Workshop on Alternative Testing Approaches in Environmental Risk Assessment 7-9 July 2004, Crécy-la-Chapelle

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The workshop was co-sponsored by

the European Centre for the Validation of Alternative Methods (ECVAM)

and the Cefic Long-range Research Initiative (LRI)

CONTENT

EXECUTIVE SUMMARY

This 2-day workshop, sponsored and organised by ECETOC, co-sponsored by ECVAM and Cefic LRI, took place at Crécy-la-Chapelle, near Paris, on July 7-9, 2004.

The aim of the workshop was to facilitate an active dialogue amongst industry, regulators and academia on the pragmatic use of alternative approaches to animal testing. The workshop addressed *in vivo, in vitro* and *in silico* approaches, in line with the 3Rs (Replacement, Refinement and Reduction), for the generation of hazard and exposure information within the context of environmental risk assessment.

There were 37 attendees, with people from academia, industry as well as the regulatory community. Appendices 1 and 2 give the full list of attendees and the meeting programme.

The primary outcome of the meeting was the development of research networks/programmes to develop alternative methodologies to address the environmental safety of chemicals.

The workshop first identified methodology for generating information for environmental risk assessment, in line with the 3Rs. Then it identified research to address knowledge gaps in the proposed methodology and finally drafted research plans including potential funding opportunities, collaborations and timelines.

The workshop was very successful in that everyone present expressed their desire to remain in contact, developing the research needs and participating in networks.

Assessing the effect of chemicals on fish

There were a number of issues raised and one key question was whether it was necessary to test fish for acute effects. Thus while approaches for limiting fish numbers (e.g. the step-down approach) were seen as helpful, they may prove to be redundant if other species (with or without Quantitative Structure Activity Relationship (QSAR) support) prove to be sufficiently protective when addressing acute effects in the early stages of assessing environmental safety.

The key areas of research identified were development of fish embryo, fish cell line tests and (Q)SARs.

i) Fish embryo test - This requires research into the acceptability of embryonic tests as non-animal alternatives and into cross species differences (in applicability as well as sensitivity). This is seen as a crucial issue, as there will be limited benefit developing an alternative method that would not be acceptable to all regulators, including those in other regions than

Europe, and for whom fish acute tests would then have to be conducted. Future innovative research on genomic and proteomic developments might offer mechanism-related approaches in the fish embryo model for hazard and risk assessment. These could later be linked to cellular developments (see ii).

ii) Fish cell lines – The aim of this research would be to investigate whether a battery of fish cell toxicity tests could be used as a screening test. In the longer term such research should address whether fish cells toxicity would be sensitive and accurate enough to replace fish *in vivo* tests.

iii) (Q)SAR - These need further development, establishing which of the current models are useful, their domain and extending the models to cover more specifically acting chemicals. There was also a clear message that more knowledge was needed to address the mechanisms of effects in fish. In this context, the research into fish embryo and cell toxicity tests could be useful.

Assessing the fate of chemicals in fish (bioconcentration)

Two strategies for addressing chemicals for a 'B' assessment were developed. The first approach was developed to address groups of chemicals when a definitive value is not required, but a value less than some pre-agreed bright-line. Such an approach, for example in the context of the EU-PBT strategy, could lead to significant reduction in the number of chemicals tested. This approach starts by identifying the chemicals within a group. This may be based on similar chemical structures or presumed bioconcentration behaviour. The purpose of the grouping would be to allow for key members of the group to be identified and tested. Interpolation within the group would then allow for bioconcentration factor (BCF) values to be assigned to the other group members via a local QSAR. The second strategy, a single chemical approach, starts with a worst-case assumption and then allows further refinement depending upon subsequent actions. Several of the alternative approaches discussed in the workshop could help move a chemical through the proposed scheme (see Figure 1).

There were four main alternatives addressed, which could be further developed to form research projects.

i) OECD 305 - the standard *in vivo* method assessing the bioconcentration potential of a chemical in fish needs investigation to clearly identify uncertainties in measurements derived by this protocol and clarifying its domain of application.

ii) Other *in vivo* experimental approaches, e.g. the dietary biomagnification factor (BMF) protocol and abbreviated OECD 305, need to be further investigated to define and possibly extend their domain and limits of applicability.

iii) *In vitro* assays, expert systems and models need further development and evaluation, especially those that are capable of incorporating absorption, distribution, metabolism, elimination, (ADME) concepts and physiology based pharmacokinetics (PBPK) modelling.

iv) Finally the ADME processes involved in bioconcentration need further research. The workshop addressed the key processes and identified research priorities, including the establishment of a gold standard database of BCF values and a review of available data concerning the potential for chemicals to degrade or be metabolised, both valuable short-term projects.

BACKGROUND

Animal welfare and the use of animals for environmental safety assessments is an area of growing public concern and political sensitivity. Concern about animal welfare issues is driving legislative (e.g. REACH, DGEE, 2003 and the 6th and 7th Amendment to the Cosmetics Directive EC, 1976; 1993; 2003) and regulatory changes that impact on the conduct of animal experiments and the requirements for product registration. For example the European Commission White Paper on the future EU Chemicals Policy (DGEE, 2003) defines as one of its objectives the 'promotion of non-animal testing'. As currently envisioned the proposed legislation would mean that 30,000 chemicals in volumes greater than 1 tonne per year would need further assessment. Unless suitable alternatives can be developed and validated, this would mean testing involving 13 million animals, of which 8 million would be fish.

To address these issues a workshop was organised to facilitate an active dialogue amongst industry, regulators and academia on the pragmatic use of alternative (non-animal) approaches. The workshop participants were charged with addressing *in vivo, in vitro* and *in silico* approaches, in line with the 3Rs (Replacement, Refinement and Reduction), for the generation of hazard and exposure information within the context of environmental risk assessment.

Aims

- Identify methodology for generating information for environmental risk assessment, in line with the 3Rs;
- identify research to address knowledge gaps in the proposed methodology;
- draft research plans including potential funding opportunities, collaborations and timelines to develop alternative methodologies to address the environmental safety of chemicals.

Programme

Thirty-seven participants with backgrounds in ecotoxicology and environmental fate assessment representing governments, academia and industry (Appendix 1), met for a 2-day workshop to address ways of using alternatives to animal testing for the evaluation of environmental fate and effects of chemicals. The workshop was divided into sessions that consisted of presentations and breakout group discussions (Appendix 2). Conclusions were discussed in plenary at the end of each session and overall conclusions were discussed in a final plenary session at the end of the second day.

The workshop was held on 7-9th July 2004. It was organised by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC, http://www.ecetoc.org) and co-funded by the European Centre for the Validation of Alternative Methods (ECVAM, http://ecvam.jrc.it/) and Cefic Long-range Research Initiative (LRI, http://www.cefic-lri.org).

WORKSHOP OVERVIEW

1. Effect of chemicals on fish

T. Braunbeck and H. Witters described how and when fish are used for product and effluent assessment in two of the opening presentations. In summary, fish are used for acute, prolonged and chronic toxicity assays, mainly for effluent testing, classification of chemicals and environmental risk assessment of chemicals, biocides, plant protection products and pharmaceuticals. Depending on future legislation, the number of fish used in chronic toxicity tests might increase, but at present the majority of fish are used for acute toxicity tests. In both presentations the issue of the relative sensitivity of algae and daphnids versus fish was discussed. Algae and daphnids are considered to be more sensitive than fish for the testing of effluents or chemicals. However, in a significant number of cases, fish appear to be the most sensitive organisms.

A survey carried out by the European Chemicals Bureau (Weyers *et al*, 2000) addressed acute toxicity data for newly notified chemicals, for the three test species. The algal growth inhibition test was the most sensitive in 43.5% of the cases, the daphnid immobilisation test in 22.3% of the cases and fish in 15.6% of the cases. For 18.6% of the chemicals, the organisms were all equally sensitive. In a study addressing effluents, fish, especially trout, were the most sensitive organisms in approximately 20% of the samples (H. Witters' presentation in plenary session).

Two syndicates, chaired by T. Hartung and G. Whale, were then asked to list and prioritise tests in terms of fish usage/resource/potential savings. This was done by brainstorming and generating a list of possible alternative methods for measuring each acute/chronic endpoint or alternatives for chronic endpoints. For each alternative method the current stage of development versus a list of criteria was discussed and agreed (Appendix 3).

The syndicate discussions concluded that, of the different vertebrate animal species used for environmental evaluation (fish, birds, amphibians), priority should be given to the development of alternative methods to fish toxicity assays. In consequence, participants of the syndicate groups considered applying the 3R approach to the acute fish test as a priority.

The following sections describe these discussions in more detail.

1.1 Identification of potential non-animal alternative methods

Following the early presentations, a number of different approaches were discussed (see Appendix 4) and compared according to the following criteria:

- Relevance;
- reliability (reproducibility/repeatability);
- state of development;
- resource needs;
- regulatory acceptance;
- time to acceptability/use.

Of the approaches discussed, four were considered to be the most promising:

- Reduction:
    - The acute threshold (step-down) approach (Hutchinson *et al,* 2003).

- Replacement:
    - Quantitative Structure Activity Relationships (QSARs);
    - fish cells tests (e.g. Castaño *et al*, 2003);
    - fish embryo tests (e.g. Nagel, 2002).

These approaches are discussed in Sections 1.2 - 1.5. Section 1.6 briefly discusses further research needs.

1.1.1 Other approaches - the future?

Genomic and transgenic technologies applied to fish, although not operational at the moment for routine testing, were mentioned as promising methods. The development of these methods should be followed and their applicability and potential kept under review.

These methods could allow further replacement, refinement and/or reduction of *in vivo* studies. This might include the use of additional useful mechanism-related endpoints in existing studies such as the early life-cycle test.

The application of genomics and proteomics should be explored to identify more sensitive and/or specific biomarkers using diverse model systems, including fish cell lines and embryos. Some projects are already ongoing (e.g. the GenDarT project).

Wider application of DNA microarray technology could provide more information, but would not, at present, result in any replacement, refinement or reduction of studies with fish. It would require more fundamental work first, before further development in the context of fish studies can be considered. In the future, DNA arrays may become a useful tool for diagnostic and classification purposes.

1.2 The acute threshold (step-down) approach

The 'acute threshold (step-down) test', as proposed by Hutchinson *et al* (2003), is a strategy to reduce the numbers of fish used in acute ecotoxicity testing of pharmaceuticals. The objective of this strategy is to reduce the numbers of fish used by applying comparative threshold data obtained from the most conservative data set from algal and daphnid acute tests.

Participants agreed that the threshold approach had merits to reduce the number of fish used for acute fish toxicity tests. However, flexibility was needed with respect to the choice of further studies. According to this tiered approach, algae and daphnids should be tested first. After these studies are conducted, a decision should be made on the need to conduct a fish acute toxicity test. Options include:

- Continuing with the fish step-down test, conducting a single-level toxicity test;
- avoiding the need for fish testing, for example if there was clear evidence of high toxicity in the non-vertebrate species;
- performing a Limit Test to fish;
- using fewer test concentrations to determine the fish LC_{50} value;
- using the data in conjunction with a validated alternative approach, for example the fish embryo toxicity test (see below).

Participants agreed that this method should be extended to a wider group of chemicals to confirm and validate its reliability and efficiency in reducing the number of fish used for fish acute toxicity assessment. In particular it was felt that there should be a clearer identification of those groups of chemicals for which fish are more sensitive. This would help refine the strategy. The applicability to the step-down approach to assessment of the toxicity of effluents to fish was also recommended.

1.3 (Q)SARs

(Q)SARs are proposed as a first step for predictions, relating biological activity to the physico-chemical and structural properties of chemical compounds.

Reliable (Q)SARs require accurate data for biological and physico-chemical parameters. The extent to which (Q)SARs can be used as alternatives to fish testing depends on the state of knowledge of mechanisms of toxicity and the characterisation of their domain of applicability.

At present, this approach can be used to make certain limited predictions of toxicity within clearly defined groups of compounds known to share a common mode of action (Verhaar *et al,* 1992).

However, for prediction of fish toxicity for a wider range of chemicals with unknown mechanism of action, there is a general agreement that (Q)SAR models are still at an early stage of development, so it is necessary to include more data to refine these models.

A proposition was made that the results of the algal and daphnid acute toxicity tests could be used to assess the correct estimation of the (Q)SAR models. If the mathematical model correctly estimated the experimental toxicity for both organisms, then the estimation for fish could be accepted. However, if there is no agreement, a fish acute toxicity test or a validated alternative fish test should then be performed.

1.4 Fish cell (cytotoxicity) tests

In vitro fish cell (cytotoxicity) tests have already been suggested by several authors as a possible alternative or as a supplementary bioassay for the conventional fish acute toxicity test. Several fish cell lines and protocols are proposed (Babich *et al*, 1991; Segner and Lenz, 1993; Brüschweiler *et al*, 1995; Castaño *et al,* 2003; Fent and Hunn, 1996).

Fish cells are often considered as less sensitive than adult fish used for acute toxicity testing but good *in vitro-in vivo* correlations have been reported (Castaño *et al*, 2003). One possible short-term project would be to collate the current knowledge of how fish cell lines and fish acute toxicity data correlate and compare the relative sensitivities. Such a project would also collect data pertaining to how many chemicals were compared, what kind of cell lines and the extent to which cell lines were less sensitive.

False negative responses can be generated with fish cell line tests especially with moderately toxic chemicals or effluents. However, this reduced sensitivity may reduce the risk of false positive results. Thus certain participants proposed that fish cell line tests could be considered as a predictive tool for the identification of the most toxic chemicals to fish.

To characterise their reliability and applicability domain more precisely, fish cell line tests should be optimised and validated on a wider range of chemicals. Further work should also address the correlation within existing fish cell line and *in vivo* data. It is possible this may highlight where and why differences occur in cell lines, e.g. the protocol (e.g. exposure, temperature, use of metabolic system); variability of *in vivo* data, etc. The workshop discussed a proposal based on these thoughts that would lead to data being supplemented using a battery of fish cell lines and the relationship between LC_{50} (*in vivo*) and the battery cell line data established (Appendix 5).

1.5 Fish embryo tests

The European Council Directive 86/609/EEC (EEC, 1986) defines a protected laboratory animal as 'any live non-human vertebrate, including free-living larval and/or reproducing larval forms, but excluding foetal or embryonic forms'. Therefore the fish embryo test has been proposed as a replacement for the fish acute toxicity test OECD 203 (OECD 1992).

Most published data on the fish embryo test relate to zebrafish (*Danio rerio*). Discussions among participants pointed out that other fish species should be considered (especially medaka, *Oryzias latipes,* and fathead minnow, *Pimephales promelas*) for the international acceptance of such a test. Given the global needs of the chemical industry, it was seen as counter-productive to develop an alternative test that would not be acceptable to other regulatory authorities. Differences between species of embryonic development should then be taken into account, for example the duration of the embryonic period and occurrence of hatching vary between fish species.

In particular, the post hatch embryonic period (called eleutheroembryo) before complete resorption of the yolk sac is much shorter for zebrafish than for medaka and fathead minnow. It was proposed that the fish experts participating at the workshop would share their experience on the different durations of the embryonic periods of these fish species.

Some participants considered that a fish embryo test with zebrafish should be limited to the egg phase, when the embryo is protected by the chorion, because after hatching the yolk sac is quickly resorbed, and external feeding is required. This statement should however not be applied to other fish species where embryonic development period continues beyond hatch and when yolk sac resorption takes a few days during which exposure can still take place. Therefore certain participants proposed that the term ‘egg test’ should be restricted to zebrafish and not extended to other fish species such as medaka and fathead minnow.

Such considerations, relative to embryonic development, equally apply to amphibians where embryonic development after hatch is generally longer than in fish. For example in *Xenopus laevis*, hatch occurs at the end of the first day of embryonic development, and external feeding is required 4 to 5 days after hatch just before complete resorption of the vitellogenic reserves (the length of time may fluctuate according to temperature). In the 4-day FETAX (Frog Embryo Teratogenesis Assay Xenopus), the first day of exposure is concerned with the embryo before hatch, and the following 3 days of exposure are concerned with the embryo while it is reliant on its vitellogenic reserves, for its calorific supply.

It was mentioned that the chorion of fish eggs could reduce the availability of some chemicals to the fish embryo. Thus several participants reported false negative results obtained with the fish

embryo test when compared to the results from adult fish tests, for certain chemicals or effluents tested in both tests. The test was also capable of generating false positives, for example methanol, for which fish eggs are more sensitive than adult fish.

The fish embryo test (DarT, *Danio rerio* toxicity, Nagel, 2002*)* has been accepted by the German authorities to replace the fish acute toxicity test required in the testing of effluents. Some participants proposed that recommendations could be made to replace the fish test with a fish embryo test, in those countries where fish tests for effluents are required by law.

In conclusion, the workshop agreed that for international acceptance, especially for the testing of chemicals, the fish embryo test should be validated on a broader range of chemicals, with different fish species. In addition, differences in sensitivities should also be further evaluated between different stages of embryonic development, i.e. before hatch versus after hatch.

In addition to lethal endpoints, the fish embryo tests can be used to look for sub-lethal endpoints, i.e. malformations. Therefore, this potential alternative method could also improve the ecological relevance of the test by providing information on chronic effects.

1.6 Strategy for further activities

Each of the potential alternative methods described above could be proposed for further validation exercises. Two specific actions are worthy of further consideration:

i) A combined research project, to evaluate the reliability and to compare predictions between QSARs, fish cell line tests and the fish embryo test (see Appendix 5) was developed. The aim of this combined project would be to develop an optimised test battery to replace the fish acute toxicity test within three years. For the purpose of these validation studies, the reference chemicals data should be scientifically robust. They should cover a wide range of chemicals differing in, for example, solubility, persistence and mode of action.
ii) Secondly, data relating the relative toxicity of chemicals to fish, algae and daphnids should be collated and applied for modelling specific mechanisms of toxicity in fish. In particular those classes or groups of chemicals that are more toxic to fish should be identified. In this way the strategy incorporating the step-down approach would be better evaluated and the need for testing fish reduced. The broader applicability of this approach should also be validated by use of available data sets of effluent testing.

2. Fate - identification of BCF/IE information for environmental risk assessment

The currently accepted regulatory methods and approaches for identification of bioconcentration factors/indirect exposure (BCF/IE) information and their use in risk assessment, classification and labelling and persistent, bioaccumulative, toxic/very persistent, very bioaccumulative (PBT/vPvB) assessments were introduced in a plenary presentation by W. de Wolf. Subsequently, in two separate sessions chaired by S. Bintein and J. Tolls, syndicates were asked to list and prioritise tests in terms of fish usage/resource/potential savings. In a brainstorm session they listed possible alternative methods for obtaining relevant data pertaining to, or measuring of BCF, bioaccumulation factor (BAF) and biomagnification (BMF) (e.g. biotransformation, bioavailability, uptake kinetics). For each alternative method the stage of development was assessed against a list of criteria and the most promising alternative methods in terms of immediate use and longer-term development were agreed. The outcome of the two syndicate discussions was in very good agreement and is presented in Appendix 4.

During the second day, the bioconcentration testing strategy developed by the ECETOC Task Force on Alternative Approaches in Environmental Testing was introduced by S. Gimeno and served as starting point for subsequent discussions in two syndicates. The syndicate chaired by D. Sijm focused on the testing strategy and identified tiers and decision points. Using the table shown in Appendix 4, the syndicate chaired by J. Tolls focused its efforts to identify the priorities for further research specifically for bioconcentration mitigating factors, and addressed in more detail a potential read-across strategy.

In the syndicate discussions, it was quickly agreed that the aquatic compartment was of most interest in the context of the workshop, hence, terrestrial and food chain effects were not further discussed. Furthermore, the availability of a test guideline for BCF determination for the aquatic environment OECD 305 (OECD, 1996) helped further focus the discussion on fish as the main target species, instead of chemical movement in the aquatic food chain to higher organisms.

It was discussed why fish were used in the BCF-assessments and not mussels, a species that may have a lower biotransformation potential as well as increased particle intake. Whether development of more standardised test guideline(s) with other species such as mussels or other invertebrates has relevance in its own right, or should be considered as replacement tests only, was discussed. Since the former was outside of the scope of the overall workshop it was agreed to only consider the replacement, refinement and/or reduction potential of the fish bioconcentration test.

It was suggested that for current and possible future regulations (e.g. REACH), there remained a need to derive a BCF value for chemicals. Categorical data can be used for classification and labelling as well as PBT/vPvB assessments. However, for environmental risk assessment purposes a numerical value will be required.

2.1 OECD TG 305

OECD Test Guideline 305 (OECD, 1996) covers the measurement of an aquatic BCF value. This test can be considered as the 'gold standard' with which to compare alternative approaches due to its immediate regulatory acceptance and general state of development (Appendix 4). However, several issues were discussed in relation to the question of standardisation of the OECD 305. It was concluded that there is a lack of agreed reference values and high quality results from this test that can be used for alternative test performance benchmarking. In this context the development of a 'gold standard' database was recommended. Other general questions with regards to the OECD test guideline dealt with reproducibility and highlighted current unknown intra-laboratory and inter-laboratory variability and reliability.

The guideline's relevance for use with all types of chemicals was also questioned. Although it was considered that the applicability domain covers a wide variety of chemicals, there was agreement that certain chemicals are difficult to test. Specifically the reliability of the BCF value for highly hydrophobic materials is questionable based on the potential for reduced bioavailability in association with determination of the total water concentration in OECD 305, as well as the increased importance of the oral exposure route for these materials. The relevance of real BCF values as opposed to BCF values derived from laboratory studies for risk assessment remains a matter of debate specifically for these hydrophobic types of materials, i.e. free dissolved or total water concentrations, as well as ionisable materials, i.e. dissociated and undissociated concentrations.

Prior to testing a chemical or its key metabolites, information relating to their respective toxicity and potential for exposure should be obtained. In this way the most relevant chemical with which to conduct a bioconcentration test will be identified.

There was also discussion about whether the guideline's requirement to test bioconcentration at two water concentrations actually added value or reduced uncertainty. Specifically in cases where biotransformation was not considered to be highly influential on the BCF result it was argued that a single concentration test was probably sufficient. The proposal to develop a database with agreed reference values and the availability of high quality data would help develop this approach, and lead to reductions in the use of animals.

2.2 Other experimental approaches

The dietary BMF test, abbreviated OECD 305 and static exposure mass-balance approaches were discussed as relevant test methods for estimating BCF values. The first two tests were identified as the most promising, and though still using fish, they use fewer animals compared to OECD 305, thus increasing their ethical acceptance and reducing their adverse animal welfare impact (Appendix 4). While regulatory acceptance appears to be high, further evaluation of their reliability and chemical applicability domain is needed.

2.3 (Q)SAR/models/read-across

QSAR models, which quantitatively describe the relationship between octanol-water partition coefficient (log K_{ow}) and BCF, are useful and should help in reducing animal testing. Discussions are ongoing at OECD to better understand the domain of applicability. For instance, most such models cannot appropriately describe the bioconcentration of surface-active materials and metals. It is probable that QSARs will be best used within a strategy developing the required information and directing the type of testing, if any, that is required.

A read-across strategy can play a significant role in reducing bioconcentration testing. Group identification occurs on the basis of structural similarities and/or similarities with regard to properties relating to bioconcentration behaviour. Its state of development in the area of bioconcentration appears limited, however overall timings for acceptability and use in a regulatory context were considered relatively short primarily due to a high ethical acceptance. The fact that guidance on the approach can be obtained from current regulatory guidelines, including the draft OECD document on the category approach (OECD, 2004) and EPA HPV guidance document (EPA, 1999), will also contribute to quick acceptance and use.

Incorporation of existing knowledge into a rule based model, e.g. Dimitrov *et al* (2004), may lead to more widely applicable prediction models. Such models not only incorporate correlations between physico-chemical parameters and BCF values, but also mechanistic understanding of processes governing the overall bioconcentration process such as absorption, distribution and metabolism. This was recognised by the workshop as being a valuable future tool for assessing the bioconcentration potential of chemicals. However, as discussed in the next section there is still much work to be done to improve the level of understanding of these processes.

A read-across strategy was discussed in the workshop to be used on the basis of *a priori* test design where representative members of the category or group are tested for their bioconcentration behaviour. The results would then be extrapolated to the remainder of the group, or *a posteriori* where existing available information is used. Extrapolation from tested to non-tested chemicals would be performed by deriving a local category QSAR; a local QSAR has a limited domain of applicability, normally of a few chemicals with a very close structural relationship.

2.4 ADME

It was agreed that investigation of the relevant processes that lead to increased or decreased absorption was key to understanding bioconcentration mitigation. Uptake restrictions through the membrane due to molecular size (diameter, length), adsorption to proteins or ionisation may be modelled via QSAR approaches or estimated from mammalian absorption information. Information on occurrence of substances in fish or wildlife is an indication that absorption will take place. Furthermore, distribution of a substance into fat tissue may be modelled by an

assessment of fat solubility, using octanol solubility as a surrogate measure. Physiology based pharmacokinetics (PBPK) models may help in predicting the transfer process. These approaches are considered relevant, require limited resources, and are met with high regulatory and ethical acceptance (Appendix 4).

Metabolism or transformation information may be obtained from mammalian, fish, bacterial, or abiotic systems. Information on rate constants and metabolites obtained from such systems can be used as inputs into models that support BCF prediction. These approaches using fish (Dyer *et al*, 2003) are still in their infancy with regards to their state of development and this is reflected in their reliability and regulatory acceptance level (Appendix 4). As ethical acceptance for fish *in vitro* biotransformation approaches is considered high, further work is needed, although it was recognised that acceptability and general use was probably many years away.

The magnitude of the research efforts and their urgency and priorities for further work for the above mitigation processes were identified as shown in Table 1. How these processes would be measured and incorporated into a quantitative process also requires further assessment.

Table 1: Research needs and priorities

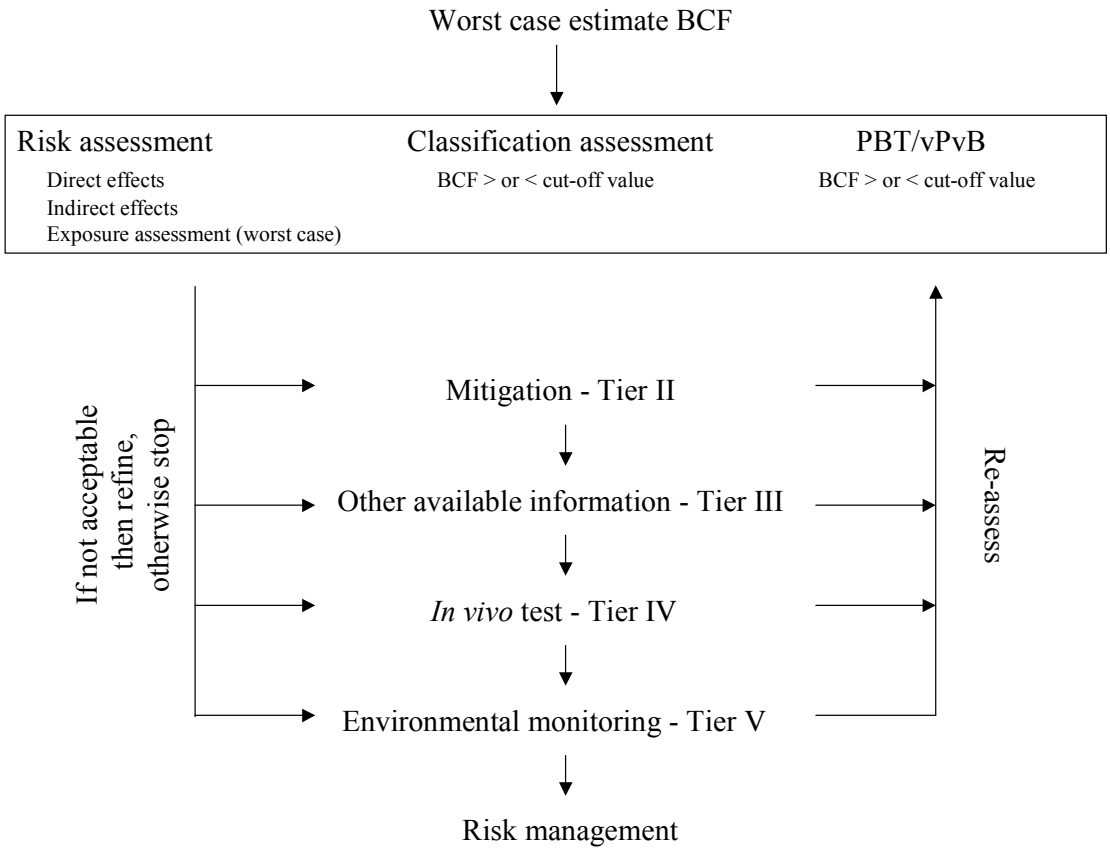
Item	Information obtained	Magnitude of research effort [a]	Urgency [a]
Adsorption and distribution			
Uptake mechanisms	Molecular size (diameter (effective or maximum), length)	3	3
Octanol solubility	Solubility in octanol	1-2	3
Mammalian absorption	Toxicokinetic data, etc.	1-2	3
Monitoring data	Evidence of uptake in environmental biota	1-2	2
Transformation (metabolism)			
Fish biotransformation data	Rate constant, metabolites	3	3
Bacterial biotransformation data	Rate constant, metabolites	1-2	3
Mammalian biotransformation data	Rate constant, metabolites	2-3	3
Abiotic transformation data	Rate constant, metabolites	1	2-3
Incorporation of (bio)transformation data into BCF	Model	2	3

[a] 1 = low, 2 = medium, 3 = high

2.5 Proposed testing strategy

A general strategy was developed that started with estimation of the worst-case situation and subsequent refinement based on the development of further information and experimentation. Figure 1 depicts this generic approach.

Figure 1: Testing strategy for establishing BCF, BAF and BMF



For most chemicals the first tier can be based on log K_{ow}. Within this tier, substances with log $K_{ow} < 3$ would be set aside from further consideration as materials of low concern. For chemicals with log K_{ow} between 3 and 4.5 the worst-case approach may lead to an acceptable result and may require no further work, depending on the regulatory context. Chemicals with a log $K_{ow} > 4.5$ will normally need to go to Tier II.

When log K_{ow} is not applicable, read-across approaches, including the use of local (Q)SAR models, should be applied. It was discussed that if such approaches are not available it may be appropriate to use the highest-ever experimentally determined BCF value, a BCF_{max} as an absolute worst case.

The next tier (Tier III) in the bioconcentration testing strategy would be to assess the influence of mitigating factors such as reduced absorption or biotransformation. Available information derived by read-across from related chemicals, other species or monitoring data would also be applied at this level.

If the risk assessment, classification or PBT/vPvB assessment was still unresolved, then *in vivo* testing (Tier IV) may be needed. Which *in vivo* test to use would depend on a number of factors including the applicability domain of test protocols. Test protocols that used fewer animals than OECD 305 would be preferred (see Section 2.2).

For risk assessment purposes, the final step (Tier V) in the strategy would be to design and perform a focused environmental monitoring programme. The objective of such monitoring would be to establish accumulation patterns in a well-defined food chain. The environmentally realistic BCF, BAF and BMF values from the monitoring would then be used in the risk assessment.

CONCLUSIONS AND RECOMMENDATIONS

The workshop reached the following conclusions and made the following recommendations:

1. Assessing effect of chemicals on fish

There were a number of issues raised and one key question was whether it was necessary to test fish for acute effects. Thus while approaches for limiting fish numbers (e.g. the step-down approach) were seen as helpful, they may prove to be redundant if other species (with or without (Q)SAR support) prove to be sufficiently protective when addressing acute effects.

The key areas of research identified were development of fish embryo and fish cell line tests as well as (Q)SARs.

i) Fish embryo test - This requires research into the acceptability of embryonic tests as non-animal alternatives and into cross species differences (in applicability as well as sensitivity). This is seen as a crucial issue, as there will be little point developing an alternative method that would not be acceptable to all regulators, including those in other regions than Europe, and for whom fish acute tests would then have to be conducted. Future innovative research on genomic and proteomic developments might offer mechanism-related approaches in the fish embryo model for hazard and risk assessment. These could later be linked to cellular developments (see ii).
ii) Fish cell lines - The aim of this research would be to investigate whether a battery of fish cell toxicity tests could be used as a screening test. In the longer term such research should address whether fish cell toxicity tests would be sensitive enough to replace fish *in vivo* tests. One possible short-term project would be to collate the current knowledge of how fish cell lines and fish acute toxicity data correlate and compare the relative sensitivities. Such a project would also collect data pertaining to how many chemicals were compared, what kind of cell lines and the extent to which cell lines were less sensitive.
iii) (Q)SAR - These need further development, establishing which of the current models are useful, their domain, and extending the models to cover more specifically acting chemicals. There was also a clear message that more knowledge was needed to address the mechanisms of effects in fish. In this context, the research into fish embryo and cell toxicity tests could be useful.

2. Assessing the fate of chemicals in fish (bioconcentration)

Two strategies for addressing chemicals for a 'B' assessment were developed. The first approach was developed to address groups of chemicals when a definitive value is not required, but a value

less than some pre-agreed bright-line. Such an approach, for example in the context of the EU-PBT strategy, could lead to significant reduction in the number of chemicals tested. This approach starts by identifying the chemicals within a group. This may be based on similar chemical structures or presumed bioconcentration behaviour. The purpose of the grouping would be to allow for key members of the group to be identified and tested. Interpolation within the group would then allow for BCF values to be assigned to the other group members via a local QSAR. The second strategy, a single-chemical approach, starts with a worst-case assumption and then allows further refinement depending upon subsequent actions. Several of the alternative approaches discussed in the workshop could help move a chemical through the proposed scheme (see Figure 1).

i) OECD 305 - the standard *in vivo* method assessing the bioconcentration potential of a chemical in fish needs investigation to clearly identify uncertainties in measurements derived by this protocol and clarifying its domain of application.
ii) Other *in vivo* experimental approaches, e.g. the dietary BMF protocol and abbreviated OECD 305, need to be further investigated to define and possibly extend their domain and limits of applicability.
iii) *In vitro* assays, expert systems and models need further development and evaluation, especially those that are capable of incorporating ADME concepts and PBPK modelling.
iv) Finally the ADME processes involved in bioconcentration ADME need further research. The workshop addressed the key processes and identified research priorities, including the establishment of a gold standard database of BCF values and a review of available data concerning the potential for chemicals to degrade or be metabolised. These were both seen as good short-term projects.

ABBREVIATIONS

3 'R's	Replacement, Refinement and Reduction
ADME	Absorption, Distribution, Metabolism, Elimination
BCF	Bioconcentration Factor
BMF	Biomagnification Factor
BAF	Bioaccumulation Factor
IE	Indirect exposure
PBPK	Physiology Based Pharmacokinetics
PBT	Persistent, Bioaccumulative, Toxic
QSAR	Quantitative Structure Activity Relationship
vPvB	very Persistent, very Bioaccumulative

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APPENDIX 2: WORKSHOP PROGRAMME

Chairman: **Peter Pärt**

Rapporteurs: **Mike Comber**
Martin Holt

Wednesday, 7 July 2004

12.00 – 13.00	Registration and buffet lunch	
13.00 – 13.10	**Introduction**	
13.10 – 13.30	**Background and product assessment**	**Mike Comber**
13.30 – 13.50	**Hazard - overview**	**Thomas Braunbeck**
13.50 – 14.10	**BCF/IE - overview**	**Watze de Wolf**
14.10 – 14.30	**OSPAR WEA**	**Hilda Witters**
14.30 – 16.30	**Syndicate Session I**	
A and B	Identification of hazard information for environmental risk assessment	
C and D	Identification of BCF/IE information for environmental risk assessment	
16.30 – 17.30	**Plenary feedback from Syndicate Session I**	
19.30	Dinner	

Thursday, 8 July 2004

08.15 – 08.35	**Human Health approach**	**Bob Combes**
08.35 – 08.55	**ECVAM approaches**	**Thomas Hartung**
08.55 – 09.15	**Acute and chronic ecotoxicity**	**Adam Lillicrap**
09.15 – 09.35	**Bioconcentration**	**Sylvia Gimeno**
09.35	Coffee	
09.45 – 11.45	**Syndicate Session II**	
E and F	Propose strategies for generating hazard information for environmental risk assessment	
G and H	Propose strategies for generating BCF/IE information for environmental risk assessment	
11.45 – 13.15	**Plenary feedback from Syndicate Session II**	
13.15 - 14.15	Lunch	
14.15 – 14.45	**Re-cap on sessions 1 and 2 and assign syndicates**	
14.45 – 16.15	**Syndicate Session III - Identification of research programmes to deliver strategies**	
ALL	Identify research needs and agree priority for action/funding	
16.15 – 17.30	**Plenary feedback from Syndicate Session III**	
17.30 – 18.00	**Plenary discussion**	
19.30	Dinner	

Friday, 9 July 2004

08.30 – 08.45	**Funding opportunities**	**Jan van der Valk**
08.45 – 08.55	**Validation needs**	**Thomas Hartung**
08.55 – 09.05	**Research topics for discussion**	**Peter Pärt**
09.05 – 11.00	**Syndicate Session IV - Developing research networks**	
	ALL Developing research networks	
11.00 – 12.00	**Feedback from Syndicate Session IV**	
12.00 – 13.00	**Plenary**	

APPENDIX 3: POTENTIAL ALTERNATIVE METHODS TO ACUTE FISH TEST

	48 hr embryo - effluent [a]	**Fish embryo toxicity test for chemicals** [a]	**Cytotoxicity fish cells** [a]	**Cytotoxicity mammalian** [a]	**Threshold concept** [a,b]	**QSARs** [a]	**Test battery (bacteria, algae, Daphnia)** [a]
Relevance of method[a]	+++	+++	+	+	+++	++(+)	++(+)
Reliability (reproducibility, repeatability)	+++	+++	+++	+++	Not applicable	++(+)	++(+)
State of development (available now or research tool practical applicability, etc.)	DIN ISO +/-	OECD submitted	DIN proposal consensus document exists	ECVAM transatlantic validation study (Balb 3T3 and human keratinocytes) will be finalised next year	Completed for human pharmaceuticals; 'new' chemicals ongoing at ECVAM; veterinary pharmaceuticals, biocides and pesticides should follow	Some proposals made	+++ *Vibrio fischeri*, algal and daphnia tests are fully worked out (DIN and OECD guidelines)
Stage versus ECVAM criteria [c,d]	+++	++	++	++(+)	Individual tests validated – not complete, needs further work	Some (Q)SARs acute tox fish and ED (AR and ER receptor binding assays) about to enter prevalidation stage	+ Individual tests validated battery approach needs further work

APPENDIX 3: POTENTIAL ALTERNATIVE METHODS TO ACUTE FISH TEST (CONT'D)

	48 hr embryo - effluent [a]	**Fish embryo toxicity test for chemicals** [a]	**Cytotoxicity fish cells** [a]	**Cytotoxicity mammalian** [a]	**Threshold concept** [a,b]	**QSARs** [a]	**Test battery (bacteria, algae, Daphnia)** [a]
Regulatory acceptance (suitability for risk assessment purposes)	At present accepted in Germany only	OECD project set up for this purpose	Not	Not	Uses accepted individual test methods but requires wider application as a concept, needs to be discussed with regulators	Limited in EU	+ Uses accepted individual test methods but requires wider application as a concept, needs to be discussed with regulators
Time to acceptability/use (1y/5y/next generation)	3-5 years	3-5 years	> 5 years	> 5 years	Regulatory discussion 2 years	> 5 years	Regulatory discussion for use as screening tests for fish. 2 years
Resource needs (e.g. equipment, experienced staff, investment to set-up)	Same as for acute fish	Same as for acute fish	More than for acute fish test	More than for acute fish test	More than for acute fish	Much lower than acute fish test	Less than acute fish test

[a] Scores range from + (low) to +++ (high).

[b] This should form part of a reduction strategy rather than replacement.

[c] ECVAM stages are: test under development, pre-validated, validated, independent assessment, regulatory acceptance.

[d] Definition of validation: reliability and relevance for a specific purpose.

APPENDIX 4: OUTCOME SYNDICATE SESSION I ON IDENTIFICATION OF BCF/IE INFORMATION FOR ENVIRONMENTAL RISK ASSESSMENT

	Gold standard	Data from:			K_{ow}-based approaches		Mitigating factors			
	OECD 305	**Other test methods (e.g. uptake from a dietary test)**	**Other chemicals (e.g. read-across, category approach)**	**Other species algae, mussels, oyster, mammals (BCF, BAF, metabolism)**	**K_{ow} measures/ QSAR or molecular structure approach**	**Experimental partitioning methods; SPME-SPMD dialysis, solubility in lipids**	**Biotransformation (*in vitro* fish liver systems), binding to proteins or lipids**	**Pertinent existing information on photochemical degradation, microbial degradation, mammalian biotransformation**	**Low bioavailability, mitigating properties (log K_{ow}, solubility in octanol, MW, steric parameters, long chain length, cross sectional area, rapid hydrolysis, readily biodegradable, volatile – as part of the strategy**	**'omics'**
Relevance of method[a]	3	3	2	2	2-3	1-2	1	1	3	1
Reliability	3	2	2	2		1-2	1	1	2	1
Regulatory acceptance	3	1-3	2	2		1	1	1-3	3	1
Ethical acceptance	1	1-2	3	2	3	3	2-3	3	3	2
State of development	3	2	1	1	2-3	1-2	1-2	1	2	1
Stage versus ECVAM criteria [b]	Not applicable	1		Not applicable	Not applicable	1	1	Not applicable		Not applicable
Resource needs	3	3	2	3	1	2-3	2-3	2	1	3
Time to acceptability/ use (1y/5y/next generation)	Now	5y	1-5y	5y	Now + need to extend domain	>5y	>5y-next generation	>5y	Now	>5y
Other criteria: domain	3	1		3	2	2	3			3
Potential as alternative	n.a	3		2	3	2	2			?

[a] 1 = low, 2 = medium, 3 = high

[b] ECVAM stages are: test under development, pre-validated, validated, independent assessment, regulatory acceptance.

APPENDIX 5: NOFISHTOX PROJECT

Aim

Replacement/reduction of acute fish toxicity tests

Research deliverables

- Validated test protocol to replace fish test for substances (embryo fish test; cell line battery for Tier I/screening and for high throughput screening purposes).
- Validated test protocol to replace acute fish test for whole effluent testing (cell line battery).
- Tiered testing scheme for fish acute toxicity testing (cell line + (Q)SAR + embryo).
- Reference set of experimental data.

Approach

- Statistical design (select set of compounds with help of (Q)SAR, aiming at careful coverage of chemical domain).
- Collect *in vivo* fish data from existing databases, checking internal (industry) *in vivo* data.
- Generate fish embryo data with various fish species.
- Generate cell line battery data (RTL-W1, RTG-2, gill cell line, etc.).
- Evaluate bioavailability of chemicals in the tests.
- Establish relationship between fish embryo, fish cell line and *in vivo* data.
- Evaluate statistics, predictivity and applicability domain.
- Develop harmonised test protocols (fish embryo, cell line battery, QSAR) for replacing acute fish toxicity testing.

Timeframe

3 years

APPENDIX 6: ORGANISING COMMITTEE

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ECETOC WORKSHOP REPORTS

No.	Title
No. 1	Workshop on Availability, Interpretation and Use of Environmental Monitoring Data 20-21 March 2003, Brussels
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
No. 3	Workshop on Use of Human Data in Risk Assessment. 23-24 February 2004, Cardiff
No. 4	Influence of Maternal Toxicity in Studies on Developmental Toxicity. 2 March 2004, Berlin