999) and Dinan *et al* (2001) and have low ACR values in chronic ecotoxicity tests with aquatic arthropods (i.e. ACR < 100) (Hutchinson, 2002; Breitholz *et al*, 2006). A second (but less well studied) example of receptor mediated compounds is the mammalian beta-adrenoreceptor blockers ('beta-blocker drugs') that appear to have low ACRs in crustaceans when compared with fish (Huggett *et al*, 2001; Owen *et al*, 2007).

- Enzyme targets – some enzymes appear to be widely conserved across the chordates (e.g. mammals and fish) and Ecdyzoa, for example, acetylcholinesterase that occurs in all animal species that have a nervous system. In contrast, aromatase (cytochrome CYP19) is found in chordates, echinoderms and molluscs but appear to be absent in the Ecdyzoa. Similarly, the steroid enzyme synthetic pathways can be affected by azole fungicides – see the ketoconazole case study. These contrasting examples illustrate the complexity of comparative enzyme function studies between difference groups of animals. Moreover, the same enzyme may have different roles in different species (e.g. prolactin controls lactation in mammals but controls osmoregulation in fish (Norris, 1997)).
- Ion channel targets – ion channel proteins (e.g. sodium, chloride and calcium ion channels) are a key component of the nervous system (Ray and Fry, 2006). As the basic design of the nervous system is conserved across all the invertebrate phyla, there is no reason to expect that any one group of invertebrates would be especially sensitive to chemicals that target ion channels (e.g. pyrethroids). Any marked interspecies of inter-life stage differences might be more likely due to differences in absorption (possibly due to varying tissue lipid content) or metabolism (e.g. levels of cytochrome P450).
- Transporter protein targets – proteins that act as trans-membrane transporters (e.g. sodium pump (Na-K-ATPase) and proton pump) (Lucu and Towle, 2003; Horisberger, 2002). In general, such essential cellular processes are expected to occur throughout the animal kingdom, hence there is no reason to expect that any one group of invertebrates would be especially sensitive to transporter protein inhibitors (e.g. SSRIs). Any marked interspecies of inter-life stage differences might be more likely due to differences in absorption (possibly due to varying tissue lipid content) or metabolism (e.g. levels of cytochrome P450). However, there is currently no available empirical evidence with which to test this hypothesis.
- This suggests three 'MOA rules of thumb': (i) for specifically MOA chemicals, marked interspecies may be expected if the protein target is involved in development or reproductive processes; (ii) for proteins that have a fundamental cellular function (e.g. neurotransmitter regulation, trans-membrane ion transport) inherent interspecies differences are not expected to be major; (iii) chemicals may operate by more than one MOA, depending on the concentration [for example, a low concentration of tamoxifen acts on the oestrogen receptor

whereas high experimental concentrations can induce DNA damage (Pagano *et al*, 2001), while in mammals there is evidence of a similar patter for carbamates (Pope *et al*, 2005)].

Table 4.3: Use of invertebrate cell lines can give valuable information for mammalian-type receptor mediated substances (adapted from Dinan et al, 2002)

Substance	Max. conc.	Agonist activity	Antagonist activity
Bisphenol A	10^{-3}M	Inactive	Active; $EC_{50} = 1.0x10^{-4}$M
Diethystilbestrol	10^{-3}M	Inactive	Inactive
Oestrone	10^{-5}M	Inactive	Inactive
Oestradiol	10^{-5}M	Inactive	Inactive
Ethinylestradiol	10^{-3}M	Inactive	Weak at $\geq 2.5x10^{-5}$M
20-hydroxyecdysone[1]	10^{-3}M	Active; $EC_{50}=7.5x10^{-9}$M	Inactive
Lindane	10^{-3}M	Inactive	Active; $EC_{50} = 3.0x10^{-5}$M

[1] In contrast, the natural ligand 20-hydroxyecdysone shows activity at 5 x 10^{-8}M.

4.4 Excretion

Livingstone (1998) concluded that overall, excretory mechanisms in invertebrates are very limited. He therefore essentially made a 3-part conclusion(s) for different aquatic organisms:

- In crustaceans: xenobiotics readily taken up into soft tissues → moderate Phase I and II biotransformation → moderate excretion of metabolites;
- in echinoderms and molluscs: xenobiotics readily taken up into soft tissues → low Phase I and II biotransformation → slow excretion of parent compounds mostly via diffusion;
- in fish: xenobiotics readily taken up into soft tissues → rapid Phase I and II biotransformation → rapid excretion of metabolites.

There is also evidence that several invertebrates contain multi-drug resistance (MDR) proteins, for example in clams, sponges, mussels, oysters and worms (as well as in plants and fish) (Bard, 2000). Both natural products and anthropogenic contaminants found in the aquatic environment appear to be substrates and inducers of the multi-xenobiotic resistance transporter in aquatic organisms. MDR proteins are a protein superfamily (among the largest and most widespread protein superfamilies known) and are responsible for the active transport of a wide variety of compounds across biological membranes including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, drugs and other xenobiotics. In mammalian studies, the 190 kDa multi-drug resistance protein 1 (MRP1) is a founding member

of a subfamily of the ATP binding cassette (ABC) superfamily of transport proteins and was originally identified on the basis of its elevated expression in multi-drug resistant lung cancer cells. In addition to its ability to confer resistance in tumour cells, it is ubiquitously expressed in normal tissues and is a primary active transporter of GSH, glucuronate and sulfate conjugated and unconjugated organic anions of toxicological relevance. MRP homologues have been described in yeast and nematodes and the vascular plant *Arabidopsis thaliana* (Leslie *et al*, 2001).

4.4.1 Excretion in arthropods

There are few studies available to quantify the different excretion processes across the invertebrates. For arthropods, chemical excretion (either the parent compound or a metabolite) can theoretically occur via respiration, diffusion over the integument, ecdysis (moulting), defaecation or reproduction (either gamete production in sexually reproducing animals or neonate production in clonal animals). Experimental studies on xenobiotic excretion in aquatic arthropods are rare, however, Landrum *et al* (2001) showed that the elimination rate constant (k_e) declined exponentially for organochlorines with increasing log K_{ow} (and as log K_{ow} increased the influence of amphipod mass on k_e was greatly reduced). As a methodological example for aquatic arthropods, recent work on the terrestrial beet armyworm (Lepidoptera: Noctuidae) showed that use of ^{14}C-labelled methoxyfenozide, defined the pattern of absorption in body tissues and excretion via faeces in last-instar armyworm larvae of a greenhouse-selected strain and compared the results with those from the laboratory susceptible strain of armyworm (Smagghe *et al*, 2003). This method allowed measurement of a rate of excretion was about twice as high in the greenhouse-selected strain, resulting in a more rapid clearance of insecticide amounts from the insect body.

4.4.2 Excretion in non-arthropods

For non-arthropod invertebrates, chemicals could theoretically be excreted via respiration, diffusion across the soft external tissues, defaecation or reproduction (either gamete production in sexually reproducing animals or neonate production in clonal animals). In all cases, invertebrate eggs are typically lipid rich (Table 4.1) and this act as a potentially significant excretion mechanism for lipophilic contaminants. Livingstone (1998) concluded that overall, excretory mechanisms in molluscs are very limited. This may be species specific, for example, and Kurelec (1998) observed that many marine invertebrates are known to live and reproduce in environments rich in potentially toxic chemicals. Chemical concentrations in tissues of these organisms are often maintained at levels below those in the surrounding environment. Kurelec (1995) suggested that the primary molecular detoxification mechanism, the multi-xenobiotic response (MXR), could be implicated in these systems. This phenomenon is similar to multi-drug

resistance (MDR) in mammalian cells described above. Recently, Hamdoun *et al* (2002) have provided further evidence that the differential sensitivity of different echinoderms species appears to be linked to the activity of multi-drug resistance proteins.

4.5 Summary

In current regulatory ecotoxicity testing, a variety of invertebrate species are used to assess the toxicity of chemicals. Perhaps most widely used is the crustacean *Daphnia magna* (OECD, 1998a). Standard test methods are available for a range of other species, including the insect *Chironomus* sp. (OECD, 1998b) (see Table 4.4). For estuarine and marine species, examples of sublethal testing protocols include bivalve molluscs (ASTM, 2004c), echinoderms (ASTM, 2004b) and mysid crustaceans (ASTM, 2003) (see Table 4.5). The availability of these regulatory methods for marine invertebrates may be a reflection of the wider biodiversity of marine versus freshwater ecosystems (Wilson, 1999). In an ERA context, this is recognised by the need to better understand the scientific basis for freshwater to saltwater ecotoxicity extrapolations and *vice versa* (Leung *et al,* 2001; Wheeler *et al,* 2002).

To summarise, for practical reasons, Chapter 4 is not intended to be an exhaustive discussion of all aspects of invertebrate phyla used in ecotoxicology but rather seeks to focus on key principles using examples from taxa already used in regulatory ecotoxicology and water quality eco-assessments. Where possible, the following widely studied species can be used for illustrative purposes: *Americamysis bahia* (marine crustacean), *Antedon mediterranea* (echinoderm; crinoid), *Brachionus calyciflorus* (freshwater rotifer), *Chironomus riparius* (freshwater insect), *Ciona intestinalis* (marine tunicate) *Daphnia magna* (freshwater crustacean); *Mytilus edulis* (marine bivalve mollusc), *Paracentrotus lividus* (echinoderm; echinoid), *Platynereis dumerilii* (marine annelid), *Potamopyrgus antipodarum* (freshwater prosobranch mollusc) and *Tubifex tubifex* (freshwater annelid). These examples seek to reflect the distinct biochemical, morphological and physiological features that distinguish arthropods from non-arthropods invertebrates. Readers are referred to De Fur *et al* (1999), Barnes *et al* (2001) and Schubert *et al* (2006) for a wider view of aquatic invertebrate zoology, their evolutionary linkages and the use of invertebrates to address priority issues in ecotoxicology (e.g. reproductive health and population sustainability).

Table 4.4: Examples of freshwater invertebrate regulatory test guidelines for acute and chronic ecotoxicity assessment (adapted from Ingersoll et al, 1999)

Taxonomic group	Species	Description	Reference
Annelids	*Lumbriculus* sp.	96 h acute lethality test	ASTM guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (E729-96) (ASTM, 2007)
		28 d Sediment-water toxicity test using spiked sediment	OECD, 2004a
Crustaceans	*Daphnia magna*	48 h acute lethality test 21 d chronic (survival, development and reproduction) test	OECD, 2004b OECD, 1998a
Insects	*Chironomus riparius*	28 d sediment-water toxicity test using spiked water	OECD, 1998b
Rotifers	*Brachionus rubens*	48 h acute test	ASTM Standard Guide E1440 (ASTM, 2004a)

Table 4.5: Examples of saltwater invertebrate regulatory test guidelines for acute and chronic ecotoxicity assessment (adapted from Ingersoll et al, 1999)

Taxonomic group	Species	Description	Reference
Annelids	Polychaetes	28 d chronic test	ASTM Standard Guide E1562-00 (ASTM, 2006)
Crustaceans	Mysids	60 d life-cycle test	ASTM Standard Guide E1191-03a (ASTM, 2003)
Echinoderms	*Paracentrotus lividus*	48 h embryo development test	ASTM Standard Guide E1563-98 (ASTM, 2004b)
Molluscs	Mussels and oysters	48 h embryo development test	ASTM Standard Guide E724-94 (ASTM, 2004c)
Rotifers	*Brachionus plicatilis*	48 h acute test	ASTM Standard Guide E 1440 (ASTM, 2004a)

5. MICROORGANISMS AND PLANTS

Both microorganisms and plants are of extraordinary importance for the ecosystem – representing destruents and primary producers, respectively. Both groups of organisms are also well suited to demonstrate principles of absorption, distribution (often terms 'translocation' in plants), metabolism and excretion (ADME) of xenobiotics. In many cases, these ADME processes are often less complex for these organisms than for higher organisms. An important aspect of the testing and risk assessment concerning microorganisms and plants is the focus on the population and community function, rather than the individual species *per se* (Walker *et al*, 2001). For example, for microorganisms, typically used tests examine summary parameters such as oxygen consumption and do not differentiate effects on different groups of microorganisms.

5.1 Microorganisms

Microorganisms are either unicellular microscopic organisms or, if multi-cellular, their tissues are relatively undifferentiated. It is this relatively simple organisation which sets them apart from true plants and true animals: Microorganisms include certain groups which have plant like characteristics (cyanobacteria), some with animal like characteristics (*protozoa*) and some with the characteristics of both kingdoms (*fungi*). The term 'microorganism' includes eukaryotes and prokaryotes. Most prokaryotes are bacteria and the two terms are often treated as synonyms. However, Woese *et al* (1990) proposed dividing prokaryotes into Bacteria and Archaea (originally *Eubacteria* and *Archaebacteria*) because of the significant genetic differences between the two.

5.1.1 Uptake

Absorption or uptake processes are mainly determined by the properties of the layer that forms the boundary of the organism to the environment. For microorganisms, which have, in the main, no special border zone and clearly no border tissue, the cell wall and the membrane are the decisive factors. All cells, except bacteria, have been shown to contain complex and well-differentiated organelles surrounded by membranes. Bacterial cells contain only protoplasm bounded by a semi-permeable cytoplasmic membrane, which, in turn, is covered by a porous cell wall. The cytoplasmic membrane selectively regulates transport of chemicals into and out of the cell. Different mechanisms are available to do this, which may or may not require cellular energy (e.g. a chemical can pass directly through the lipid layer or via proteins). Passive transport processes (not requiring energy) include diffusion, osmosis and facilitated diffusion. Unassisted movement of molecules from a higher concentration to lower concentration (concentration gradient) until equilibrium is reached is called passive diffusion. Some solutes after moving into

the cell bind with some other proteins or are metabolically transformed. Therefore the chemicals' concentration is not built up in the cell and the diffusion process continues at a faster rate. Passive diffusion is relatively slow with the rate depending on membrane permeability and solute concentrations (e.g. glucose and tryptophan have diffusion rates of 10,000 x less than for water) and it is therefore not enough for cellular growth and reproduction. Facilitated diffusion enhances the rate of diffusion and is found mainly in eukaryotic cells but rarely in bacteria (e.g. glycerol is the only known substrate that undergoes facilitated diffusion in some bacteria). Facilitator proteins (specific membrane proteins) selectively increase the permeability of the membrane for certain solutes.

Active transport processes require energy as it takes place against a concentration gradient. The processes include, group translocation, binding protein transport and cytosis. The molecule is not modified during transport. Permeases are very specific membrane protein transport carriers (Ioannou, 2001). Uniporters (carry one chemical at a time), cotransporters (carry more than one type of chemical), symporters (two chemicals carried in the same direction simultaneously [e.g. lactose and proton H^+]) and antiporters (chemicals are transported across the membrane in opposite directions [e.g. Na^+ are pumped outside the cell at the same time H^+ are transported inside the cell]). Examples for active transport systems are the proton motive force (PMF), the sodium-potassium pump, the group translocation-phosphenol pyruvate: phosphotransferase system (PEP:PTS) and binding protein transport. In general, all active transport systems are quite specific. The Task Force thus considers that it is currently not possible to predict uptake by active transport systems for unknown chemicals such as industrial chemicals or pharmaceuticals. In addition there is a special, less specific, active uptake system referred to as cytosis (a transport process in which a chemical is engulfed by the cytoplasmic membrane to form a vesicle such that transport is not through but rather around the cytoplasmic membrane).

5.1.2 Metabolism

There are no major morphological differences between the different prokaryotes, but there are major differences in their metabolic capabilities (e.g. aerobic versus. anaerobic, autotrophic versus heterotrophic, oxidation of inorganic compounds, fixation of molecular nitrogen). Bacteria exhibit an extremely wide variety of metabolic types and in general have the capacity to be very adaptive and the mechanisms of cellular regulation are of major importance. Certain enzymes essential for different types of conversion of nutrients can be induced under specific environmental pressures. It is widely accepted that eukaryotic metabolism is largely a derivative of bacterial metabolism with mitochondria having descended from a lineage within the α-Proteobacteria and chloroplasts from the Cyanobacteria by ancient endosymbiotic events. Bacterial metabolism can be divided broadly on the basis of the kind of energy used for growth, electron donors and electron acceptors and by the source of carbon used. Most bacteria are

heterotrophic; using organic carbon compounds as both carbon and energy sources. In aerobic organisms, oxygen is used as the terminal electron acceptor. In anaerobic organisms other inorganic compounds, such as nitrate, sulphate or carbon dioxide as terminal electron acceptors are leading to the environmentally important processes of denitrification, sulphate reduction and acetogenesis, respectively. Non-respiratory anaerobes use fermentation to generate energy and reducing power, secreting metabolic by-products (such as ethanol in brewing) as waste. Facultative anaerobes can switch between fermentation and different terminal electron acceptors depending on the environmental conditions in which they find themselves. As an alternative to heterotrophy many bacteria are autotrophic, fixing carbon dioxide into cell mass. Energy metabolism of bacteria is either based on phototrophy or chemotrophy, i.e. the use of either light or exergonic chemical reactions to derive energy. Lithotrophic bacteria use inorganic electron donors for respiration (chemolithotrophs) or biosynthesis and carbon dioxide fixation (photolithotrophs), whereas organotrophs use organic compounds as electron donors for biosynthetic reactions (and mainly as carbon sources). Common inorganic electron donors are hydrogen, ammonia (leading to nitrification), iron and several reduced sulphur compounds (Chatterjee and Yuan, 2006).

Table 5.1 shows the classification scheme proposed by Atlas (1988) based on the type of nutrition exhibited by various microorganisms. No single organism is capable of utilising all of the wide variety of inorganic and organic compounds found in the environment and consequently a diverse ecosystem will develop. The exact composition of this community will depend on the outcome of competition for the limited and varied food supply and will be influenced by environmental parameters such as redox potential and temperature.

Table 5.1: Nutritional types of microorganisms (Atlas, 1988)

Type	Generic description
Chemoautotrophs (Chemolithotrophs)	Use carbon dioxide as their principle carbon source Energy is obtained by the oxidation of inorganic compounds, such as NH_4^+, NO_2^-, H_2, H_2S, S and Fe^{2+}
Chemoheterotrophs (chemoorganotrophs)	Use organic compounds as their principle source of carbon and energy
Photoautotrophs (Photolithotrophs)	Use carbon dioxide as their principle carbon source and light as their source of energy
Photochemotrophs (Photoorganotrophs)	Use organic compounds as their principle source of carbon and light as their source of energy

The metabolism of xenobiotics in microorganisms (as in plants and animals) may involve a three-phase process: in phase I the parent compound undergoes oxidation, hydroxylation or reduction.

In phase II the parent compound or its metabolite is conjugated. In phase III the final metabolic stage is reached by further conjugation or compartmentalisation (for a full discussion see Van Eerd *et al*, 2003).

5.1.3 Sites of pharmacological and toxicological action

The principal targets for antibacterial agents reside at the cytoplasm and cytoplasmic membrane, damage to other structures often arising from initial events at these loci (Table 5.2). The Gram-negative bacteria offer a complex barrier system to biocides and antibiotics, regulating, and sometimes preventing, their passage to target regions. Routes of entry differ between hydrophobic and hydrophilic agents, often with a structure dependency; specialised uptake mechanisms are exploited and portage transport can occur for pro-drug antibacterials. Uptake isotherms offer insight into the sorption process and can sometimes shed light on biocide mechanisms of action. The multi-component barrier system of Gram-negative bacteria offers opportunities for phenotypic resistance development where partitioning or exclusion minimises the delivery of an antibacterial agent to the target site. Active efflux processes are recognised as increasingly relevant mechanisms for resistance, potentially offering routes to biocide: antibiotic cross-resistance.

The phenomenon of cellular impermeability is particularly important in the resistance of mycobacteria and Gram-negative bacteria (Hancock, 1997), since target sites for biocides (Denyer and Stewart, 1998; Maillard, 2002) are often situated within the cell and particularly, at the cytoplasmic membrane level or within the cytoplasm. The wall structure of Gram-negative bacteria, and specifically the presence of an outer envelope, is often responsible for the impermeability of these microorganisms to antimicrobial agents (Denyer and Maillard, 2002). Among vegetative bacteria, mycobacteria are deemed to be the least sensitive to biocides, followed by Gram-negative and then Gram-positive bacteria. As with biocides, many clinically useful antibiotics have a target site in the cytoplasmic membrane (e.g. b-lactam antibiotics) or in the cell cytoplasm (e.g. inhibitors of protein, DNA and RNA synthesis). Therefore, these molecules also have to cross the outer membrane and/or the cytoplasmic membrane to reach their primary target site (Denyer and Maillard, 2002).

5.1.4 Excretion

The processes of excreting chemicals out of the cell are less well understood than those involved in uptake. It is supposed that diffusion as well as active transport is involved, if the concentration of a chemical within a cell is higher than that outside the cell (Schlegel, 1985). Metabolism of xenobiotics in microorganisms can lead to mineralisation or conversion to volatile metabolites,

which are released. Microorganisms may be capable of complete oxidisation of xenobiotics to CO_2. If complete mineralisation cannot be performed by one type of organism, breakdown products (metabolites) may be released to the surrounding environment, where they may undergo further metabolism by other bacteria and/or fungi (Van Eerd *et al*, 2003).

Table 5.2: Examples of mechanisms of toxic action in microorganisms

Effect	Example chemical	Mechanism
Growth inhibition Reproduction usually occurs through dichotomy. After replication of the chromosome the cell is divided by development of cell walls from the outside to the centre. The death of a cell is defined by the irreversible loss of the ability to grow and reproduce. Various types of cell damage are reparable.	Poylene antibiotics e.g. nystatin, candicidin.	Interact with sterols and destabilising eukaryotic and cell wall-less bacterial membranes (but do not inhibit growth of cell wall containing bacteria).
Cell wall damage	Phenols, cresols, neutral-soaps and detergents affect cell membranes and destroy semi-permeability.	Accumulate in the lipoprotein membrane and disrupt their functions.
	Penicillin	Transpeptidase enzyme builds peptide bridges in actively dividing cells. Penicillin binds to it stopping cell wall synthesis.
Inhibition of cell wall synthesis	Lysozyme (an enzyme produced by organisms that consume bacteria, and contained in normal body secretions such as tears, saliva, and egg white to protect against possible pathogenic bacteria).	Digestion of beta 1,4 glycosidic bonds decomposes glycoside bonding of murein resulting in reducing polysaccharide chains to disaccharides. Lysozyme only acts as a toxicant to gram-positive bacteria; gram-negative bacteria can be affected, if complex forming compounds (EDTA) are added before to remove Ca^{2+}-ions.
Damage to enzyme or metabolism	Antimycin A	Inhibits electron transport in the respiration chain due to blocking of cytochrome c-reductase.
	2,4-Dinitrophenol	Uncoupler of phosphorylation in the respiration chain.

Table 5.2: Examples of mechanisms of toxic action in microorganisms *(cont'd)*

Effect	Example chemical	Mechanism
Damage to enzyme or metabolism (cont'd)	Fluoracetate	Inhibits the tricarboxylic acid cycle. It first activates in the same way as acetate and gets attached to citrate resulting in fluorocitrate, which represses aconitase and consequently the further conversion of citrate.
Competitive inhibition The uptake of structural analogues into the cell especially affects bio-synthesises in different ways.	Malonate.	Inhibition of the conversion of succinate to fumarate.
Inhibition of cell component synthesis. Antagonists can block the attachment of the intrinsic molecule, which results in the inhibition of synthesises of certain cellular compounds. They can also be bound into polymers and thus reduce or eliminate the activity of a certain enzyme or a nucleic acid.	Sulfanilic acid derivates	Can bind to coenzyme tetrahydrofolate, instead of 4-aminobenzoate, which stops growth.
Inhibition of protein synthesis	Streptomycin and Neomycin	Block linking of amino acids.
	Erythromycin	Reduce the function of the 50S-ribosome subdivision.
	Tetracyclines	Inhibit the attachment of aminoacyl-tRNA to the ribosomes.
	Chloramphenicol	Block the attachment of amino acids by inhibiting peptidyltransferase. It does not affect any other parts of the metabolism. Note these chemicals also affect ribosomes in mitochondria and chloroplasts of Eukaryotes. But since the mitochondrial membrane cannot be easily passed, low concentrations are not toxic.
Inhibition of nucleic acid synthesis	Mytomycin C	Inhibits selectively DNA-synthesis by causing breaks within the double helix and prevent it of building up, without influencing RNA- and protein-synthesis.
	Actinomycin D	Forms complexes with the DNA double helix by attaching to guanine. All three types of RNA cannot be synthesised.

5.1.5 Summary

A number of bacterial assays have been developed, including test methods based on bacterial luminescence, growth inhibition, respiration, nitrification inhibition, bacterial viability and motility, for assessing the toxicity of chemicals in the environment. The advantages in testing microorganisms are that they are easy to handle, their use is not restricted due animal welfare reasons and chronic tests can be done within short periods because of their rapid proliferation. Table 5.3 summarises the currently available national and international guidelines used in a regulatory framework for effects testing with microorganisms.

Tests based on the inhibition of bacterial growth. One approach to toxicity testing using bacteria is to measure the impact of toxicants on the growth of pure or mixed bacterial cultures isolated from various environments (Trevors, 1986; Alsop *et al*, 1980). As microbial growth relies upon the coordination of a large number of metabolic events a test toxicant has a large number of potential sites that can lead to an observed toxicological effect (i.e. reduction in population growth). Growth studies are therefore considered to be more sensitive and environmentally relevant than respirometric test methods. Toxicity assays based on the inhibition of bacterial growth, measure changes in bacterial densities through adsorbance measurements or the determination of inhibition zones on solid growth media (Anderson and Abdelghani, 1980; Liu, 1981; Liu and Kwasnieska, 1981; Slabbert, 1986; Trevors *et al*, 1981; ISO, 1995a).

Inhibition of bacterial luminescence. A bacterial bioluminescence test was developed in the late 1970s under the name of Microtox™, as a rapid screening alternative to toxicity tests with higher aquatic organisms such as fish and invertebrates (Bulich, 1979; Bulich *et al,* 1981). The test measures reductions in the bioluminescence of *Vibrio fischeri* due to the presence of specific toxicants. The underlying mechanisms and chemistry of the Microtox™ have been described by Isenberg (1993). Several publications (Blum and Speece, 1991; Tarkpea *et al*, 1986; Qureshi *et al*, 1982; Bulich *et al*, 1981) have reported on comparisons between Microtox™ and other toxicity tests using fish, invertebrates and bacteria. Blum and Speece (1991) compared the toxicity obtained from bacterial toxicity tests to published fathead minnows. The heterotrophs and methanogens showed very similar sensitivities to the test toxicants, whereas Microtox™ and *Nitrosomonas* species and fathead minnows were an order of magnitude more sensitive to the same toxicants. Comparison of Microtox™ and fish acute toxicity is best attempted only on narcotic or polar narcotic MOA chemicals (Dearden *et al*, 1995). The Microtox™ test has been reported as a good substitute for fish acute toxicity for chemicals acting by a non-polar narcotic MOA. For other MOAs, notably polar narcosis and for chemicals capable of forming covalent bonds with macromolecules through electrophilic reactions, poorer relationships are observed and thus Microtox™ is less suitable as a substitute for fish toxicity for these MOAs.

Protozoan toxicity tests. At present there are no standardised test methods for protozoan species, although a number of test methods have been reported in the scientific literature and a test guideline for a *Tetrahymena* sp. bioassay is under priority development in OECD. Roberts and Berk (1990; 1993) have described an assay measuring changes in the motility of the freshwater ciliate *Tetrahymena pyriformis* in the present of specific toxic chemicals. Slabbert and Morgan (1982) and Slabbert (1986) have described a short-term test using *Tetrahymena pyriformis*, which measures changes in its respiration rate due to the presence of toxic chemicals. Schultz and Applehans (1985) reported acute toxicity data for multiple nitrogen substituted aromatic compounds. The assay proposed by Schultz (1997) is a short-term, static protocol using the common freshwater ciliate *Tetrahymena pyriformis* (strain GL-C). The 50% impairment growth concentration (IGC_{50}) is the recorded endpoint.

In response to the recommendations for the Ecotoxicology Session of TestSmart™ − A Humane and Efficient Approach to Screening Information Data Sets (SIDS) Data Workshop, Sinks and Schultz (2001) compared population growth impairment data using the 2 d *Tetrahymena pyriformis* growth assay (log [IGC_{50}−1]) with data for the 96 h *Pimephales promelas* mortality assay (log [LC_{50}−1]) for 100 chemicals. Each chemical was *a priori* assigned a mode of action. Toxicities for narcotics showed an excellent relationship between endpoints with the coefficient of determination (r^2) being 0.93. A weaker relationship, $r^2 = 0.78$, was observed for the electro(nucleo)philes. The poorer fit for the covalent-reacting electro(nucleo)philes is attributed to differences in protocol, in particular, to test-medium composition and exposure scheme. Those chemicals whose potency is mediated by metabolism in fish (diesters and proelectrophiles) as well as the acids exhibited poor correlation between endpoints, with toxicity in the fish assay being greater than that predicted from the ciliate data. A result for the present investigation supports earlier findings that, with noted exceptions, there is a strong relationship between toxicity potency as quantified by *Pimephales promelas* mortality and *Tetrahymena pyriformis* growth impairment.

Protoxkit F™ is a commercially available 24 h chronic (growth inhibition) toxicity test based on the ciliate protozoan *Tetrahymena thermophila*. Tetratox™ is another commercially available assay. Population density is measured spectrophotometrically and the 50% inhibitory growth concentration in mg/L (IGC_{50}) and the 95% fiducial interval are determined for each test compound. The Tetratox database is a collection of toxic potency data for more than 2,400 industrial organic compounds of which more than 1,600 have been published. Jawacki and Sawicki (2002) compared the sensitivity of the ciliate protozoan *Spirostomum ambiguum* with standard toxicity tests and concluded that *Daphnia magna* and *Pimephales promelas* were in general six to eightfold more sensitive than the 24 h LC_{50} for *Spirostomum ambiguum*. The best agreement was for nonpolar narcotics.

In summary, whilst a number of commercially available tests have been assessed for their suitability to replace testing with higher organisms no clear and consistent relationship has been reported for MOA4 type chemicals.

Table 5.3: Examples of standardised test protocols for microbial function

Guideline	Organism	Test	Endpoint	Use for	Specific sensitivity for / specific insensitivity for
OECD 209 (OECD, 1992b)	Wide range of aquatic microorganisms present in sewage plants	Activated sludge respiration inhibition test	EC_{50} O_2 uptake	Short term impact of chemicals and waste waters on aerobic microorganisms	-
OPPTs 850.6800 (US EPA, 1996c)	Wide range of aquatic microorganisms present in sewage plants	Modified activated sludge, respiration inhibition test	EC_{50} O_2 uptake	-	-
ISO 9509 (ISO, 1989)	*Nitrosomonas* species, *Nitrobacter* species	Toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms	-	Wastewater treatment systems to achieve water quality standards for ammonia removal	Inhibitory effects of chemicals to nitrifying bacteria
ISO 15522 (ISO, 1995a)	Pure or mixed bacterial cultures	Determination of the inhibitory effect of water constituents on the growth of activated sludge microorganisms	EC_{50} O_2 uptake, adsorbance measurements, determination of inhibition zones	Cultures isolated from various environments; growth of sewage bacteria	Large number of potential sites leading to toxicological effect, thus, more sensitive
ISO 10712 (ISO, 1995b)	*Pseudomonas putida*	*P. putida* cell multiplication inhibition test	EC_{50} O_2 uptake	Screening test to identify surface water, ground water, wastewater and specific chemicals that adversely affect the growth of *P. putida*	-

***Table 5.3: Examples of standardised test protocols for microbial function** (cont'd)*

Guideline	Organism	Test	Endpoint	Use for	Specific sensitivity for / specific insensitivity for
ISO/DIS 11348-2 (ISO, 1996)	*Vibrio fischeri*	Water quality – determination of the inhibitory effect of water samples on the light emission of *V. fischeri* (luminescent bacteria test durations range from 5 to 30 minutes)	EC_{50} for bioluminescence	Impact of toxic chemicals on *V. fischeri*	-
OPPTs 850.5100 (US EPA, 1996d)	Total soil microflora	Soil microbial community toxicity	EC_{50} for ammonification, nitrification, CO_2 evolution	Small soil samples (up to 100g)	Determination of the activity of the total soil microflora, thus, effects on single species and overall effects might not be noticed
ISO 14240-1 (ISO, 1997)	-	Determination of soil microbial biomass: Substrate-induced respiration method	-	-	-
OECD 217 (OECD, 2000b)	-	Soil microorganisms: Carbon transformation test	O_2 uptake, deviation to control	-	The test appeared to be quite sensitive to heavy metals
OECD 216 (OECD 2000c)	-	Determination of nitrogen (and facultatively) ammonium formation	Development of NO_3^- and/or NH_4^+ deviation from control	-	-

Table 5.3: Examples of standardised test protocols for microbial function *(cont'd)*

Guideline	Organism	Test	Endpoint	Use for	Specific sensitivity for / specific insensitivity for
ISO 14238 (ISO, 1995C)	Microorganisms involved in ammonification and nitrification	Determination of nitrogen mineralisation and nitrification in soils and the influence of chemicals on these processes	Release of inorganic nitrogen from soil organic matter; conversion of ammonia to nitrate	-	Fewer species of microorganisms are involved in this process

5.2 Plants

Because plants are important as primary producers, ecologically important effects on plants will not only be important on plant species *per se*, but could have a wider influence on higher trophic levels. Due to their photo-autotrophy, plants show some unique metabolic pathways (mainly photosynthesis, but also synthesis of some amino acids). In these cases, MOA knowledge may contribute to substantially narrow the number of species to be considered in testing. For example, a review of existing data on the effect of human pharmaceuticals on various aquatic species by Crane *et al* (2006) gives helpful hints on prioritised environmental testing. The following conclusions can be drawn out of the data comparison: (1) Cyanobacteria are likely to be sensitive surrogates for algae and other unicellular organisms; (2) microalgae and Cyanobacteria are considerably more sensitive to antibiotics than standard algal toxicity test species. Green algae and higher aquatic plants (macrophytes such as *Lemna* sp.) may show differential sensitivity to xenobiotics, although higher plants tend to be more sensitive in the limited number of comparisons for human pharmaceuticals (Crane *et al*, 2006). Algal species were sensitive to several different human pharmaceuticals, such as fluoroquinolone and sulfonamide antibacterials, selective serotonin reuptake inhibitors, β-adrenergic receptor blockers and oestrogens. Overall, the work of Crane *et al* (2006) demonstrates that plants are not only sensitive to herbicides, but also to several fungicides and pharmaceuticals. In some cases, for unexplained reasons there can be marked interspecies differences in the sensitivity of algae to chemicals (Blanck *et al*, 1984).

5.2.1 Uptake

For the simplest plant species, unicellular algae, only cellular uptake and transport processes have to be considered, but no intercellular transport. More complex processes are involved for the

uptake in aquatic macrophytes, since, depending on the species, different kinds of plant tissues are in contact with the chemical and a need for transport may arise. Transport does not, however, play a crucial role in *Lemna* sp., the standard aquatic macrophyte species for ecotoxicity testing. It is notable that a more extensive process is observed in terrestrial plants since, besides different sites of uptake (e.g. root, leaf) and two types of transport (xylem, phloem), an additional barrier (the cuticle) comes into play.

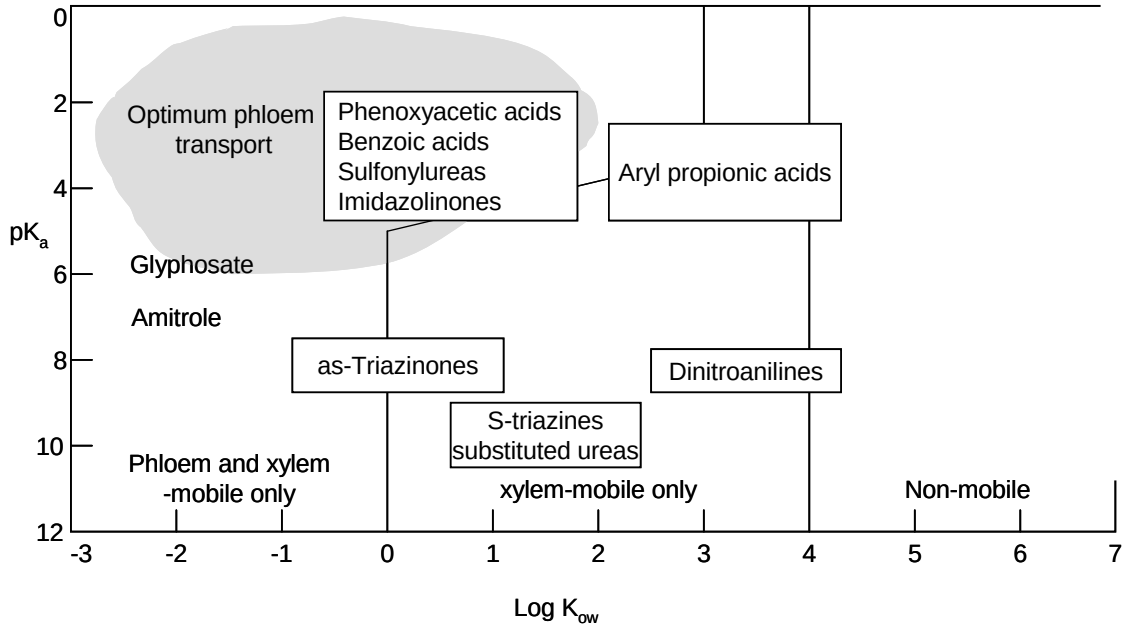
In algae, the first barrier to a chemical reaching the inner parts of the plant cell, the cytoplasm, is the cell wall. The cell wall is a very polar and relatively porous medium (mainly consisting of cellulose; pectins, xylans, lignin, suberin, phenolic acids and some proteins), which offers little resistance against infusion of xenobiotics. Thus the uptake generally is regulated by the passage through the next barrier, the cell membrane. This membrane is of different permeability in different species depending on the relation between the area of phospholipids and that of pores and proteins. However, such species differences are not well studied to date and should not be used to reliably predict uptake of chemicals. In contrast, the most important process for chemicals to reach the cytoplasm is by passive diffusion. As a general rule, diffusion of a compound into algal cells increases with reduction of molecule size and increase of lipophilicity (Fent, 2001). However, a very high lipophilicity may result in accumulation in the algal cell membrane, thus reducing penetration across the polar cell wall. Thus there is an optimum lipophilicity for uptake into plant cells (root cells) of log K_{ow} between 0.5 and 3.5 (Fent, 2001). In addition to diffusion, uptake in general can occur along a concentration gradient *via* carrier proteins or transporters, which are selective to sucroses, hexoses, amino acids and peptides. Carriers may also be of importance for xenobiotics. There seem to be a few plasma membrane carriers that handle small-sized hydrophilic xenobiotics. Other carriers translocating relatively large xenobiotics (approx. 350 Dalton) with a lipophilic moiety have also been detected (Délétage-Grandon *et al*, 2001). However, these carrier processes are largely unknown and tend to be highly dependent on molecule properties making them more or less unpredictable. Active transport against the concentration gradient can also occur through specifically acting carrier proteins. As for the transport along a concentration gradient, these processes are largely unknown yet and thus nearly unpredictable. Overall, the uptake of xenobiotics in algae is mainly influenced by the ability to pass the cell membrane. As the most common uptake is by diffusion the physico-chemical properties of the molecule are decisive. Thus, as a general rule, uptake is best for small, non-polar, lipophilic chemicals. A better permeation of neutral than charged species is observed, due to the higher affinity of the cellular phospholipids for chemicals in their neutral forms.

The general principles described for algae are also valid for macrophytes. However, different to algae, aquatic macrophytes consist of different tissues and thus may have zones of different uptake (leaves, roots, rhizoids). For example, the uptake of chlorinated hydrocarbons is variable in different aquatic plant species; a preferential uptake through the root can be observed for

Myriophyllum sp. (Hock and Elstner, 1995). For 2,4,6-trinitrotoluene (TNT) uptake through roots was also highest in *Myriophyllum aquaticum* (Vanderford *et al*, 2005). Transport to stem and leaves can be observed. Stems, primarily acting as transporters, contain large quantities of extractable residues (Vanderford *et al*, 2005). In the case of chlorinated hydrocarbons a positive correlation between accumulation and lipid content consists (Hock and Elstner, 1995). In the case of cyanobacterial toxins, uptake in *Phragmites australis* is highest into the stem followed by the rhizome (Pflugmacher *et al*, 2001). However, in the higher aquatic plant species routinely used for ecotoxicological testing, *Lemna* sp., differences in uptake and transport processes are negligible. In general, xenobiotic uptake in macrophytes such as *Lemna* sp. is relatively rapid due to a very thin cuticle on the underside of the fronds (Saunders, 2004); hence specific differences in the uptake compared to algae are likely to be minor. Thus *Lemna* sp. appears to serve as a worst case model for the uptake into higher plants, provided the chemical to be tested is available in the water phase and not adsorbed to the sediment.

In summary, there is extensive knowledge relating to the uptake and transport of chemicals based upon their physico-chemical characteristics (especially lipophilicity (log K_{ow}), acidity (pK_a) and molecule size) (see Figure 5.1 for key principles).

Figure 5.1: Effects of lipophilicity (log Kow), acidity (pKa) on chemical uptake in plants



More specific uptake and transport processes are summarised in Table 5.4.

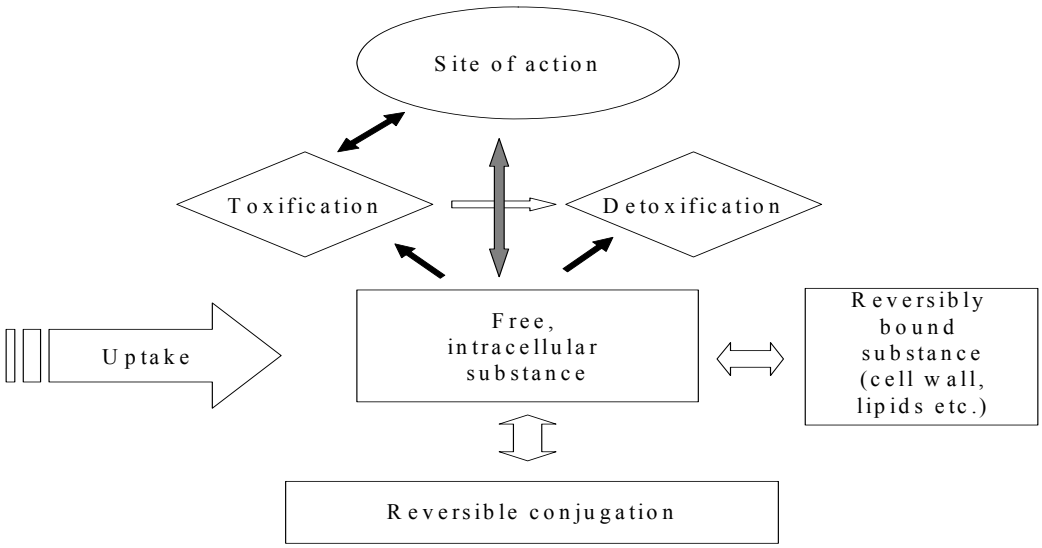
Table 5.4: Uptake and transport characteristics of chemicals in plants

	Algae	**Aquatic Macrophyte**	**Terrestrial Macrophyte**
Main barrier for uptake	Cell membrane	Cell membrane	Cuticle, cell membrane
Chemical specific factors with biggest influence on uptake processes	Log K_{ow} (optimum between 0.5 and 3.5 for uptake into roots) (cell membrane: dissolved chemicals and those with a high lipophilicity), pK_a (optimum for acidic chemicals: pH<pK_a; for alkalines: pH>pK_a, as neutral/undissociated species pass membranes more easily), molecular size (less than 500 d)		
Importance of plant specific uptake processes	Low	Low	Medium
Relevance of active transport processes	Low	Low / medium	High

5.2.2 Metabolism

The elimination of chemicals in plants includes metabolism, excretion and permanent binding to biological structures. In principle, the processes and enzymes involved in metabolism of xenobiotics are the same in algae, mosses, terrestrial and aquatic plants (Schäffner *et al*, 2002), and are summarised in Figure 5.2. This gives a scientific basis to limiting the number of plant species to be tested.

Figure 5.2: Schematic layout of metabolic behaviour of chemicals in plants (adapted from Devine et al, 1993)



Metabolism can generally be broken down into three phases before a detoxification is reached (Tables 5.5 and 5.6). Those phases can occur in succession or independently (Schäffner *et al*, 2002; Van Eerd *et al*, 2003).

Table 5.5: Phases of detoxification in plants (Schäffner et al, 2002; Van Eerd et al, 2003)

	Phase	Main processes	Main involved enzymes	Outcome
Phase I	Transformation (altered chemical structure)	Oxidation Reduction Hydrolysis	Cytochrome P450 dependent monooxygenases, peroxidases, reductases, dehydrogenases, dehalogenases, esterases, nitroreductases	Amphophilic, modified, commonly (but not always) less toxic than parent
Phase II	Conjugation (frequently less toxic metabolites)	With carbohydrates, glutathione, carboxylic acids	Glucosyltransferases, glutathione-S-transferases, acyltransferases	Hydrophilic, greatly reduced toxicity or nontoxic
Phase III	Compartmentation	Into vacuole or apoplast/cell wall	Exocytosis, ATP binding cassette (ABC) and multi-drug and toxic compound extrusion (MATE) transporters	Hydrophilic or insoluble, nontoxic

Table 5.6: Comparison between plant and microbial pesticide metabolism (Van Eerd et al, 2003)

Biotransformation	Plants	Microorganisms
General pesticide metabolism	Detoxification	Mineralisation
Oxidation	P450 mediated	Not generally P450 mediated Mediated by various oxidoreductases
P450 oxidation	Microsomal membrane bound	Soluble form, not membrane bound
Hydrolytic transformation	Predominantly via esterases, amidases, aryl acylamidases and nitrilases	Greater enzyme diversity
Bond cleavage	None known	Diverse C-P lyases and hydrolytic enzymes
Aromatic nitro-reductive processes	Nitroreductases, GSH conjugation	Nitroreductases, no GSH conjugation
Reductive dehalogenation	None known	Halo-respiration
Conjugation	With sugar and amino acids Compartmentalised or sequestered GSH conjugation	With xylose, methyl, or acetyl groups Conjugates formed extracellularly No known GSH conjugation

GSH-glutathione-S-transferase.

5.2.3 Excretion

Both unicellular and higher plants have several processes for excreting metabolites of xenobiotics where they are no longer of physiological use or may lead to phytotoxicity. Principles for excretion are similar to those for uptake (i.e. physico-chemical properties of the chemical) and may theoretically be modified by species-specific excretion processes. In the absence of specific uptake mechanisms (carriers) in aquatic algae, equilibrium between environmental and cellular chemical concentration would be reached rapidly. In algae, the high ratio of surface to volume quickly causes a balance between uptake and elimination of a chemical.

In algae as well as in higher plants xenobiotics and/or their metabolites (e.g. conjugates with glutathione or glucose) can reach the vacuole as an intermediate or final location through active transport. In algae, pulsating vacuoles (which are especially active in osmoregulation) are assumed as being able to excrete xenobiotics, often in the form of glutathione-conjugates (Pflugmacher *et al*, 2001).

A very common type of detoxification is the immobilisation of toxic chemicals by preventing translocation to the target tissues (e.g. vacuoles can serve as a permanent location for toxic chemicals such as glycosides). A further possibility exists in toxic chemicals being transformed to insoluble salts. Also the 'ion trap mechanism', mentioned above, prevents weak acids from leaving the cytoplasm. Both unmodified parent chemicals and metabolites, can bind to plant polymers, including protein or lipoidal components of the cell membrane, and proteins or nucleic acids in the protoplasm. Most commonly conjugated compounds (e.g. with or without sugar, with glutathione) can be accumulated in the cell wall. Peroxidases and laccases cause covalent binding of compounds, which results in accumulation within the cell wall (Saunders, 2004). Amino acid conjugates are mainly excreted into the cell wall. In *Lemna* sp., for example, those bound residues in cell walls and tissues are a very common process of eliminating toxic compounds. This can even contribute to removing contaminants, e.g. chlorinated phenols, fluorophenols and mixed chloro-fluoro-phenols, from wetlands (Saunders, 2004). In *Myriophyllum aquaticum* an increasing percentage of original compound (TNT) and metabolites becomes intimately bound to the plant structural material over time, with the largest amount of bound residues in root tissues (Vanderford *et al*, 2005).

5.2.4 Summary

In ecotoxicology, effects on aquatic plants are normally studied by conducting tests with algae. However, it is currently unclear which groups of algae or aquatic plants are most sensitive to different chemicals (Brock *et al*, 2000). Sensitivity varies between toxicants, between taxonomic groups and species within taxa, including crop species versus non-crop species and algae versus

Lemna sp. (Wang and Freemark, 1995; Boutin and Rogers, 2000; Ma *et al,* 2003). Moreover, the sensitivity of algae to a toxicant appears to be somewhat species-specific (Table 5.7). Differences in effects of the same toxicant to algal species can be several orders of magnitude for metals, pesticides, surfactants and effluents (Lewis, 1995), especially in the case of toxicants having specific MOAs (Nyholm and Peterson, 1997; Brock *et al*, 2000; Shigeoka *et al*, 1988; Junghans *et al*, 2003). Interestingly, a study dealing with the toxicity of 21 herbicides with different modes of action to the green alga *Scenedesmus quadricauda* led to the following order of high to low toxicity for the groups of herbicides: photosynthesis-inhibiting herbicides > lipid synthesis inhibitor > protox inhibiting herbicides > glutamine synthase inhibiting herbicides > EPSP synthase inhibiting herbicides > auxin herbicides. This general trend can also be seen for other alga species (Ma *et al,* 2003).

Testing insecticide combinations on algae showed inconsistent sensitivities to green algae and Cyanobacteria (Megharaj *et al,* 1989a,b). Comparing results from different types of multi-species tests for the same chemicals (surfactants, metals) to those conducted with laboratory single species tests also gave no consistent trend of sensitivity (Lewis, 1995). A comparison of toxicity data for 488 chemicals showed that for nearly 200 chemicals the differences in toxicity between various algae species was 1 - 10, for 100 it was 10 - 99, for 50 the difference in toxicity lay between 100 and 1000 and for several chemicals the differences in toxicity were >1000. Even the toxicity differences of different strains of the same algae species exposed to the same chemical were >10 and >100 in several cases (Lewis, 1995).

In terms of difference of sensitivities between algae and macrophytes, auxin-stimulating chemicals exert a much higher toxicity to macrophytes. In contrast, values from algae toxicity tests for photosynthesis- and growth-inhibiting herbicides seem to be adequate for higher aquatic plants (Lewis and Wang, 1997; Ma *et al*, 2003). Toxicity tests with *Lemna* sp. resulted in values of the same order of magnitude as those for standard algae for most photosynthesis- and growth-inhibition herbicides. The EC_{50} for Lemna of sulfonylurea (metsulfuron-methyl) was up to a factor of 400 lower than for algae (Brock *et al*, 2000). For phenoxycarbon acids (2,4-D), clopyralid Lemna were less sensitive than algae, even though field experiments showed that rooting aquatic plants are very sensitive to these compounds (Brock *et al*, 2000). Herbicides (dalapon, naptalam, propham) that are absorbed by or have their primary effect on the roots have been shown to be clearly more toxic to higher plants than to algae (Kirby and Sheahan, 1994). As described in an analysis of pesticide data by Fletcher (1990) algae tests did not detect a response in 20% of the cases in which vascular plants were affected.

Macrophytes tend to be less sensitive compared to algae for metals (Powell, 1997). According to the literature review by Lewis (1995) no consistent trend exists of relative toxicities of duckweed species compared to algae and fungal species. Conclusively, algae cannot always act as substitute for higher

aquatic plants and thus should not be used as a surrogate (Ma *et al*, 2003; Powell,1997; Brock *et al*, 2000).

Table 5.7: Sensitivity of algae to different chemicals

Chemical group	Exemplary chemical	Effects	Reference
Insecticides	Parathion	Small amounts are sufficient to produce changes in the physiology and structure of green algae cells	Lohmann and Hagedorn, 1985
	Quinalphos, Monocrotophos, Cypermethrin, Fenvalerate, Carbofuran and Carbaryl	Small amounts may adversely affect different algae species	Megharaj *et al,* 1989a,b
Surfactants	General	Vary widely and the effect levels are compound and species-specific. Toxicity is less when determined for natural algal communities under natural conditions	Lewis, 1990
	Surface-active quaternary ammonium compounds and betaines (trimethylammonium acetates)	Strongly inhibit the growth of *Selenastrum capricornutum*	Nyberg, 1988
	Amphoterics and cationics	Most algae are very sensitive	Nyberg, 1988
	Anionic surfactants	At least fairly toxic	Nyberg, 1988
	Nonionics	May stimulate or inhibit growth of *Selenastrum capricornutum*	Nyberg, 1988
Fungicides	Prochloraz (inhibitor of ergosterol biosynthesis in fungi)	Most potent inhibitor of the reproduction of *Chlorella* populations, comparable only to the photosynthesis inhibitor chlortoluron	Faust *et al,* 1994
	Anilazine (a multi-site thiol and amino-group inactivator)	As toxic as some tested herbicides	Faust *et al,* 1994
	Cytochrome P450-interacting plant growth retardants, fungicides	Strong toxic properties against unicellular green algae	Thies and Grimme, 1996

Table 5.8: Aquatic plant species used in regulatory protocols

Guideline	Ecosystem	Plant taxa	Plant species	Endpoint	Observed insensitivities/sensitivities[1]
OPPTS 850.5400 (US EPA, 1996e) OECD 201 (OECD, 2006b)	Freshwater	Microalgae, Chlorophyta, Chlorophyceae	*Pseudokirchneriella subcapitata* (=*Selenastrum capricornutum* = *Monoraphidium capricornutum* = *Raphidocelis subcapitata* Korsikov)	EC_{50} for inhibition of algal growth	Mitotic processes, cellulose synthesis. Standard tests with algae do not offer adequate protection in case of auxin-stimulating chemicals, which exert a much higher toxicity to macrophytes compared to algae (Ma *et al*, 2003, Powell 1997, Brock *et al*, 2000). Risk estimations based on algae toxicity tests for photosynthesis- and growth-inhibiting herbicides seem to be adequate for higher aquatic plants (Brock *et al*, 2000). In case of metals macrophytes tend to be less sensitive compared to algae (Powell, 1997). Triazine and phenylurea herbicides (photosynthesis-inhibiting) are very toxic to algae (Lewis and Wang, 1997).
	Freshwater		*Desmodesmus subspicatus (= Scenedesmus subspicatus)*[2]		
	Freshwater	Microalgae/ Diatom, Heterokontophyta, Bacillariophyceae	*Navicula pelliculosa*		
	Marine		*Skeletonema costatum*[3]		
	Freshwater	Prokaryota, Eubacteria, Cyanobacteria, Cyanophyceae	*Anabaena flos-aquae*		
	Freshwater		*Synechococcus leopoliensis*[2]		
OECD 221 (OECD, 2006c) OPPTS 850.4400 (US EPA, 1996f) ISO 20079 (ISO, 1995d)	Freshwater	Macrophytes	*Lemna minor* L.	EC_5, EC_{50}, EC_{90}, LOEC, NOEC for inhibition of growth (total frond number, growth rate and/or frond mortality; frond number, total frond area, dry weight or fresh weight)	Lemna might underestimate the risk of auxin-simulators to aquatic plants (Brock *et al*, 2000). Testing of sulfonylurea (metsulfuron-methyl) led to an EC_{50} for Lemna up to a factor of 400 lower than algae (Brock *et al*, 2000). In case of phenoxycarbon acids (2,4-D) and clopyralid Lemna were less sensitive than algae, even though field experiments showed that rooting aquatic plants are very sensitive to these compounds. Little difference in the sensitivities of *Lemna minor* and *Lemna gibba* was found (Lewis, 1995).
			Lemna gibba L.		

Table 5.8: Aquatic plant species used in regulatory protocols *(cont'd)*

Guideline	Ecosystem	Plant taxa	Plant species	Endpoint	Observed insensitivities/sensitivities[1]
OPPTS 850.4450 (US EPA, 1996g)	Freshwater	Macrophytes	Representatives of several groups required		
OPPTS 850.4100 (US EPA, 1996h) OPPTS 850.4225 (US EPA, 1996i) and OECD 208 (OECD, 1984) Seedling emergence	Terrestrial	Macrophytes	Crop species	EC_x for emerged seedlings, shoot height, shoot dry weight, visual phytotoxicity	
OPPTS 850.4150 (US EPA, 1996j) OPPTS 850.4250 (US EPA, 1996k) OECD 227 (OECD, 2003) Vegetative vigor	Terrestrial	Macrophytes	Crop species	EC_x for plant height, plant dry weight, dead plants, visual phytotoxicity	
OPPTS 850.4300 (US EPA, 1996m) Terrestrial plants field study	Terrestrial	Macrophytes	Dicots (repr. 3 fam.) Monocots (repr. 3 fam) Vascular Cryptogamae (repr. 2 fam.) Bryophyta or Hepatophyta (1 repr.) Gymnospermae (1 repr.)	50% detrimental effect level. Recommendations are more general nature and document is more a guidance than a guideline.	

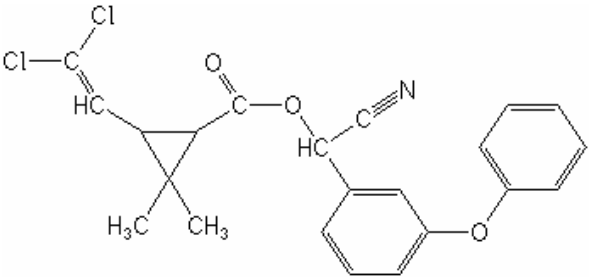
[1] Note: the observed insensitivities may not apply to all species and/or chemicals but may be highly selective [2] only according to OECD guideline 201 [3] only according to OPPTS 850.4400

6. CASE STUDIES

A series of case studies have been generated using the following approach: (1) gather background information (including standard physico-chemical property data, use and release patters and also a short indication of the intended biological activity, if applicable); (2) assess MOA information (if applicable) taking the protein pharmacology approach outlined in Figure 2.3 (after Rang *et al*, 2003); and (3) recommend the priorities for chronic testing in aquatic species (as per Figure 2.2).

6.1 Cypermethrin (ion channel mediated pharmacology)

6.1.1 Structure and physico-chemical properties



CAS number:	52315-07-8
Molecular weight:	416.3
Water solubility:	0.004 mg/L
Half-life:	Aquatic 7 d; soil 28 d
Melting point:	61-83^{o}C
Log K_{ow}:	6.6
Vapour pressure:	2 x 10^{-4}mPa (20^{o}C)

6.1.2 Use and release pattern

Cypermethrin is an alpha-cyano pyrethroid insecticide used for the control of a wide range of insects, especially Lepidoptera, but also Coleoptera, Diptera, Hemiptera, and other classes, found in fruit (including citrus), vines, vegetables, potatoes, cucurbits, lettuce, capsicums, tomatoes, cereals, maize, soya beans, cotton, coffee, cocoa, rice, pecans, oilseed rape, beet, ornamentals, forestry, etc. It is used for the control of flies and other insects in animal houses and mosquitoes,

cockroaches, houseflies and other insect pests in public health. It is also used as an animal ectoparasiticide (Tomlin, 2006).

6.1.3 Intended biological activity

Cypermethrin acts on the nervous system of the insect, and disturbs the function of neurons by interaction with the sodium channel. It is a non-systemic insecticide with contact and stomach action that also exhibits anti-feeding action. There is good residual activity on treated plants.

6.1.4 Efficacy MOA information

Cypermethrin is classified as MOA4 according to Verhaar *et al,* (1992) and ion channels target (Rang *et al,* 2003). The mechanism of action of the pyrethroids is through the nervous system. Their primary mode of action is through interference with ion channels in the nerve axon, resulting in hyperactivity of the nervous system with subsequent lack of control of normal function.

In terms of further aspects influencing toxicity, two types of modes of action of pyrethroids have been observed in mammals. That associated with the Type I pyrethroids (non-cyano, non-halogen-substituted pyrethroids, i.e. pyrethrum) is characterised by tremors (the T syndrome) while that associated with the Type II pyrethroids (halogen-substituted acid moiety and cyano-substituted alcohol moiety) is characterised by choreoathetotic writhing and salivation. Cypermethrin is a Type II pyrethroid. The Type II pyrethroids appear to have a primary mechanism of action that involves an action at the pre-synaptic membrane that involves increased release of synaptic vesicles through an effect on voltage-dependent calcium channels (Laskwoski, 2002). As a consequence, the symptomology of poisoning by Type I and Type II pyrethroids in non-mammals is not as distinct but similar depletion of pre-synaptic vesicles has been observed in insects. Symptoms of poisoning appear rapidly with all pyrethroids and this is characteristic of this class of compounds. The rapid onset of poisoning and the lack of persistence of the pyrethroids in the environment increase the importance of acute toxicity data for assessing environmental exposures.

Importantly, in addition to their action in the nervous system, pyrethroids have been reported to interfere with certain ATPase enzymes associated with maintaining ionic concentration gradients across membranes. It has been speculated that this may increase the sensitivity of freshwater aquatic organisms to these insecticides through the addition of osmotic stress. This has also been suggested as the reason why pyrethroids generally appear to be more toxic at median (isotonic) salinities in euryhaline species than at either high or low salinities. In general, susceptibility to

the pyrethroids is dependent on sensitivity at the site of action and toxicokinetics. Included in the latter are bioavailability and rates of biological transformation. It has been suggested that biotransformation may play a role in differential toxicity of the pyrethroids to fish. This same mechanism may explain the general lower sensitivity to pyrethroids of fish compared to arthropods.

Since cypermethrin acts on nerve cells, any organism with a well-developed nervous system may potentially be susceptible to pyrethroid intoxication. However susceptibility may be influenced by route of exposure, uptake and distribution, and affinity to the target site. It is note-worthy that cypermethrin is highly lipophilic and will bind readily to organic matter. This indicates that bioavailability through non-aqueous media is likely to be very low, and this is borne out by studies of bioavailability studies in the aquatic insect *Chironomus riparius* (Maund *et al*, 2002).

All animals potentially have the target site since the sodium channel is well conserved among the Animalia. Although plants also have sodium channels, it is thought that they are more selective than animal ion channels (Hua *et al*, 2003), which may explain why cypermethrin and other pyrethroids are of low toxicity to crop and non-target plants.

6.1.5 *In vitro* toxicity

Fish cell lines showed no cytotoxicity following 24 h exposure to cypermethrin levels up to 18 mg/L (Babin and Tarazona, 2005).

6.1.6 *In vivo* toxicity

Acute toxicity species sensitivity distributions are available for cypermethrin with aquatic arthropods and fish (Solomon *et al*, 2001). For arthropods, the median acute toxicity value from a range of species is 0.095 µg/L, whereas for fish the median acute toxicity value is 2.1 µg/L. Aquatic arthropods are therefore clearly very much more sensitive than fish.

Table 6.1: In vivo toxicity data

Mammalian (Tomlin 2006)[1]	**Non-mammalian**
LD_{50} (mouse oral) = 138 mg/kg	[1]Acute oral LD_{50} mallard duck >10 000 mg/kg
LD_{50} (rat oral) = 250-7180 mg/kg	[1]Fish 96 h LC_{50} for rainbow trout = 0.69 µg/L
LD_{50} (dog i.v.) = 23.2 mg/kg NOEL (2 y) dog = 5 mg/kg; rat = 7.5 mg/kg ADI = 0.05 mg/kg b.w.	[2]Fish 30 d ELS NOEC = 0.08 µg/L and 330 d full life-cycle NOEC = 0.08 µg/L [3]*Daphnia* 48 h LC_{50} = 0.15 µg/L, and 21 d LOEC = 0.007 µg/L [1]Algal 48 h growth EC_{50} = 44000 µg/L

[1] Tomlin, 2006 - [2] Hill, 1989 - [3] Maund *et al,* 2001.

6.1.7. Biomarker data

Fish cell lines showed no significant EROD induction following 24 h exposure to cypermethrin levels up to 18 mg/L (Babin and Tarazona, 2005).

6.1.8. Base set (acute) aquatic toxicity interspecies sensitivity ratio

For arthropods, the median acute toxicity value from a range of species is 0.095 µg/L, whereas for fish the median acute toxicity value (HC_{50}) is 2.1 µg/L (Solomon *et al,* 2001), hence the median interspecies sensitivity ratio is 22.1 (aquatic animals) and much higher for algae (72 h EC_{50} = 44,000 µg/L) versus invertebrates (ISR = 29,333).

6.1.9. Recommended priority for chronic testing

In a prospective sense, cypermethrin would have a high priority for chronic testing, due to mode of action and very high acute toxicity. Since aquatic arthropods are substantially more sensitive than fish, and considering the rapidity of onset of effects following intoxication with cypermethrin suggesting that incipient toxicity values are likely to be achieved fairly rapidly, chronic testing should be focused on aquatic arthropods.

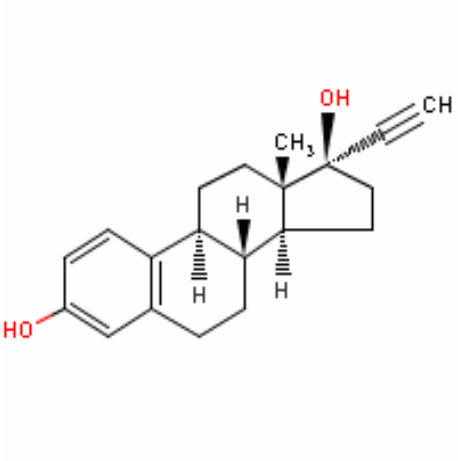
6.1.10. MOA Overview

Current knowledge shows that sensitive species (arthropods) are characterised by a highly active nervous system, ready uptake of chemical over body surface and have limited metabolic and

excretory processes. For similar chemicals, suggests that aquatic arthropods should be included in chronic effects assessment for ERA.

6.2 17α-ethinylestradiol (EE2) (receptor-mediated pharmacology)

6.2.1. Structure and physico-chemical properties



Chemical name:	19-nor-17alpha-pregna 1,3,5(10)-triene-20-yne-3,17-diol
CAS number:	57-63-6
Molecular weight:	296.44
Melting point:	142-146°C
Boiling point:	411°C
Water solubility:	4.75-4.83 mg/L
Log K_{ow} :	3.67
Vapour pressure:	3.6 x 10^{-7} Pa

6.2.2. Use and release pattern

EE2 is a synthetic oestrogen for birth control with the potential for continuous entry into surface waters but is subject to removal during sewage treatment.

6.2.3. Intended biological activity

EE2 is an endocrine active compound (sex hormone) which targets oestrogen receptors in humans. In contrast to the natural analogue 17β-oestradiol, EE2 is less susceptible to liver metabolism (Rang *et al*, 2003).

6.2.4. Efficacy MOA information

EE2 is classified as MOA4 according to Verhaar *et al*, (1992). It is known to act as an oestrogen receptor agonist (Rang *et al*, 2003).

6.2.5. *In vitro* toxicity

No data are available for aquatic animal cell lines. Mutagenic potential of ethinylestradiol has been studied in assays using standard Salmonella strains, cultured human lymphocytes, Chinese hamster cells and bone marrow from mice. These results show that ethinylestradiol does not directly interact with DNA, but under some circumstances (very high, non-physiological concentrations), it may produce nonspecific chromosomal damage. (Hundal *et al*, 1997) reported bacterial reverse mutation assay *Salmonella typhimurium* strains - negative with and without S9. Chromosomal aberration cultured human lymphocytes, *in vitro*, 1-100 mg/mL showed significant increase in aberration frequencies ±metabolic activation, sister chromatid exchanges cultured human lymphocytes, *in vitro*; 1, 10 and 100 mg/mL showed significant increase in SCEs frequencies ±metabolic activation and DNA damage or point mutation in host-mediated assay liver from mice injected with strains of *S. typhimurium* – no significant DNA damage or point mutations.

Cell-mediated V-79 cell mutation V79 Chinese Hamster, *in vitro* – no mutagenic effects with or without metabolic activation (Drevon *et al*, 1981).

Mitotic inhibition and aneuploidy induction Chinese Hamster cells, *in vitro*, 10-100 mM. At high doses (75-100 mM) metaphase arrest, abnormal cell divisions and aneuploidy were observed by Wheeler *et al*, 1986.

Chromosome aberration, Chinese Hamster cells, *in vitro*. Increase in chromatid breaks and exchanges. At the highest level (10^{-4}M) chromosome shrinkage observed (Kochar, 1985).

6.2.6. *In vivo* toxicity

Acute toxicity:

- Fathead minnow (*Pimephales promelas*) 96 h EC_{50} = 1.6 mg/L (Schweinfurth *et al*, 1997).
- Zebrafish (*Danio rerio*) 96 h EC_{50} = 1.7 mg/L (Versonnen *et al*, 2003).
- *Daphnia magna* 48 h EC_{50} = 5.7 mg/L and EC_{10} = 3.2 mg/L (Kopf, 1995 cited in Halling-Sorensen, 1998).

- Green alga (*Selenastrum capricornutum*) 72 h EC_{50} = 0.84 mg/L and EC_{10} = 0.054 mg/L (Kopf, 1995 cited in Halling-Sorensen, 1998).

Chronic toxicity:

- Fathead minnow (*Pimephales promelas*) full life-cycle test to EPA protocol 301 d NOEC = 1 ng/L and LOEC = 4 ng/L (Länge *et al*, 2001). Effects observed included growth retardation in developing fish; sexual maturation (i.e. female: male ratio 84:5 (11% ovotestes) at 56 dph with 100% phenotypic females at 172 d (i.e. no testicular tissue in any fish) and only 50% of depurated females exposed to 4 ng/L were functionally reproductive.
- Fathead minnow (*Pimephales promelas*) full life-cycle test 150 d NOEC = 0.32 ng/L and at 0.96 ng/L showed changes in male secondary sex characteristics. At 150 d, 3.5 ng/L resulted in 100% feminisation and no eggs (Parrott and Blunt, 2005).
- Zebrafish (*Danio rerio*) 2-generation test gave 210 d NOEC = 0.5 ng/L (5 ng/l for first generation) and LOEC = 5 ng/L based on growth retardation in developing fish; affected sexual maturation (i.e. female:male ratio 84:5 (11% ovotestes) at 56 dph with 100% females at 172 dph (i.e. no testicular tissue in any fish). Only 50% of depurated females exposed to 4 ng/L were functionally reproductive (Nash *et al,* 2004).

Other ecotoxicity data:

- *Daphnia magna* reproduction 21 d EC_{50} for reproduction = 0.105 mg/L and EC_{10} for reproduction = 0.0125 mg/L; while 21 d NOEC reproduction = 0.01 mg/L ((Kopf, 1995 cited in Halling-Sorensen, 1998).
- Bioconcentration factor (BCF) probably <500 based on experimental data in fathead minnows (*Länge et al,* 2001) while moderate BCF of 1000-1995 (calculated from log K_{ow}).

Mammalian data:

- Chromosomal aberrations and micronucleus test - bone marrow from mice treated with 0.002 and 0.200 mg/kg for 15 days gave no significant increased chromosome aberrations (breaks, exchanges, multiple aberrations or stickiness and pulverization). No significant increases in frequency of micronuclei or increase in micronucleated erythrocytes at doses up to 40 times the human therapeutic dose were found (Shyama and Rahiman, 1996).

6.2.7 Biomarker data

In vivo studies show induction of vitellogenin and other oestrogen related biomarkers in low ng/L range (Länge *et al*, 2001; Nash *et al*, 2004; Parrott and Blunt, 2005).

6.2.8 Base set (acute) aquatic toxicity interspecies sensitivity ratio

Base set toxicity EC_{50} values are 0.84 mg/L (algae), 5.7 mg/L (daphnids) and 1.6 mg/L (fish), hence the base set median interspecies sensitivity ratio (ISR) is 6.8.

6.2.9 Recommended priority for chronic testing

Based on available acute toxicity data, the three tested taxa (fish, crustaceans, and algae) appeared to be equally sensitive to EE2 so the ISR concept does not raise an alert for chronic effects testing. However, the oestrogen receptor mediated MOA would likely be expected to produce effects in fish and other aquatic organisms which have the oestrogen receptor (daphnids have an ecdysteroid and juvenile hormone based endocrinology and no analogous receptor has been identified in algae). The fish vitellogenin studies support this approach. In a prospective sense, this suggests the need for chronic fish studies for such compounds.

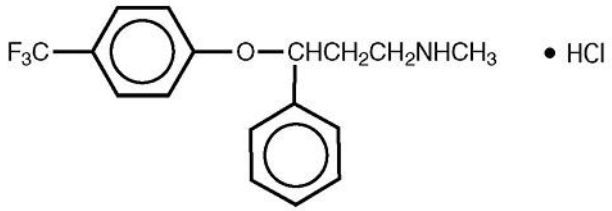
Today, there is a growing database of longer-term studies in multiple species, including full life-cycle fish studies, that shows acute to chronic ratios (ACR) that correlate with the presence of the oestrogen receptor in the organism. Specifically, multiple studies demonstrate reproductive effects in fish to be the most sensitive endpoint for exposure to EE2 in the aquatic environment with corresponding ACR of greater than one million. For example, the fathead minnow LC_{50} of 1.6 mg/L (Schweinfurth *et al*, 1997) divided by the chronic NOEC of 1 ng/L from the two-generation full life-cycle study conducted in fathead minnows over 305 days (Lange *et al*, 2001) results in an ACR of 1.6×10^6. A full life-cycle study of shorter duration (150 days) reported a LOEC of 0.96 ng/L based on changes in secondary sex characteristics in male fish with a NOEC of 0.32 ng/L for this endpoint; however, reproductive success was not affected at 0.96 ng/L (Parrott and Blunt, 2005). In zebrafish, the ACR derived from the LC_{50} of 1.7 mg/L (Versonnen *et al*, 2003) and the two-generation full life-cycle study NOEC of 0.5 ng/L (Nash *et al*, 2004) is 3.4×10^6. Further, all of the full life-cycle studies had a steep dose response, with a LOEC between 3.5 to 5 ng/L that resulted in 100% feminisation of fish. The ACR for daphnia and algae are 570 and 16, respectively, based on one study each (Kopf, 1995 cited in Halling-Sørensen *et al*, 1998). In a representative study from a non standard species, *Nitocra spinipes*, a sexually reproducing harpacticoid copepod, a 96 h LC_{50} of 0.5 mg/L was reported; there were no effects on mortality, larval development rate, fecundity, or sex ratio from long-term (18d) exposure of this species to 0.05 mg/L EE2, which was a NOEC. Thus, the ACR in this crustacean was 10 (Breitholtz and Bengtsson, 2001).

6.2.10 MOA overview

Current knowledge shows that sensitive aquatic species are characterised by an oestrogen (and androgen)-based developmental and reproductive biology, including fish and molluscs. Daphnids and other arthropods have not evolved mammalian-type oestrogen receptors (Hutchinson, 2007). For similar chemicals, suggests that fish or molluscs should be included in chronic effects assessment for ERA.

6.3 Fluoxetine SSRI (transporter mediated pharmacology)

6.3.1 Structure and physico-chemical properties



Chemical name:	(±)-N-methyl-3-phenyl-3-[((alpha),(alpha),(alpha)-trifluoro-*p*-tolyl)oxy]propylamine hydrochloride
CAS number:	56296-78-7
Molecular weight:	345.79 (PDR)
Water solubility:	14 mg/L (PDR)
Half-life:	1-3 days, Met. 4-16 days (PDR)
Log K_{ow}:	1.0, 1.8, 2.6 at pH 5, 7 and 9 (MSDS 2005), high degree of protein binding (> 94.5%) (PDR), however, Caminda *et al* (2006) quote log K_{ow} = 4.09
pK_a:	8.7 (MSDS)

6.3.2 Use and release pattern

Chronic use as a human pharmaceutical as a psychotropic drug and is also marketed for the treatment of premenstrual dysphoric disorder. Fluoxetine is extensively metabolised (Rang *et al*, 2003; see also http://www.rxlist.com) in human patients and laboratory mammals.

6.3.3 Intended biological activity

Alleviation of depression and anxiety by inhibition of serotonin reuptake, prolonging availability at CNS neuronal synapse. Studies at clinically relevant doses in man have demonstrated that fluoxetine blocks the uptake of serotonin into human platelets. Studies in animals also suggest that fluoxetine is a much more potent uptake inhibitor of serotonin than of norepinephrine. Antagonism of muscarinic, histaminergic, and α1-adrenergic receptors has been hypothesised to be associated with various anticholinergic, sedative, and cardiovascular effects of classical tricyclic antidepressant drugs (Rang *et al*, 2003; see also http://www.rxlist.com).

6.3.4 Efficacy MOA information

Fluoxetine is classified as MOA4 according to the conceptual scheme proposed by Verhaar *et al*, (1992), specifically inhibiting the enzyme (protein-phosphatase-1 or 'PP-1') controlling serotonin reuptake at the CNS neuronal synapse.

More broadly, in terms of the target site biochemistry, serotonin regulates the phosphorylation of an intracellular protein essential for neurotransmission, DARPP-32, at three specific sites on the very large protein, adding phosphate groups to two of the sites, and removing phosphate at the third specific site. This combination of changes in DARPP-32 directly leads to a decrease in the activity of a key intracellular enzyme, PP-1. Decreasing the activity of PP-1 leads to changes in how neurotransmitters bind or are blocked from binding to receptors, changes in movements of ions like sodium, potassium, and calcium into and out of the nerve cell, and changes in how much or how little of certain proteins the neuron produces.

6.3.5 *In vitro* toxicity

Caminada *et al*, (2006) tested fluoxetine for cytotoxicity in two different fish cell lines, giving 24 h EC_{50} values of between 0.0242 and 0.0107 mM (3.31 and 7.48 mg/L fluoxetine, respectively).

6.3.6 *In vivo* toxicity

Acute toxicity (Eli Lilly Material Safety Date Sheet, 2005):

- Fish (rainbow trout) 96 h LC_{50} = 1570 μg/L.
- Water flea *(Daphnia magna)* 48 h EC_{50} = 940 μg/L.

- Algae (*Selenastrum capricornutum*) 72 h EC_{50} = 30.5 µg/L (based on average specific growth rate).

Toxicity to microorganisms:

- Fungus (*Chaetomium globosum*): MIC = 64 mg/L.
- Mold (*Aspergillus flavus*): MIC = 64 mg/L.
- Soil bacteria (*Pseudomonas acidovorans*): MIC = 1000 mg/L.
- N-fixing bacteria (*Azotobacter chroococcum*): MIC = 64 mg/L.
- Blue-green algae (*Nostoc* sp.): MIC = 250 mg/L.

Additional ecotoxicity data:

- *Ceriodaphnia dubia* 8 d $^{\text{reproduction decrease}}$NOEC = 89 µg/L (Henry *et al*, 2005) but stimulation of *D. magna* reproduction at 36 µg/L (Flaherty *et al*, 2005; note this effect was not shown by Brooks *et al*, 2003). Induced spawning in freshwater mussels at 150 - 3000 µg/L (Fong, 1998) and at 309 µg/L (Cunha and Machado, 2001).
- Juvenile fish development inhibited at 60 µg/L suggesting fish LC_{50}/LOEC ratio >9900 but 28 d medaka $^{\text{reproduction}}$NOEC = 5 µg/l (Foran *et al,* 2004). Nakamura *et al* (2007) report an LC_{50} in the Japanese medaka of 5.5 mg/L at pH 7.

6.3.7 Biomarker data

To determine its potential to disrupt teleost reproductive function, Foran *et al* (2004) exposed Japanese medaka (*Oryzias latipes*) to fluoxetine at aqueous nominal concentrations of 0, 0.1, 0.5, 1 and 5 µg/L for 4 weeks. The last 14 days of this exposure included a reproductive assessment in which no significant changes were observed in egg production, rate of fertilisation and spawning, or hatching success of fertilized eggs. A low incidence (1.97-2.53%; 4.02-5.16-fold greater than controls) of developmental abnormalities was observed in offspring from all fluoxetine treatments. Adult gonadal somatic index, hepatic vitellogenin, and *ex vivo* gonadal steroidogenesis were also unaffected. Circulating plasma oestradiol levels in females were significantly increased by 0.1 and 0.5 µg/L fluoxetine treatments.

6.3.8 Base set (acute) aquatic toxicity interspecies sensitivity ratio

Base set toxicity EC_{50} values are 30.55 µg/L (algae), 940 µg/L (daphnids) and 1570 µg/L (fish), hence the base set median interspecies sensitivity ratio (ISR) is approximately 58.

6.3.9 Recommended priority for chronic testing

Further research into the toxicity of fluoxetine to macrophytes could potentially be useful in giving insight into the mechanisms of SSRI phytotoxicity.

6.3.10 MOA Overview

For fluoxetine, aquatic animal studies show complex pattern of inhibiting and stimulating effects on development and reproduction that are difficult to rationalise. This may reflect the inherent complexity of neurophysiology and the fact that the precise mechanism of SSRI action remains unclear in mammals. As far as we are aware, the mechanistic basis for the phytotoxic effects of fluoxetine has not been reported and this could be a fruitful line of research. Pragmatically, the 14 d algal growth study gives the most sensitive endpoint to date underscores the need to maintain a multi-species approach in the aquatic testing of MOA4 chemicals.

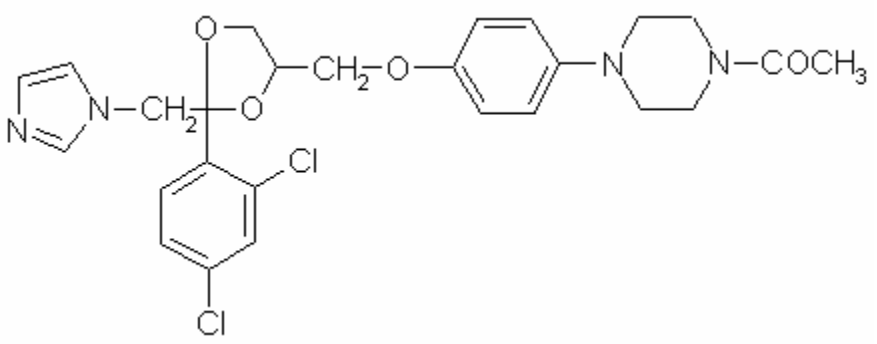
6.3.11 Data for PNEC calculation

- Green algae (*Pseudokirchneriella subcapitata*): 14 d NOEC = 1.1 μg/L.
- Cladoceran (*Ceriodaphnia dubia*): NOEC = 56 μg/L (Brooks *et al*, 2003).
- Japanese medaka (*Oryzias latipes*): No reproductive effects up to the highest concentration tested, 5 μg/L, and no concentration-related effects on any other physiological measurements were found by Foran *et al* (2004). However, the potential for selective serotonin reuptake inhibitors to act as endocrine disruptors in fish is not known. These data would suggest a $PNEC_{freshwater}$ of 0.11 μg/L (derived from the NOEC of 1.1 μg/L for green algae, divided by a safety factor of 10) and a $PNEC_{marine}$ of 0.01 μg/L ($PNEC_{freshwater}$ divided by 10).

All the above data were taken from www.fass.se.

6.4 Ketoconazole (enzyme mediated pharmacology)

6.4.1 Structure and physico-chemical properties



Chemical name:	Imidazolderivative (2 nitrogens in the azole ring) ($C_{26}H_{28}Cl_2N_4O_4$)
CAS number:	65277-42-1
Molecular weight:	531.4 (Römpp Chemie Lexikon)
Water solubility:	0.0866 mg/L (drugbank redpoll.pharmacy.ualberta.ca)
Melting point:	146°C (Römpp Chemie Lexikon), 148-152°C (European Pharmacopoeia), 148°C (pharmazie.uni-marburg)
Log K_{ow}:	4.35 at pH 7 (Leutz, 2005)

6.4.2 Use and release pattern

Ketoconazole is a broad-spectrum antimycotic used in human and veterinary medication to treat a variety of fungal skin and systemic infections. Alot of fungal diseases can be treated, e.g. *Pityriasis ersicolor*, Dermatomycosis, Onychomycosis, Paracoccidioidomycosis, Doccidioidomycosis, Candidiasis, as well as those caused by *Cryptococcus* sp., *Aspergillus* sp., *Fusarium* sp., *Trichospron* sp. and *Pseudallescheria* sp.

6.4.3 Intended biological activity

Ketoconazole inhibits the synthesis of ergosterol or interacts directly with ergosterol, which is the predominant component of the fungal cell membrane. Ergosterol serves as a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells. Cytochrome P450-oxidase is inhibited in the biosynthesis of ergosterol.

6.4.4 Efficacy MOA information

Ketoconazole is classified as MOA4 according to Verhaar *et al* (1992) and enzyme target inhibitor (Rang *et al*, 2003). The applicable mechanism of action of ketoconazole is its negative impact on membrane fluidity, asymmetry and integrity in fungi, as the synthesis of ergosterol, which is the predominant component of fungal cell walls, is inhibited. The mode of action is described as an inhibition of cytochrome P450-oxidase within the biosynthesis of ergosterol. Integrity of the cell membrane requires that inserted sterols, i.e. ergosterol, lack C-4methyl groups (Hector, 1993). In ergosterol synthesis, 14α-demethylation of lanosterol leads to ergosterol. Ketoconazole has been found to target the heme protein, which catalyses cytochrome P450 (CYP) dependent 14α-demethylation of lanosterol (Hector, 1993; Vanden Bossche, 1988). This happens due to interaction of azole nitrogens with the heme iron ion in CYP. The geometric orientation of the azole moiety to the ferrous heme iron is regulated by the interaction between the N1-substituent and the heme environment. Interference or disruption of numerous membrane and barrier functions occurs as a result of the ergosterol depletion. Membrane permeability increases, metabolism is disrupted and inhibition of growth follows, leading to death of the cell. Besides inhibition of ergosterol biosynthesis ketoconazole inhibits synthesis and activity of several membrane-bound enzymes, as well as membrane lipid biosynthesis. Further consequences of ketoconazole exposition are a higher degree of lipid unsaturation and an inhibition of fatty acid desaturation and elongation. Additionally, an altered mitochondrial activity can follow.

Besides the inhibition of ergosterol formation by 14α-demethylation, ketoconazole can inhibit a variety of P450s. Cytochrome P450 can be found throughout nature (Guengerich, 2001). This highly diversified set of hemethiolate proteins is present in eukaryotic, as well as in prokaryotic cells (Guengerich, 2001; Van Eerd *et al*, 2003).

CYPs are divided into three classes:

- Class I P450s are flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) dependent and require reduced nicotinamide adenine dinucleotide phosphate (NADPH). They are microsomal membrane bound in plants and filamentous fungi. Class I P450s of non-filamentous fungi and bacteria are soluble.
- Class II P450s are similar to Class I P450s, but they only occur in bacterial and animal mitochondria.
- Class III P450s are located in plant plastids and do not require auxiliary redox partners (Van Eerd *et al*, 2003).

Plants and animals have similar enzyme systems and gene families to metabolise a wide range of xenobiotics, including the CYP monooxygenases, which have been detected universally in animal

and higher plant species. CYP systems have also been detected in unicellular green algae (Thies and Grimme, 1996) and marine macroalgae (Chlorophyta, Chromophyta, and Rhodophyta) (Pflugmacher and Sandermann, 1998). Cytochrome P450 51 (CYP51), which is known as sterol 14α-demethylase and Erg11p in yeast, is found in different kingdoms of life, i.e. yeast, mammals and plants.

The involvement of Cytochrome P450, the target site for ketoconazole in different kinds of organisms is described below:

- In humans and mammals CYPs are necessary for phase I metabolism of drugs/xenobiotics. Steroid biosynthesis is CYP-dependent at the stage of 14α-demethylation (various adrenal and gonadal steroids, e.g. cholesterol, testosterone, oestradiol). Inhibition of synthesis of those cholesterol hormones occurs at usual doses for antifungal treatments in humans. CYP is involved in further enzyme induction and inhibition (meiosis-activating sterols in sperm and egg production), fatty acid metabolism and other processes. Besides gynecomastia and adrenal insufficiency, rare cases of fatal hepatotoxicity have been reported in humans/mammals as possible side effects.
- In fish CYPs are involved in drug/xenobiotic metabolism, further CYP-mediated enzyme activities have been reported. For example 21 d exposure to ketoconazole inhibited fecundity at 0.4 mg/L in fathead minnow (Kahl *et al*, 2005). Interaction of ketoconazole with CYP1A and CYP3A activities and protein expression in Atlantic cod has also been observed (Hasselberg *et al*, 2005). *In vivo* and *in vitro* studies showed inhibition of CYP1A and CYP3A enzyme activities in rainbow trout and killifish (Hegelund *et al*, 2004). *In vitro* exposure of rainbow trout hepatocytes and *in vivo* exposure of rainbow trout to ketoconazole led to significant effects upon CYP1A (Myrbaeck *et al*, 2002).
- In birds, crustaceans, amphibians and insects CYPs are involved in xenobiotic and fatty acid metabolism. In recent studies ketoconazole led to significant reductions in reproduction of *Caenorhabditis elegans* (Breger *et al*, 2007). In plants CYPs are involved in colouration, defense, xenobiotic metabolism (Guengerich, 2001) and phytosterol biosynthesis (Vanden Bossche, 1988).

6.4.5 *In vitro* toxicity

Table 6.2: In vitro toxicity data

Organism / cellular fractions	Observation parameter / substrate	Endpoint / effective value	Citation
Rat - subcellular fraction liver	[^{14}C]mevalonate incorporation into cholesterol	$IC_{50} = 2x10^{-6}M$	Vanden Bossche, 1988
Rat - subcellular fraction testis	Inhibition of cytochrome P450 dependent 17,20-lyase	$IC_{50} = 2x10^{-7}$ M	Vanden Bossche, 1988
Bovine adrenal microsomes	Inhibition of cytochrome P450 dependent 17,20-lyase	$IC_{50} = 4x10^{-7}$ M	Vanden Bossche, 1988
Human - *Baculovirus supersomes*	Insect cell-expressed aromatase supersomes and DBF as substrate	IC_{50} = 0.9 µM to 5.6 µM	Stresser *et al,* 2000b and Kragie *et al,* 2002 cit. in Trösken *et al,* 2006
Human - *Baculovirus supersomes*	Insect cell-expressed aromatase supersomes and testosterone as substrate	IC_{50} = 281 µM	Trösken *et al,* 2006
Human placental microsomes	Tritiated androstenedione as substrate	IC_{50} = 65 µM	Mason *et al,* 1987 cit. in Trösken *et al,* 2006
Human placental microsomes	Tritiated testosterone as substrate	IC_{50} = 6 µM	Ayub and Levell 1988 cit. in Trösken *et al,* 2006
Atlantic cod pooled liver microsomes	Inhibition of CYP1A activity	IC_{50} = 0.6 µM	Hasselberg *et al,* 2005
Atlantic cod pooled liver microsomes	Inhibition of CYP3A-mediated BFCOD	IC_{50} = 0.3 µM	Hasselberg *et al,* 2005
Human - *Baculovirus supersomes*	cDNA expressed CYP3A4	IC_{50} = 0.4 µM	Hasselberg *et al,* 2005
Fathead minnow brain and ovary tissue	Significant inhibition of aromatase activity	> 1 µM	Villeneuve *et al,* 2005
Leaping mullet (*Liza saliens*) liver microsome	Inhibition of cocaine metabolism (cyt P4503A inhibition)	IC_{50} = 20µM	Arinc *et al,* 2003
Harbour seal liver microsomes	Inhibition of chlorobornane congener metabolism (cytochrome P450 inhibition)	IC_{50} = 0.75 µM	van Hezik *et al,* 2001
Ringed seal (*Phoca hispida*) hepatic microsomes	Inhibition of hydroxylation of testosterone (CYP3A reaction)	80% at 1 µM	Wolkers *et al,* 1998
Ringed seal (*Phoca hispida*) hepatic microsomes	Inhibition of ethoxyresorufin 0-deethylation (EROD) (CYP1A reaction)	Significant inhibition at > = 5 µM	Wolkers *et al,* 1998

Table 6.2: In vitro toxicity data *(cont'd)*

Organism / cellular fractions	Observation parameter / substrate	Endpoint / effective value	Citation
Ringed seal (*Phoca hispida*) hepatic microsomes	Inhibition of carreine N_3-demethylation (CYP1A reaction)	Significant inhibition at > = 5 µM	Wolkers *et al,* 1998
Female Sprague-Dawley rats adrenal glands cells mitochondria	Inhibition of corticosterone production	IC_{50} = 0.3 µg/mL	Loose *et al,* 1983
Cultured pig kidney cell line	Inhibition of 24-hydroxylase	IC_{50} = 0.2 µg/mL	Loose *et al,* 1983
Rabbit liver microsomes	Progesterone 21 6β- and 16α-hydroxylase activities inhibition	IC_{50} = 10^{-5}M - 10^{-4}M	Senciall *et al,* 1988
Rabbit liver microsomes	Steroid acid formation	IC_{50} = approx. 10^{-5}M[1]	Senciall *et al,* 1988
Human liver microsomes	Inhibition of sildenafil metabolism (CYP3A4 inhibition)	IC_{50} < 0.05 µM	EMEA, 2005
Frog (*Rana esculenta*) brain and pituitary	Synthesis of 17-hydroxypregnenolone, 17-hydroxyprogesterone, dehydroepiandrosterone and androstenedione, respect. (Inhibition of cyt P450 17α-hydroxylase/C17, 20-lyase)	Significant inhibition at >= 10^{-9}M - 10^{-7} M	Do Rego *et al,* 2007

[1] "steroid acid formation similarly inhibited (as progesterone 21 6β- and 16α-hydroxylase activities) at 10^{-5}M".

6.4.6 *In vivo* toxicity

Table 6.3: In vivo toxicity data

Mammalian (Römpp Chemie Lexikon)	Non-mammalian (IUCLID data, 2007)
LD_{50} (mouse i.v.) = 32 mg/kg	*Fish* 96 h LC_{50} = 3900 µg/L
LD_{50} (mouse oral) = 618 mg/kg	*Daphnia* 48 h EC_{50} = 400 µg/L
LD_{50} (rat i.v.) = 86 mg/kg	*Algae* 72 h EC_{50} = 1800 µg/L
LD_{50} (rat oral) = 166 mg/kg	
LD_{50} (dog i.v.) = 23.2 mg/kg	
LD_{50} (dog oral) = 178 mg/kg	
LD_{50} (guinea pig oral) = 178 mg/kg[1]	
LD_{50} (guinea pig i.v.) = 28 mg/kg[2]	

[1] MSDS ketoconazole, sciencelab.com
[2] wisda.pharmazie.uni-marburg.de

Additional ecotoxicity data examples:

- Rainbow trout (*Oncorhynchus mykiss*), behavioural changes, 24 hours, NOEC > = 200 mg/L (ketoconazole pure product) (Tojo *et al*, 1993). *Oncorhynchus mykiss*, mortality, 24 hours, NOEC => 200 mg/L (ketoconazole pure product) (Tojo *et al*, 1993). *Oncorhynchus mykiss*, mortality, 3 hours, NOEC >= 200 mg/L (ketoconazole pure product) (Tojo *et al*, 1994).
- In male Sprague-Dawley rats 25 mg ketoconazole fed every 8 hours for 21 days caused 50% suppression of serum testosterone and prostate and seminal vesicle weights. Intratesticular testosterone concentrations decreased by 50%. Testicular 17 α-hydroxylase, 17,20-desmolase and 17 β-hydroxysteroid dehydrogenase activities were also significantly decreased (Bhasin *et al*, 1986).
- In Atlantic cod 12 mg ketoconazole/kg body weight resulted in 159% average induced increases in CYP1A-mediated EROD activities and 133% increase in CYP1A protein levels (Hasselberg *et al*, 2005).
- In urinary excretion of rabbits ketoconazole caused a reduction of 50% of acidic metabolites of [^{3}H]deoxycorticosterone and 75% of acidic metabolites of [^{14}C]progesterone (Senciall *et al*, 1988).

6.4.7 Biomarker data

For fish (female fathead minnows), Ankley *et al* (2007) report 7 d $^{\text{vitellogenin}}$NOEC and $^{\text{vitellogenin}}$LOEC values of 300 and 900 µg/L, respectively. In the same study, male fish 21 d $^{\text{reproduction}}$NOEC and $^{\text{reproduction}}$LOEC values were 25 and 100 µg/L, respectively. Hence, the acute-chronic ratio (LC_{50}/LOEC) = 3900/100 = 39 for ketoconazole. Molecular analyses also showed increased CYP11A and CYP17 activity in ketoconazole exposed fish (Ankley *et al*, 2007).

6.4.8 Base set (acute) aquatic toxicity interspecies sensitivity ratio

For ketoconazole acute endpoints for three aquatic species are available. The 72 h EC_{50} for algae is 1800 µg/L, the 48 h EC_{50} for daphnia is 400 µg/L and the 96 h LC_{50} for fish is 3900 µg/L. Hence the acute interspecies sensitivity ratio is 9.8.

6.4.9 Recommended priority for chronic testing

According to the mode of action a high priority for chronic testing would be recommended. Cytochrome P450 occurs in prokaryotic and eukaryotic cells and is involved, among other important processes like drug/xenobiotic metabolism and fatty acid metabolism, in multiple hormone regulated processes (steroid biosynthesis). As endocrine potential in vertebrates has

been observed, chronic testing of substances with inhibition of ergosterol biosynthesis or other CYP enzymes is advisable. Acute toxic effects are less likely (see toxicity data).

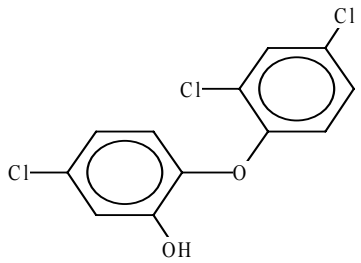
Ketoconazole is a potentially valuable reference pharmaceutical with a well-described biochemical mode of action in mammals and fish. It is known that non-arthropod invertebrates (e.g. molluscs) generally show lower CYP activity than mammals, fish or arthropods, suggesting that non-arthrpod invertebrates which have a limited CYP activity may be somewhat more sensitive to ketoconazole.

6.4.10 Data for PNEC calculation

For the estimation of the PNEC, the overall database is limited and it would be desirable to gather chronic data from a wider range of aquatic organisms. Thus a considerable uncertainty remains, which has to be considered by the assessment factor. The available studies are on the one hand short-term studies, with a lowest endpoint of 400 mg/L (48 h LC_{50} *D. magna*), and a chronic fish study with a NOEC of 25 mg/L (NOEC 21 day fathead minnow). For these studies an assessment factor of 100 (daphnia) and 10 (fathead minnow) should be applied. This would result in a $PNEC_{freshwater}$ of 4 mg/L and 2.5 mg/L, respectively. This is a quite consistent value and gives evidence of robustness of the derived PNEC.

6.5 Triclosan (a nonionic antimicrobial agent)

6.5.1 Structure and physico-chemical properties



CAS number:	3380-34-5
Molecular weight:	289.6
Water solubility:	10 mg/L (at 20ºC, Yalkowsky and Dannenfelser, 1992)
Melting point:	54-57.3°C (Syracuse Research Corporation, EpiWin Suite V 3.12)
Log K_{ow}:	4.80 (Reiss *et al,* 2002)
pK_a of the phenol:	8.14
Vapour pressure:	3.6×10^{-7} Pa

6.5.2 Use and release pattern

Triclosan is used as an antimicrobial in hand soaps and as an antigingival and antiplaque agent in toothpastes. These products are typically used in the home and disposed down the drain and into a wastewater treatment system. Triclosan is also incorporated into a variety of plastics and fabrics where it is also used as an antimicrobial.

6.5.3 Intended biological activity

Triclosan is a broad-spectrum antimicrobial agent used as an antimicrobial in hand soaps, denitrifices, mouthwashes and dishwashing liquids. It is also used in some durable goods including clothing and plastics where antimicrobial activity is beneficial (e.g. dish towels).

6.5.4 Efficacy MOA information

Triclosan is classified as MOA4 according to Verhaar *et al* (1992) and enzyme target inhibitor (Rang *et al*, 2003). Triclosan has been shown to inhibit Fab I, enoyl-acyl carrier protein in bacteria (McMurray *et al*, 1998; Slater-Radosti *et al*, 2001). The mechanism of action is unknown but presumed to involve enzyme inhibition.

6.5.5 *In vitro* toxicity

24 h EC_{50} 0.05-0.06 mM in a human gingival epithelial cell line was assessed with the neutral red assay (Zuckerbraun *et al*, 1998).

After 3 d exposure, a lag in the growth kinetics of gingival cells was observed at 0.01 mM triclosan.

Triclosan-induced cell death was apparently by apoptosis.

6.5.6 *In vivo* toxicity

Acute data

- LD_{50} (mouse i.v.) = 1090 mg/kg (Kanetoshi *et al*, 1992).
- LD_{50} (oral) = 3750 - 5000 mg/kg (mice, dog, rat) (Bhargava and Leonard, 1996).
- LC_{50} (*Oryzias latipes*) = 399 µg/L (Ishibishi *et al*, 2004).
- LC_{50} (*Pimephales promelas*) = 260 µg/L (Orvos *et al,* 2002).
- LC_{50} (*Lepomis macrochirus*) = 440 µg/L (Orvos *et al*, 2002).
- EC_{50} (*Daphnia magna*) = 344 µg/L (Orvos *et al*, 2002).
- EC_{50} (*Ceriodaphnia dubia*) = 185 µg/L (Orvos *et al*, 2002).
- EC_{50} (*Scenedesmus subspicatus*) = 0.7 µg/L (Orvos *et al*, 2002).
- EC_{50} (*Pseudokirchneriella subcapitata*) = 4.46 µg/L (Orvos *et al*, 2002).
- EC_{50} (*Lemna minor*) = > 62.5µg/L (Orvos *et al*, 2002).

Chronic data

- 21 d NOEC (*Oryzias latipes*) = 156 µg/L (Ishibishi *et al*, 2004).
- 7 d EC_{50} (*Ceriodaphnia dubia*) = 4.5 µg/L (Orvos *et al*, 2002).
- 4 d EC_{25} (*Pseudokirchneriella subcapitata*) = 2.44 µg/L (Orvos *et al*, 2002).
- 61 d post hatch NOEC (*Onchorynchus mykiss*) = 34.1 µg/L (Orvos *et al*, 2002).
- 21 d NOEC (*Daphnia magna*) = 40 µg/L (Orvos *et al*, 2002).
- 3 d $NOEC_{biomass}$ (*Scenedesmus subspicatus*) = 0.5 µg/L (Orvos *et al*, 2002).
- 4 d EC_{25} (*Navicula pelliculosa*) = 10.7 µg/L (Orvos *et al*, 2002).
- 4 d EC_{25} (*Anabaena flos-aqua*) = 0.67 µg/L (Orvos *et al*, 2002).

6.5.7 Biomarker data

A 21 d chronic medaka study a ^{vitellogenin}LOEC of 20 µg/L, suggesting that triclosan (or a metabolite) may be weakly oestrogenic with the potential to induce vitellogenin (Ishibishi *et al*, 2004).

6.5.8 Base set (acute) aquatic toxicity interspecies sensitivity ratio

Triclosan has an acute ecotoxicity profile of 2.6 µg/L (algae), 264 µg/L (daphnids) and 366 µg/L (fish), giving an ISR of 141.

6.5.9 Recommended priority for chronic testing

High priority for chronic testing, due to mode of action and the targeted taxa (microorganisms). Triclosan acts by inhibiting Fab I, enoyl-acyl carrier protein reductase (McMurray *et al*, 1998; Slater-Radosti *et al*, 2001). This enzyme is not present in higher vertebrates and the available data suggest this protein is not present in aquatic invertebrates. However due to the presence of this carrier protein in microorganisms, chronic toxicity studies in algae were conducted. Given the observation of a biomarker response (VTG and GSI) long-term studies that consider reproduction were conducted with fish.

6.5.10 MOA Overview

Triclosan represents an example of a chemical that targets microbial (fungal) enzyme pathways as the target protein for its intended MOA. In order to support the existing risk assessment, further mechanistic studies are desirable to study the biochemical MOA in fish and other aquatic species.

6.6 Acute interspecies sensitivity ratios

Since the use of base set acute tests (using algae, daphnids and fish) is the typical starting point for regulatory assessments, Table 6.4 has been prepared to illustrate some of the acute interspecies sensitivity ratios (ISRs) currently available for MOA1, 2, 3 and 4 chemicals. The on-going ECETOC Task Force on PBT Case Studies has been used as the primary source of several data (Holt, personal communication), supplemented by data obtained from IUCLID or from manufacturers' Safety Data Sheets. For MOA1 (inert chemicals), the acute ISRs range from 1.17 to 2.34, whereas for MOA2 (less inert chemicals), the acute ISRs range from 8.56-343. Lying within the same range, the acute ISR values for MOA3 (reactive chemicals) is <5 to >25 whilst the range for MOA4 (specifically acting chemicals) is greatest (6.8 to > 29000). These data suggest that it may be difficult to definitively use acute ISRs as a potential indicator of test organism susceptibility to different classes of chemicals. Moreover, the low acute ISR (6.8) of pharmaceuticals such as EE2 also suggests that acute base set data may not always be useful for indicating the test organism most vulnerable to chronic effects (in the case of EE2, algae are the most sensitive in the acute studies but other studies show that fish are much more sensitive to chronic EE2 exposures at the ng/L level). However, for MOA1 chemicals, the low acute ISRs do provide valuable insight into the likely narcotic MOA which is shared across different aquatic species. It should be noted that Table 6.4 contains an illustrative list of examples but more data are needed to explore whether these patterns of acute ISRs are typical of larger datasets.

Table 6.4: Illustrative examples of interspecies sensitivity ratios (ISR values) for MOA1 - 4 chemicals

Chemical	CAS number	Acute toxicity data (mg/L)	ISR (A/D/F)	Information source
Verhaar et al, MOA1	*Inert chemicals (assumed toxic via narcosis)*			
1,2,4-trichlorobenzene	120-82-1	Algal 72 h EC_{50} = 1.4 Daphnid 48 h EC_{50} = 1.6 Fish 96 h LC_{50} = 1.3	1.17	ECETOC PBT Case Studies TF
Trichloroethylene	79-01-6	Algal 72 h EC_{50} = 36.5 Daphnid 48 h EC_{50} = 20.8 Fish 96 h LC_{50} = 28.3	1.75	ECETOC PBT Case Studies TF
Tetrachloroethylene	127-18-4	Algal 72 h EC_{50} = 3.6 Daphnid 48 h EC_{50} = 8.5 Fish 96 h LC_{50} = 8.4	2.34	ECETOC PBT Case Studies TF
Tert butyl-methylether	1634-04-4	Algal 72 h EC_{50} = 491 Daphnid 48 h EC_{50} = 472 Fish 96 h LC_{50} = 672	1.42	ECETOC PBT Case Studies TF
Verhaar et al, MOA2	*Less inert chemicals (assumed toxic via polar narcosis)*			
Phenol	108-95-2	Algal 72 h EC_{50} = 122.8 Daphnid 48 h EC_{50} = 11.8 Fish 96 h LC_{50} = 18.2	10.4	ECETOC PBT Case Studies TF
Aniline	62-53-3	Algal 72 h EC_{50} = 35.9 Daphnid 48 h EC_{50} = 0.2 Fish 96 h LC_{50} = 68.6	343	ECETOC PBT Case Studies TF
2-ethoxy ethylacetate	111-15-9	Algal 72 h EC_{50} = 1000 Daphnid 48 h EC_{50} = 354 Fish 96 h LC_{50} = 41	24.4	ECETOC PBT Case Studies TF
Methylacetate	79-20-9	Algal 72 h EC_{50} = 120 Daphnid 48 h EC_{50} = 1027 Fish 96 h LC_{50} = 225	8.56	ECETOC PBT Case Studies TF
Verhaar et al, MOA3	*Reactive chemicals (unselective reactivity with biomolecules)*			
Epichlorohydrin	106-89-8	Algal 72 h EC_{50} = >6 Daphnid 48 h EC_{50} = 24 Fish 96 h LC_{50} = 30	<5.0	EHC document on www.inchem.org/ documents ehc/ehc/ehc33.htm
Butanal (butyraldehyde)	123-72-8	Algal 72 h EC_{50} = 44 Daphnid 48 h EC_{50} = 383 Fish 96 h LC_{50} =16	8.70	SDS from Matheson Tri-Gas
Hexachlorobutadiene [1]	87-68-3	Algal 72 h EC_{50} = >25 Daphnid 48 h EC_{50} = 0.5 Fish 96 h LC_{50} = 0.1 - 0.6	>25	Knie *et al*, EHC document on www.inchem.org

Table 6.4: Illustrative examples of interspecies sensitivity ratios (ISR values) for MOA1 - 4 chemicals *(cont'd)*

Chemical	**CAS number**	**Acute toxicity data (mg/L)**	**ISR (A/D/F)**	**Information source**
Verhaar et al, MOA4	*Specifically acting chemicals (selective protein targets)*			
Cypermethrin	52135-07-8	Algal 72 h EC_{50} = 44 Daphnid 48 h EC_{50} = 0.0015 Fish 96 h LC_{50} = 0.00069	29,333	This report
EE2	57-63-6	Algal 72 h EC_{50} = 0.84 Daphnid 48 h EC_{50} = 5.7 Fish 96 h LC_{50} = 1.6	6.8	This report
Fluoxetine	56296-78-7	Algal 72 h EC_{50} = 0.027 Daphnid 48 h EC_{50} = 0.94 Fish 96 h LC_{50} = 1.57	58	This report
Ketoconazole	65277-42-1	Algal 72 h EC_{50} = 1.8 Daphnid 48 h EC_{50} = 0.4 Fish 96 h LC_{50} = 3.9	9.8	This report
Triclosan	222-182-2	Algal 72 h EC_{50} = 0.0014 Daphnid 48 h EC_{50} = 0.39 Fish 96 h LC_{50} = 0.602	430	This report

[1] Hexachlorobutadiene acute ecotoxicity data from http://www.inchem.org/documents/ehc/ehc/ehc156.htm on 20/12/06

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Intelligent testing strategy needs

When a new chemical is registered, a suite of ecotoxicity tests is usually conducted to meet the regulatory need for data. This package typically consists of toxicity tests on fish, *Daphnia* (invertebrate), and aquatic algae, together with environmental exposure data. Testing multiple taxa provides an ability to evaluate potential effects on the ecosystem in general. Unfortunately, testing multiple taxa leads to increased animal usage and resource expenditures that could be used on more high priority chemicals (Bradbury *et al*, 2004). As our understanding of the relative sensitivity of these three taxa develop and as we learn to build a weight-of-evidence argument based on existing SARs, QSARs, *in vitro*, and mammalian toxicity data, we will be better able to evaluate the need for always having toxicity data on plants, crustaceans and fish.

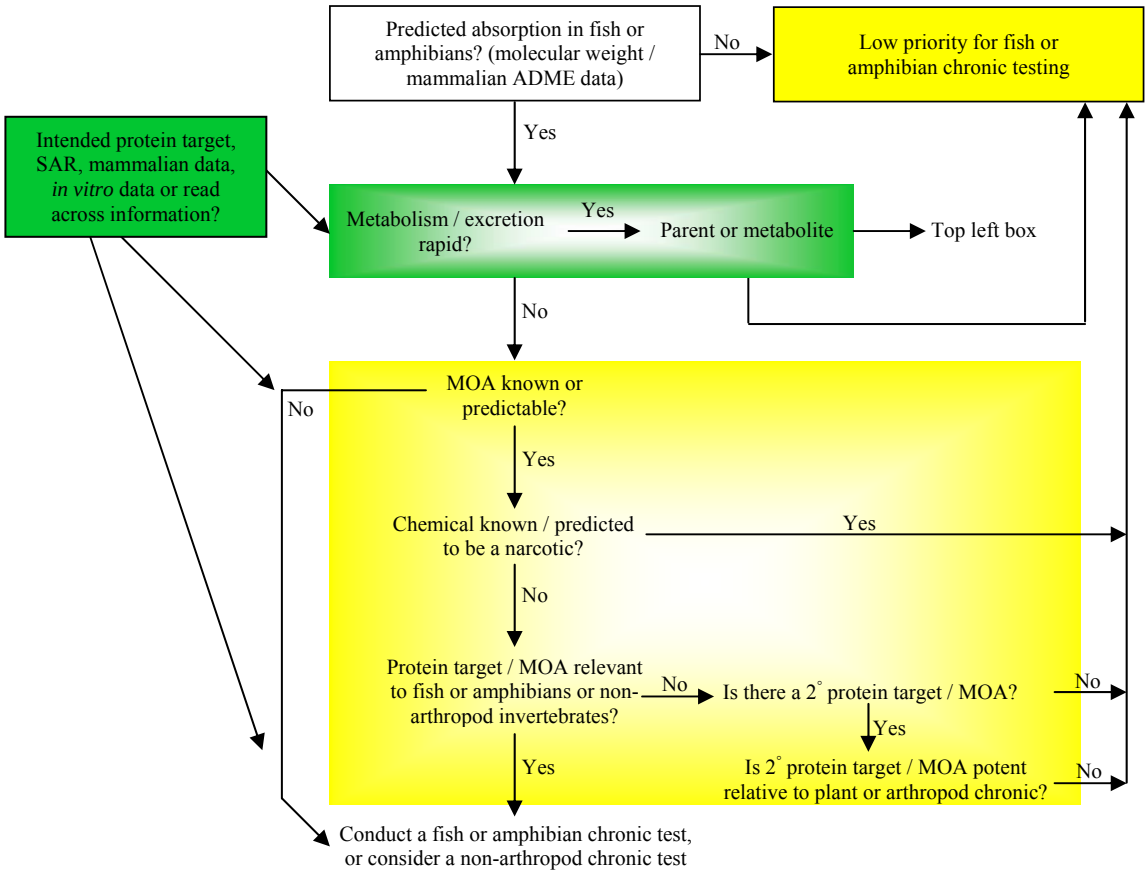
This new movement of making 'smart' choices in the development of the environmental toxicity test programmes is referred to as 'intelligent testing strategy' (ITS). If correctly implemented, ITS could support more focused testing, supporting environmental protection alongside reduced animal usage and reduced demands on test compound supply. Unfortunately, to date, relatively few agencies have proposed a decision-making scheme that moves the science closer to these goals. For the industry this represents a valuable opportunity to engage scientists and regulators in a constructive discussion of what ITS can do. For example, it could identify critical knowledge gaps that need to be filled and suggest approaches based on *in silico*, *in vitro* and *in vivo* tools (Bradbury *et al*, 2004). The Verhaar *et al* (1992) scheme offers a valuable way to use ITS for acute toxicity assessments for MOA1, 2 and 3 chemicals, augmented by the use of the $ETNC_{aq}$ (de Wolf *et al*, 2005). Using multiple lines of evidence, including recent initiatives for using MOA in human risk assessments (Clewell, 2005; ECETOC, 2006a), the Task Force has examined opportunities and limitations to develop a 'protein target ITS approach' for the aquatic hazard assessment of MOA4 chemicals. A related approach is being developed to reduce the need for fish bioconcentration testing, taking into account SAR methods, *in vitro* fish hepatocyte assays or small-scale invertebrate tests (de Wolf *et al*, 2007).

7.2 ECETOC proposed approach for MOA4 for chemicals

The ITS approach seeks to identify when chronic testing is needed and when it is sufficient to use data from an analogous chemical, structure-activity, or an acute test result in the effects assessment. This relies upon a weight-of-evidence approach for each chemical to design the appropriate test program. Initially, physico-chemical data, SAR predictions, *in vitro* tests (e.g. high throughput screening for biological target and efficacy), and data from mammalian studies for the compound of interest and related chemicals are collected and evaluated as input. Taking

the example of fish (Figure 7.1), the first step of the flow diagram is to determine if uptake is likely to occur. If not (e.g. high molecular weight polymers), the chemical should have a low priority for chronic toxicity testing. The next step in Figure 7.1 is an evaluation of metabolism and excretion (green box). If SAR, *in vitro*, or mammalian data support rapid metabolism or excretion, again, the chemical is low priority for chronic testing. Mammalian metabolism information can only be used if a similar biochemical pathway is known to exist in fish and if the rate is sufficient for metabolism to be a meaningful elimination pathway. If a significant metabolite is produced, the metabolite is brought through the assessment scheme.

Figure 7.1: ITS approach applied to fish and amphibians



The third step in Figure 7.1 is an evaluation of the MOA for efficacy or, if known, for the leading toxic effect (yellow box). If the MOA is known (or can be inferred from related chemicals) and is a narcotic (MOA1 or MOA2), the chemical is assigned a low priority for chronic testing. If the effective MOA is known but is not narcosis, the existence of the likely protein target in fish or amphibians is considered (e.g. based on comparative endocrinology or systems biology; Ekins *et al*, 2005). If an efficacy (or secondary toxicity) protein target is not present in fish, the chemical is also assigned as low priority for fish chronic toxicity testing. Finally, where the MOA justifies chronic testing in fish, the scheme recognises that the available information can be used to help in the optimal design of the fish chronic test protocol. For example, if SAR, *in vitro*, or mammalian information suggests the compound is endocrine active, chronic fish tests should be designed with this in mind (e.g. inclusion of reproductive endpoints or life-cycle testing). If the compound is not suspected of being active on the endocrine system, simpler fish chronic studies may be optimal (e.g. fish embryo-larval development and growth tests).

In Figure 7.1, the decision that a narcotic chemical does not need to be tested on fish is based on our knowledge of the sensitivity of fish, algae and invertebrates to narcotics and our high comfort level in conducting risk assessments on these compounds without chronic fish tests. In fact, chronic testing of any species is not likely to be needed for narcotics unless exposure concentrations are close to predicted effects concentrations. While narcosis is the effective MOA for few, if any, pesticides and pharmaceuticals, it is assumed that toxicity is the MOA for a large proportion of industrial chemicals, therefore helping to reduce the need for chronic fish testing. Further, as we learn more about other MOA, relative interspecies sensitivities and the assessment of risk, we will be in a position to include more diverse MOA in this approach. The Task Force concludes that the implications of this flow diagram are:

- Mammalian data on uptake, MOA and metabolism can be used intelligently to avoid or appropriately target fish and invertebrate chronic toxicity testing (and *visa versa*);
- the design of an individual chemical needs to be factored into the environmental testing. Much is usually known about the protein targets of new molecules developed in the pesticide and pharmaceutical areas and this knowledge can be used as guidance as to whether fish, other Deuterostomes or Ecdyzoans are likely to be sensitive. Genomic data will probably be of great value in this context in the future, for example, recent global projects to describe the complete genomes of several laboratory mammals, zebrafish, echinoderms and molluscs;
- as a corollary to the previous point, SARs are rapidly progressing and our ability to assign MOA should be used to design the toxicity test programme. For example, if the chemical has a biological target in plants or crustaceans that does not occur in fish, there is a low probability of fish being the most sensitive species;
- similarly, SAR procedures have been developed and refined to enable prediction of effects on multiple species with acceptable levels of error;

- finally, SARs, use of read-across, *in vitro* testing for MOA and metabolism are being developed and refined as many organisations actively seek to replace, reduce or refine (vertebrate) animal testing of chemicals. As this science progresses, we need to define a decision-making scheme that will allow this data to be used to help inform intelligent testing strategies.

7.3 Recommendations

Finally, the Task Force has a number of research recommendations relevant to regulator bodies and those industrial sectors required to undertake risk assessments of chemicals designed to have specific biological activity. Taking a pragmatic approach to present and probable future internationally accepted test guidelines (e.g. ISO and OECD), these recommendations are:

- To invest in a hierarchy of biological understanding (including genomics, proteomics and population responses) in *Daphnia magna* and other small aquatic invertebrates (freshwater and marine) frequently used in environmental risk assessment;
- for animal welfare reasons, to minimise the need for *in vivo* fish bioconcentration testing by developing *in vitro* fish protocols for chemical metabolism and also by developing a small-scale and rapid invertebrate bioconcentration test method (supported by MOA data);
- to support risk assessments of endocrine disrupters by developing a database of the normal (baseline) range in developmental and reproductive endpoints as measured across different laboratories;
- to capitalise on the learning from zebrafish biomedical research to develop a publicly available database on zebrafish toxicology;
- to develop aquatic plant ADME models with specific regard to understanding key biotransformation enzymes;
- to support continuing research into SAR validation through providing 'training data sets' based on high quality *in vivo* chronic tests with plants, invertebrates and fish;
- to critically review data from a wider range of chemicals in the proposed MOA framework, including chemicals where the mammalian MOA is less specific than for agrochemicals and pharmaceuticals.

7.4 Summary

In this chapter, we have focused on the use of mammalian data in a weight-of-evidence type of approach to inform the toxicologist of the potential for exposure and effects in fish and related taxonomic groups (e.g. amphibians and invertebrates from the Deuterosomes, such as echinoderms). We have prioritised these taxa due to their evolutionary links at the molecular

level but, more pragmatically, due to the ethical and resource challenges posed by working on fish and amphibians. As a cautionary reminder, it should be remembered that in mammalian toxicology it is known that some MOA4 chemicals may produce diverse exposure-dependent effects that suggest one MOA for biological activity (efficacy/therapeutic effect), together with another MOA for toxicity measured in other tissues (e.g. carbamates which induce neurotoxicity via cholinesterase inhibition neurotoxicity but also tumour formation and other non-neoplastic lesions by another presumed MOA (Pope *et al*, 2005)). Recent review of agricultural chemical safety assessments have also shown a 20-fold difference in chronic NOAELs to butylate for rats and dogs and a 5000-fold difference for Diazinon (Doe *et al*, 2006; Barton *et al*, 2006). It is also worth observing that in human drug development an overall 71% concordance of toxicological effects in rodents versus non-rodent species is observed (Olson *et al*, 2000).

We have discussed the application of mammalian uptake, metabolism and excretion data to help make decisions about the need for similar studies in fish. Taking protein targets as a guiding philosophy (Rang *et al*, 2003), these specific biological processes were selected for inclusion due to their importance to environmental risk assessment and the scientific basis for extrapolating their function across compounds. Addressing toxicological hazards to aquatic organisms from diverse sources such as carcinogenicity, mutagenicity and reproductive toxicity (the CMR issue) (Chengelis, 1990; Rao *et al*, 1995 and Gray *et al*, 2002), together with immunotoxicology (Sweet and Zelikoff, 2001), are also of wider relevance to this MOA4 approach. These should be considered in a weight-of-evidence approach in order to define the appropriate ITS for fish or amphibians. A flow-chart of an intelligent test strategy that applies the weight-of-evidence gathered from non-fish animal studies was proposed to extend the dialogue in this important area. Currently, we see the developing ITS strategy as continuing to evolve and improve as there are multiple programmes contributing tools to the framework (see http://www.cefic.org/lri). As these tools and the flow diagram are validated, ECETOC should seek to identify additional opportunities to avoid unnecessary testing of fish and amphibians based on use alternative methods with a MOA4 rationale and guidance from protein target conservation across invertebrate taxa.

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GLOSSARY

Biomarker	A change in a biological response (ranging from molecular through cellular and physiological responses) that can be related to exposure to, or toxic effects of, environmental chemicals (after Huggett *et al*, 1992).
Critical body burden (CBB)	The term is used in this report to encompass the various terms used by different authors, including critical body/tissue residue, residue-based toxicity, internal effects concentration etc. It relates to the highest tissue concentration having no effect.
EC_{50}	Median effect concentration (generating an effect response in 50% of the test population). Where the endpoint is lethality, this is known as the LC_{50}.
EC_{10}	Median effect concentration (generating an effect response in 10% of the test population), regarded in the TGD as being of similar value as the NOEC. The EC_{10} can be based on a population endpoint that is used to for risk assessment application, such as survival, growth or reproduction (termed the $^{adverse}EC_{10}$), or on a biomarker response (termed the $^{biomarker}EC_{10}$).
Endocrine disruptor	An exogenous substance that causes adverse effects in an organism, or its progeny, subsequent to changes in endocrine function.
Interspecies sensitivity ratio (ISR)	The ratio of acute toxicity data for different organisms (e.g. algae, daphnids and fish).
Lowest-Observed Effect Concentration (LOEC)	This can be based on a population adverse effect measurement such as decreased survival, growth or reproduction (termed the ^{adverse}LOEC) or possibly on waterbiomarker response (termed the ^{biomarker}LOEC).

Environmental Threshold of No Concern	This is a concept based on the possibility of establishing an aquatic exposure threshold value for all chemicals below which no significant risk to the freshwater environment is expected.
Mechanism of action	A complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome, underlying the MOA.
Mode of action (MOA)	A common set of physiological and behavioural signs that characterise a type of adverse biological response (Escher and Hermens, 2002), where the major (but not all) biochemical steps are understood.
Mode of action (Type 1)	Non polar narcotic substances: Narcosis (or baseline) toxicity is believed to be the result of reversible and non-specific disturbance of membrane integrity and function resulting from the partitioning of the chemical into biological membranes (Escher and Hermens, 2002). Because the effects are not specific to particular chemical structures, this can be considered the minimum (or baseline) toxicity that any chemical will display, if it is not obscured by greater toxicity through other modes of action. This MOA is therefore displayed by chemicals that are 'inert' in terms of chemical or biological reactivity, and by interactions with specific biological receptors.
Mode of action (Type 2)	Polar narcotic substances: This group consists of more polar but essentially non-reactive substances such as substituted phenols and anilines which ionise to some extent depending on pH and display slightly greater toxicity (external concentration) than would be predicted by 'baseline' toxicity QSARs. They are often characterised as possessing hydrogen bond donor acidity.

Mode of action (Type 3)	Reactive substances: Reactive substances are considered as a group that includes diverse modes of action resulting from non-selective reactions with biomolecular structures and consequently displaying enhanced toxicity (lower CBBs) compared with baseline narcotics (Verhaar *et al,* 1992). This group also includes chemicals that are metabolically activated into reactive substances. Of particular importance are electrophilic substances that react with amino, hydroxyl and sulphydryl groups within proteins and DNA (Hermens, 1990), such as certain carbonyls, epoxides, nitriles, hydrazines, acid anhydrides and aldehydes.
Mode of action (Type 4)	Specifically active receptor-active substances: Specifically acting chemicals can be classified by their interaction with one of four major protein targets i.e. (a) receptors; (b) ion channels; (c) enzymes and (d) transporters (Rang *et al,* 2003).
No-Observed Effect Concentration (NOEC)	The highest concentration below the LOEC where the stated effect was not observed. The effect can be based on a population endpoint which is used to for risk assessment application, such as survival, growth or reproduction (termed the ^{adverse}NOEC) or possibly on a biomarker response (termed the ^{biomarker}NOEC).
Predicted No Effect Concentration (PNEC)	The environmental concentration that is regarded as a level below which the balance of probability is such that an unacceptable effect will not occur.
Threshold of toxicological concern (TTC)	Is a concept that refers to the establishment of a human exposure threshold value for all chemicals, below which there would be no appreciable risk to health.

ABBREVIATIONS

ACR	Acute to chronic ratio
ADME	Absorption, distribution, metabolism and excretion (also known as absorption, disposition, metabolism and excretion)
ASTM	American Society for Testing of Materials
EE2	17α-ethinylestradiol
EHC	Environmental health criteria
EQS	Environmental quality standard
ERA:	Environmental risk assessment
ETNC	Environmental threshold of no concern
GSI	Gonadosomatic index
ISO	International Organisation for Standardisation
ISR	Interspecies sensitivity ratio
ITS	Intelligent testing strategy
K_{ow}	Octanol/water partition coefficient
LOEC	Lowest-observed effect concentration
MDR	Multi-drug resistance
MOA*	Mode of action
MTD	Maximum tolerated dose
MXR	Multi-xenobiotic response
NOEC	No-observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PNEC	Predicted no effect concentration
QSARs	Quantitative structure-activity relationships
SARs	Structure-activity relationships
SSRI	Selective serotonin re-uptake inhibitor
TTC	Threshold of toxicological concern
VTG	Vitellogenin
WEA	Whole effluent assessment
WFD	Water framework directive

* MOA1, MOA2, MOA3, MO4: see glossary.

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ECETOC PUBLISHED REPORTS

Monographs

No.	Title
No. 1	Good Laboratory Practice (Published October 1979)
No. 2	A Contribution to Strategy for Identification and Control of Occupational Carcinogens (Published September 1980)
No. 3	Risk Assessment of Occupational Chemical Carcinogens (Published May 1985)
No. 4	Hepatocarcinogenesis in Laboratory Rodents: Relevance for Man (Published October 1982)
No. 5	Identification and Assessment of the Effects of Chemicals on Reproduction and Development (Reproductive Toxicology) (Published December 1983)
No. 6	Acute Toxicity Tests, LD_{50} (LC_{50}) Determinations and Alternatives (Published May 1985)
No. 7	Recommendations for the Harmonisation of International Guidelines for Toxicity Studies (Published December 1985)
No. 8	Structure-Activity Relationships in Toxicology and Ecotoxicology: An Assessment (Summary) (Published June 1986)
No. 9	Assessment of Mutagenicity of Industrial and Plant Protection Chemicals (Published June 1987)
No. 10	Identification of Immunotoxic Effects of Chemicals and Assessment of their Relevance to Man (Published August 1987)
No. 11	Eye Irritation Testing (Published June 1988)
No. 12	Alternative Approaches for the Assessment of Reproductive Toxicity (with emphasis on embryotoxicity/teratogenicity) (Published November 1989)
No. 13	DNA and Protein Adducts: Evaluation of their Use in Exposure Monitoring and Risk Assessment (Published October 1989)
No. 14	Skin Sensitisation Testing (Published March 1990)
No. 15	Skin Irritation (Published July 1990)
No. 16	Early Indicators of Non-Genotoxic Carcinogenesis (Published June 1991)
No. 17	Hepatic Peroxisome Proliferation (Published May 1992)
No. 18	Evaluation of the Neurotoxic Potential of Chemicals (Published September 1992)
No. 19	Respiratory Allergy (Published August 1993)
No. 20	Percutaneous Absorption (Published August 1993)
No. 21	Immunotoxicity: Hazard Identification and Risk Characterisation (Published September 1994)
No. 22	Evaluation of Chemicals for Oculotoxicity (Published November 1994)
No. 23	Receptor Mediated Mechanisms in Chemical Carcinogenesis (Published December 1995)
No. 24	Risk Assessment for Carcinogens (Published July 1996)
No. 25	Practical Concepts for Dose Selection in Chronic Toxicity and Carcinogenicity Studies in Rodents (Published February 1996)
No. 26	Aquatic Toxicity Testing of Sparingly Soluble Volatile and Unstable Substances (Published September 1996)
No. 27	Aneuploidy (Published August 1997)
No. 28	Dose-response and threshold-mediated mechanisms in mutagenesis - Mutation Research Special Issue (Published January 2000)
No. 29	Skin Sensitisation Testing for the Purpose of Hazard Identification and Risk Assessment (Published September 2000)
No. 30	Genetic Susceptibility to Environmental Toxicants (Published October 2001)
No. 31	Guidance on Evaluation of Reproductive Toxicity Data (Published February 2002)
No. 32	Use of Human Data in Hazard Classification for Irritation and Sensitisation (Published July 2002)
No. 33	Application of Physiological - Toxicokinetic Modelling to Health Hazard Assessment of Chemical Substances (Published February 2003)
No. 34	Toxicogenomics in Genetic Toxicology and Hazard Determination (Published July 2005)

No. 35 Biomarkers and molecular epidemiology (Published as a special section of Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, by Elsevier, August 2006)

No. 36 Environmental Genotoxins in Children and Adults (Published as a special section of Mutation Research/Genetic Toxicology and Environmental Mutagenesis, by Elsevier, September 2006)

No. 37 Biomarkers in Children and Adults (Published as a special section of Toxicology Letters, by Elsevier, July 2007)

Technical Reports

No. Title

No. 1 Assessment of Data on the Effects of Formaldehyde on Humans (Published January 1979) (Updated by TR No. 6)

No. 2 The Mutagenic and Carcinogenic Potential of Formaldehyde (Published May 1981)

No. 3 Assessment of Test Methods for Photodegradation of Chemicals in the Environment (Published August 1981)

No. 4 The Toxicology of Ethylene Glycol Monoalkyl Ethers and its Relevance to Man (Published June 1982) (Updated by TR No. 17)

No. 5 Toxicity of Ethylene Oxide and its Relevance to Man (Published September 1982)

No. 6 Formaldehyde Toxicology: An Up-Dating of ECETOC Technical Reports 1 and 2 (Published September 1982)

No. 7 Experimental Assessment of the Phototransformation of Chemicals in the Atmosphere (Published September 1983)

No. 8 Biodegradation Testing: An Assessment of the Present Status (Published November 1983)

No. 9 Assessment of Reverse-Phase Chromatographic Methods for Determining Partition Coefficients (Published December 1983)

No. 10 Considerations Regarding the Extrapolation of Biological Data in Deriving Occupational Exposure Limits (Published February 1984)

No. 11 Ethylene Oxide Toxicology and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 5 (Published March 1984)

No. 12 The Phototransformation of Chemicals in Water: Results of a Ring-Test (Published June 1984)

No. 13 The EEC 6th Amendment: A Guide to Risk Evaluation for Effects on the Environment (Published March 1984)

No. 14 The EEC 6th Amendment: A Guide to Risk Evaluation for Effects on Human Health (Published March 1984)

No. 15 The Use of Physical-Chemical Properties in the 6th Amendment and their Required Precision, Accuracy and Limiting Values (Published June 1984)

No. 16 A Review of Recent Literature on the Toxicology of Benzene (Published December 1984)

No. 17 The Toxicology of Glycol Ethers and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 4) (Published April 1985) (Updated by TR No. 64

No. 18 Harmonisation of Ready Biodegradability Tests (Published April 1985)

No. 19 An Assessment of Occurrence and Effects of Dialkyl-o-Phthalates in the Environment (Published May 1985)

No. 20 Biodegradation Tests for Poorly-Soluble Compounds (Published February 1986)

No. 21 Guide to the Classification of Carcinogens, Mutagens, and Teratogens under the 6th Amendment (Published February 1986)

No. 22 Classification of Dangerous Substances and Pesticides in the EEC Directives. A Proposed Revision of Criteria for Inhalational Toxicity (Published January 1987)

No. 23 Evaluation of the Toxicity of Substances to be Assessed for Biodegradability (Published November 1986)

No. 24 The EEC 6th Amendment: Prolonged Fish Toxicity Tests (Published October 1986)

No. 25 Evaluation of Fish Tainting (Published January 1987)

No. 26 The Assessment of Carcinogenic Hazard for Human Beings exposed to Methylene Chloride (Published January 1987)

No. 27 Nitrate and Drinking Water (Published January 1988)

No. 28 Evaluation of Anaerobic Biodegradation (Published June 1988)

No. 29 Concentrations of Industrial Organic Chemicals Measured in the Environment: The Influence of Physico-Chemical Properties, Tonnage and Use Patterns (Published June 1988)
No. 30 Existing Chemicals: Literature Reviews and Evaluations (Fifth Edition) (No longer available) (Published May 1994)
No. 31 The Mutagenicity and Carcinogenicity of Vinyl Chloride: A Historical Review and Assessment (Published July 1988)
No. 32 Methylene Chloride (Dichloromethane): Human Risk Assessment Using Experimental Animal Data (Published May 1988)
No. 33 Nickel and Nickel Compounds: Review of Toxicology and Epidemiology with Special Reference to Carcinogenesis (Published February 1989)
No. 34 Methylene Chloride (Dichloromethane): An Overview of Experimental Work Investigating Species Differences in Carcinogenicity and their Relevance to Man (Published March 1989)
No. 35 Fate, Behaviour and Toxicity of Organic Chemicals Associated with Sediments (Published January 1990)
No. 36 Biomonitoring of Industrial Effluents (Published April 1990)
No. 37 Tetrachlorethylene: Assessment of Human Carcinogenic Hazard (Published May 1990)
No. 38 A Guide to the Classification of Preparations Containing Carcinogens, Mutagens and Teratogens (Published July 1990)
No. 39 Hazard Assessment of Floating Chemicals After an Accidental Spill at Sea (Published July 1990)
No. 40 Hazard Assessment of Chemical Contaminants in Soil (Published April 1992)
No. 41 Human Exposure to N-Nitrosamines, their Effects and a Risk Assessment for N-Nitrosodiethanolamine in Personal Care Products (Published August 1990)
No. 42 Critical Evaluation of Methods for the Determination of N-Nitrosamines in Personal Care and Household Products (Published February 1991)
No. 43 Emergency Exposure Indices for Industrial Chemicals (Published March 1991)
No. 44 Biodegradation Kinetics (Published September 1991)
No. 45 Nickel, Cobalt and Chromium in Consumer Products: Allergic Contact Dermatitis (Published March 1992)
No. 46 EC 7th Amendment: Role of Mammalian Toxicokinetic and Metabolic Studies in the Toxicological Assessment of Industrial Chemicals (Published May 1992)
No. 47 EC 7th Amendment "Toxic to Reproduction": Guidance on Classification (Published August 1992)
No. 48 Eye Irritation: Reference Chemicals Data Bank (Second Edition) (Published June 1998)
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No. 50 Estimating Environmental Concentrations of Chemicals using Fate and Exposure Models (Published November 1992)
No. 51 Environmental Hazard Assessment of Substances (Published January 1993)
No. 52 Styrene Toxicology Investigation on the Potential for Carcinogenicity (Published August 1992)
No. 53 DHTDMAC: Aquatic and Terrestrial Hazard Assessment (CAS No. 61789-80-8) (Published February 1993)
No. 54 Assessment of the Biodegradation of Chemicals in the Marine Environment (Published August 1993)
No. 55 Pulmonary Toxicity of Polyalkylene Glycols (Published December 1997)
No. 56 Aquatic Toxicity Data Evaluation (Published December 1993)
No. 57 Polypropylene Production and Colorectal Cancer (Published February 1994)
No. 58 Assessment of Non-Occupational Exposure to Chemicals (Published May 1994)
No. 59 Testing for Worker Protection (Published April 1994)
No. 60 Trichloroethylene: Assessment of Human Carcinogenic Hazard (Published May 1994)
No. 61 Environmental Exposure Assessment (Published September 1994)
No. 62 Ammonia Emissions to Air in Western Europe (Published July 1994)
No. 63 Reproductive and General Toxicology of some Inorganic Borates and Risk Assessment for Human Beings (Published February 1995)
No. 64 The Toxicology of Glycol Ethers and its Relevance to Man (Published August 1995)
No. 65 Formaldehyde and Human Cancer Risks (Published May 1995)
No. 66 Skin Irritation and Corrosion: Reference Chemicals Data Bank (Published March 1995)

No. 67 The Role of Bioaccumulation in Environmental Risk Assessment: The Aquatic Environment and Related Food Webs (Published October 1995)
No. 68 Assessment Factors in Human Health Risk Assessment (Published August 1995) (Updated by TR No. 86)
No. 69 Toxicology of Man-Made Organic Fibres (Published April 1996)
No. 70 Chronic Neurotoxicity of Solvents (Published February 1996)
No. 71 Inventory of Critical Reviews on Chemicals (Only available to ECETOC members) (Published August 1996)
No. 72 Methyl *tert*-Butyl Ether (MTBE) Health Risk Characterisation (Published June 1997)
No. 73 The Value of Aquatic Model Ecosystem Studies in Ecotoxicology (Published December 1997)
No. 74 QSARs in the Assessment of the Environmental Fate and Effects of Chemicals (Published June 1998)
No. 75 Organophosphorus Pesticides and Long-term Effects on the Nervous System (Published December 1998)
No. 76 Monitoring and Modelling of Industrial Organic Chemicals, with Particular Reference to Aquatic Risk Assessment (Published January 1999)
No. 77 Skin and Respiratory Sensitisers: Reference Chemicals Data Bank (Published August 1999)
No. 78 Skin Sensitisation Testing: Methodological Considerations (Published December 1999)
No. 79 Exposure Factors Sourcebook for European Populations (with Focus on UK Data) (Published June 2001)
No. 80 Aquatic Toxicity of Mixtures (Published July 2001)
No. 81 Human Acute Intoxication from Monochloroacetic Acid: Proposals for Therapy (Published November 2001)
No. 82 Risk Assessment in Marine Environments (Published December 2001)
No. 83 The Use of T25 Estimates and Alternative Methods in the Regulatory Risk Assessment of Non-threshold Carcinogens in the European Union (Published December 2002)
No. 84 Scientific Principles for Soil Hazard Assessment of Substances (Published July 2002)
No. 85 Recognition of, and Differentiation between, Adverse and Non-adverse Effects in Toxicology Studies (Published December 2002)
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No. 88 Environmental Risk Assessment of Difficult Substances (Published June 2003)
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No. 90 Persistence of Chemicals in the Environment (Published October 2003)
No. 91 Aquatic Hazard Assessment II (Published November 2003)
No. 92 Soil and Sediment Risk Assessment (Published December 2004)
No. 93 Targeted Risk Assessment (Published December 2004)
No. 94 Whole Effluent Assessment (Published December 2004)
No. 95 The Toxicology of Glycol Ethers and its Relevance to Man (Fourth Edition) Volume I and Volume II Substance Profiles (Published February 2005)
No. 96 Trends in Children's Health and the Role of Chemicals: State of the Science Review (Published June 2005)
No. 97 Alternative Testing Approaches in Environmental Safety Assessment (Published December 2005)
No. 98 Risk Assessment of PBT Chemicals (Published December 2005)
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No. 100 Contribution to the Methodology for the Development of Acute Exposure Threshold Levels in Case of Accidental Chemical Release (Published July 2006)
No. 101 Guidance for Setting Occupational Exposure Limits: Emphasis on Data-Poor Substances (Published October 2006)

Joint Assessment of Commodity Chemicals (JACC) Reports

No.	Title
No. 1	Melamine (Published February 1983)
No. 2	1,4-Dioxane (Published February 1983)
No. 3	Methyl Ethyl Ketone (Published February 1983)
No. 4	Methylene Chloride (Published January 1984)
No. 5	Vinylidene Chloride (Published August 1985)
No. 6	Xylenes (Published June 1986)
No. 7	Ethylbenzene (Published August 1986)
No. 8	Methyl Isobutyl Ketone (Published May 1987)
No. 9	Chlorodifluoromethane (Published October 1989)
No. 10	Isophorone (Published September 1989)
No. 11	1,2-Dichloro-1,1-difluoroethane (HFA-132b) (Published May 1990)
No. 12	1-Chloro-1,2,2,2-tetrafluoroethane (HFA-124) (Published May 1990) (Updated by JACC No. 25)
No. 13	1,1-Dichloro-2,2,2-trifluoroethane (HFA-123) (Published May 1990) (Updated by JACC No. 33)
No. 14	1-Chloro-2,2,2-trifluoromethane (HFA-133a) (Published August 1990)
No. 15	1-Fluoro 1,1-dichloroethane (HFA-141) (Published August 1990) (Updated by JACC No. 29)
No. 16	Dichlorofluoromethane (HCFC-21) (Published August 1990)
No. 17	1-Chloro-1,1-difluoroethane (HFA-142b) (Published August 1990)
No. 18	Vinyl Acetate (Published February 1991)
No. 19	Dicyclopentadiene (CAS: 77-73-6) (Published July 1991)
No. 20	Tris-/Bis-/Mono-(2 ethylhexyl) phosphate (Published May 1992)
No. 21	Tris-(2-butoxyethyl)-phosphate (CAS:78-51-3) (Published March 1992)
No. 22	Hydrogen Peroxide (CAS: 7722-84-1) (Published January 1993)
No. 23	Polycarboxylate Polymers as Used in Detergents (Published November 1993)
No. 24	Pentafluoroethane (HFC-125) (CAS: 354-33-6) (Published May 1994)
No. 25	1-Chloro-1,2,2,2-tetrafluoroethane (HCFC 124) (CAS No. 2837-89-0) (Second Edition) (Published July 1994) (Updated by JACC 46)
No. 26	Linear Polydimethylsiloxanes (CAS No. 63148-62-9) (Published September 1994)
No. 27	*n*-Butyl Acrylate (CAS No. 141-32-2) (Published August 1994)
No. 28	Ethyl Acrylate (CAS No. 140-88-5) (Published September 1994)
No. 29	1,1-Dichloro-1-fluoroethane (HCFC-141b) (CAS No. 1717-00-6) (Published December 1994)
No. 30	Methyl Methacrylate (CAS No. 80-62-6) (Published February 1995)
No. 31	1,1,1,2-Tetrafluoroethane (HFC-134a) (CAS No. 811-97-2) (Published February 1995) (Updated by JACC No. 50)
No. 32	Difluoromethane (HFC-32) (CAS No. 75-10-5) (Published May 1995)
No. 33	1,1-Dichloro-2,2,2-trifluoroethane (HCFC-123) (CAS No. 306-83-2) (Published February 1996) (Updated by JACC No. 47)
No. 34	Acrylic Acid (CAS No. 79-10-7) (Published September 1995)
No. 35	Methacrylic Acid (CAS No. 79-41-4) (Published May 1996)
No. 36	*n*-Butyl Methacrylate; Isobutyl Methacrylate (CAS No. 97-88-1) (CAS No. 97-86-9) (Published December 1996)
No. 37	Methyl Acrylate (CAS No. 96-33-3) (Published September 1998)
No. 38	Monochloroacetic Acid (CAS No. 79-11-8) and its Sodium Salt (CAS No. 3926-62-3) (Published June 1999)
No. 39	Tetrachloroethylene (CAS No. 127-18-4) (Published December 1999)
No. 40	Peracetic Acid (CAS No. 79-21-0) and its Equilibrium Solutions (Published January 2001)
No. 41	*n*-Butanol (CAS No. 71-36-3) (Published March 2004)

No. 42 Tetrafluoroethylene (CAS No. 116-14-3) (Published December 2003)
No. 43 *sec*-Butanol (CAS No. 78-92-2) (Published December 2004)
No. 44 1, 1, 1, 3, 3-Pentafluoropropane (HFC-245fa) (Published June 2004)
No. 45 1, 1-Difluoroethane (HFC-152a) (CAS No. 75-37-6) (Published September 2004)
No. 46 1-Chloro-1,2,2,2-tetrafluoroethane (HCFC 124) CAS No. 2837-89-0 (Second Edition) (Published November 2004)
No. 47 1,1-Dichloro-2,2,2-trifluoroethane (HCFC-123) CAS No. 306-83-2 (Third Edition) (Published May 2005)
No. 48 Hexafluoropropylene (HFP) CAS No. 116-15-4 (Published September 2005)
No. 49 Vinylidene Fluoride CAS No. 75-38-7 (Published November 2005)
No. 50 1,1,1,2-Tetrafluoroethane (HFC-134a) (CAS No. 811-97-2) (Second Edition) (Published January 2006)
No. 51 Synthetic Amorphous Silica (CAS No. 7631-86-9) (Published September 2006)
No. 52 Trifluoroethane (HFC-143a) CAS No. 420-46-2 (Published October 2006)
No. 53 Cyanides of Hydrogen, Sodium and Potassium, and Acetone Cyanohydrin (CAS No. 74-90-8, 143-33-9,151-50-8 and 75-86-5) (Published September 2007)

Special Reports

No. Title
No. 8 HAZCHEM; A Mathematical Model for Use in Risk Assessment of Substances (Published October 1994)
No. 9 Styrene Criteria Document (Published June 1995)
No. 10 Hydrogen Peroxide OEL Criteria Document (CAS No. 7722-84-1) (Published July 1996)
No. 11 Ecotoxicology of some Inorganic Borates (Published March 1997)
No. 12 1,3-Butadiene OEL Criteria Document (Second Edition) (CAS No. 106-99-0) (Published January 1997)
No. 13 Occupational Exposure Limits for Hydrocarbon Solvents (Published August 1997)
No. 14 *n*-Butyl Methacrylate and Isobutyl Methacrylate OEL Criteria Document (Published May 1998)
No. 15 Examination of a Proposed Skin Notation Strategy (Published September 1998)
No. 16 GREAT-ER User Manual (Published March 1999)
No. 17 Risk Assessment Report for Existing Substances Methyl *tertiary*-Butyl Ether (Published December 2003)

Documents

No. Title
No. 32 Environmental Oestrogens: Male Reproduction and Reproductive Development (Published January 1996)
No. 33 Environmental Oestrogens: A Compendium of Test Methods (Published July 1996)
No. 34 The Challenge Posed by Endocrine-disrupting Chemicals (Published February 1996)
No. 35 Exposure Assessment in the Context of the EU Technical Guidance Documents on Risk Assessment of Substances (Published May 1997)
No. 36 Comments on OECD Draft Detailed Review Paper: Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals (Published August 1997)
No. 37 EC Classification of Eye Irritancy (Published December 1997)
No. 38 Wildlife and Endocrine Disrupters: Requirements for Hazard Identification (Published January 1998)
No. 39 Screening and Testing Methods for Ecotoxicological Effects of Potential Endocrine Disrupters: Response to the EDSTAC Recommendations and a Proposed Alternative Approach (Published January 1999)
No. 40 Comments on Recommendation from Scientific Committee on Occupational Exposure Limits for 1,3-Butadiene (Published October 2000)
No. 41 Persistent Organic Pollutants (POPs) Response to UNEP/INC/CEG-I Annex 1 (Published January 2000)

No. 42 Genomics, Transcript Profiling, Proteomics and Metabonomics (GTPM). An Introduction (Published April 2001)

No. 43 Contact Sensitisation: Classification According to Potency. A Commentary (Published July 2003)

No. 44 Guidance for the Interpretation of Biomonitoring Data (Published November 2005)

Workshop Reports

No. Title

No. 1 Workshop on Availability, Interpretation and Use of Environmental Monitoring Data
20-21 March 2003, Brussels (Published December 2003)

No. 2 Strategy Report on Challenges, Opportunities and Research needs arising from the Definition, Assessment and Management of Ecological Quality Status as required by the EU Water Framework Directive based on the workshop EQS and WFD versus PNEC and REACH - are they doing the job? 27-28 November 2003, Budapest (Published March 2004)

No. 3 Workshop on the Use of Human Data in Risk Assessment
23-24 February 2004, Cardiff (Published November 2004)

No. 4 Influence of Maternal Toxicity in Studies on Developmental Toxicity
2 March 2004, Berlin (Published October 2004)

No. 5 Workshop on Alternative Testing Approaches in Environmental Risk Assessment
7-9 July 2004, Paris (Published December 2004)

No. 6 Workshop on Chemical Pollution, Respiratory Allergy and Asthma
16-17 June 2005, Leuven (Published December 2005)

No. 7 Workshop on Testing Strategies to Establish the Safety of Nanomaterials
7-8 November 2005, Barcelona (Published August 2006)

No. 8 Workshop on Societal Aspects of Nanotechnology
7-8 November 2005, Barcelona (Published October 2006)

No. 9 Workshop on the Refinement of Mutagenicity/Genotoxicity Testing
23-24 April, Malta (Published September 2007)

No. 10 Workshop on Biodegradation and Persistence
26-27 June, Holmes Chapel (Published September 2007)