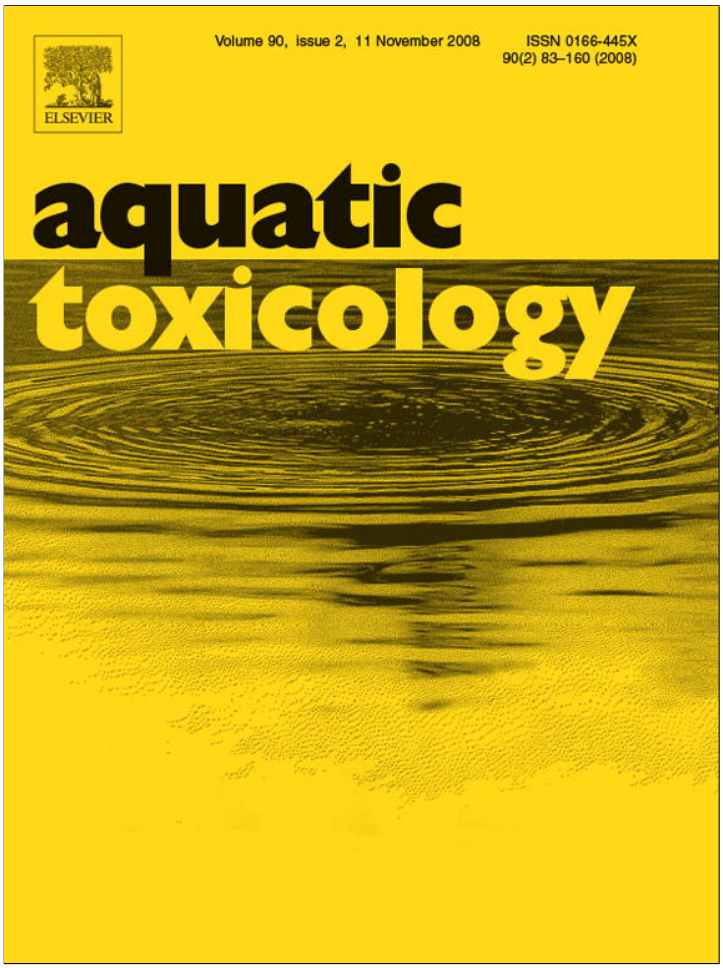

Aquatic Toxicology 90 (2008) 128–137

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Developing a list of reference chemicals for testing alternatives to whole fish toxicity tests

Kristin Schirmer[a,b,*,1], Katrin Tanneberger[a,1], Nynke I. Kramer[c,1], Doris Völker[d], Stefan Scholz[d], Christoph Hafner[e], Lucy E.J. Lee[f], Niels C. Bols[g], Joop L.M. Hermens[c]

[a] *Eawag - Swiss Federal Institute of Aquatic Science and Technology, Department of Environmental Toxicology, 8600 Dübendorf, Switzerland*
[b] *ETH Zürich, Institute of Biogeochemistry and Pollutant Dynamics, 8092 Zürich, Switzerland*
[c] *Department of Environmental Toxicology and Chemistry, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, Netherlands*
[d] *Department of Cell Toxicology, UFZ-Helmholtz Centre for Environmental Research, Leipzig, Germany*
[e] *Hydrotox GmbH, Freiburg, Germany*
[f] *Department of Biology, Wilfrid Laurier University, Waterloo, ON, Canada*
[g] *Department of Biology, University of Waterloo, Waterloo, ON, Canada*

ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 5 August 2008
Accepted 9 August 2008

Keywords:
Alternatives to animal testing
Chemical database
Cell lines
Fish embryo
Acute fish lethality test

ABSTRACT

This paper details the derivation of a list of 60 reference chemicals for the development of alternatives to animal testing in ecotoxicology with a particular focus on fish. The chemicals were selected as a prerequisite to gather mechanistic information on the performance of alternative testing systems, namely vertebrate cell lines and fish embryos, in comparison to the fish acute lethality test. To avoid the need for additional experiments with fish, the U.S. EPA fathead minnow database was consulted as reference for whole organism responses. This database was compared to the Halle Registry of Cytotoxicity and a collation of data by the German EPA (UBA) on acute toxicity data derived from zebrafish embryos. Chemicals that were present in the fathead minnow database and in at least one of the other two databases were subject to selection. Criteria included the coverage of a wide range of toxicity and physico-chemical parameters as well as the determination of outliers of the *in vivo*/*in vitro* correlations. While the reference list of chemicals now guides our research for improving cell line and fish embryo assays to make them widely applicable, the list could be of benefit to search for alternatives in ecotoxicology in general. One example would be the use of this list to validate structure–activity prediction models, which in turn would benefit from a continuous extension of this list with regard to physico-chemical and toxicological data.

1. Introduction

One of the most commonly applied animal tests in regulatory ecotoxicology to this day is the fish acute lethality test. It is standardized under the Organization for Economic Co-operation and Development (OECD testing protocol 203, 1992) and is used to test chemicals as well as industrial effluents. The test involves the exposure of fish to the chemical or effluent sample for up to 96 h. At least 10 fish are exposed per concentration and a minimum of 5 different concentrations are tested in addition to a control. The information obtained is the concentration or dilution that causes 50% of the fish to die. Although simple, the fish acute lethality test has limitations. The number of fish required is quite large and the endpoint, death within 96 h of exposure, reflects an integrative, acute endpoint, which requires high concentrations of toxicants and cannot differentiate routes of mechanisms or subtle, more long-term effects. For these reasons, alternatives to the fish acute lethality test have been sought for years. These potential alternatives have been of two general types. On one hand are mathematically derived

Abbreviations: DIN, German Institute for Standardization; FET, fish embryo toxicity test; IC_{50}, inhibitory concentration causing a 50% decline in cell vitality; log *P*, partition coefficient between water and octanol; log HLC, Henry's law constant—partition coefficient between water and air; LC_{50}, lethal concentration which kills 50% of the fish or the fish embryos; MEIC, Multicentre Evaluation of *In vitro* Cytotoxicity; MOA, mode of toxic action; NPN, non-polar narcotic chemical; OECD, Organization for Economic Co-operation and Development; PN, polar narcotic chemical; QSAR, quantitative structure–activity relationship; REACh, Registration, Evaluation and Authorization of Chemicals; SDS, sodium dodecyl sulphate; UBA, German Environmental Protection Agency; U.S. EPA, United States Environmental Protection Agency.

* Corresponding author at: Department of Environmental Toxicology, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Überlandstrasse 133, 8600 Dübendorf, Switzerland. Tel.: +41 44 823 5266; fax: +41 44 823 53 11.
E-mail address: Kristin.Schirmer@eawag.ch (K. Schirmer).
[1] These authors contributed equally to this work.

0166-445X/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2008.08.005

quantitative structure activity relationships (QSARs), which aim to predict toxicity outcomes based on physico-chemical properties of the test chemicals, and on the other are experimental alternatives, which involve vertebrate cell lines and very early life-stages of fish. The latter are considered non-protected stages based on the definition provided by EU Directive 86/609/EEC (Art. 2a). This was also one of the arguments to revise the German wastewater regulation to accept the zebrafish embryo as an alternative to the acute fish test for effluent testing (Federal Law Gazette, 2005). Early life-stages of fish are also considered for their potential to reduce or replace prolonged or chronic fish toxicity tests (Voelker et al., 2007; Scholz et al., 2008). The commencement of the new EU regulation for industrial chemicals, REACh (Registration, Evaluation and Authorisation of Chemicals), has provided further impetus and spurred new initiatives to apply and further develop current knowledge to provide alternative testing strategies.

A key issue for developing non-animal alternatives is deciding on a list of chemicals for comparing alternative methods with conventional test results. The conventional approach used to test alternative methods against acute fish toxicity tests has been to select test chemicals in a seemingly random manner. These test chemicals, either of similar structure or simply differing in modes of action, were subsequently tested under identical conditions by the investigating lab and the data compared to available *in vivo* data. A comprehensive table of studies applying this approach for the comparison of toxicity in cell lines to that obtained in fish is provided in Schirmer (2006). The described selection approach has also been followed in the derivation of acute embryo toxicity data for comparison to the acute fish lethality test (Braunbeck and Lammer, 2006; Nagel, 2002). At first sight, this unsystematic approach has led to an impressive list of chemicals being tested both in cell lines or fish embryos. A more in-depth analysis, however, reveals several shortcomings of the study-by-study random chemical selection approach.

Among the encountered shortcomings, four seem particularly important. First, relatively few chemicals have been tested across the test systems, e.g. in cell lines, embryos and fish. Second, although an OECD guideline exists with regard to the fish acute toxicity test, available data vary in terms of quality control. Comparing effect concentrations from different sources and different species of fish may introduce considerable and impeding variability. Third, physico-chemical characteristics are not sufficiently taken into account. For example, although the partition coefficient between water and octanol ($\log P$) has previously been considered (e.g. Saito et al., 1993), the distribution between water and air (Henry's law coefficient—log HLC) has not yet been included for alternatives to fish tests. As pointed out by Riedl and Altenburger (2007) in an algal test system, partitioning into air may impact on the bioavailability of chemicals in open static batch systems, which include the tissue culture plates routinely used to expose cell lines and fish embryos. Finally, narcotic chemicals dominate the lists of compounds tested. This reflects the notion that the majority of industrial chemicals acts through narcosis (Russom et al., 1997). Narcosis is thought to result from non-specific chemical–membrane interactions, which can be reasonably well predicted using $\log P$ or, even better, biomembrane/water partition coefficients (Escher and Hermens, 2002; Vaes et al., 1998). On the other hand, the over-representation of narcotic chemicals means that very little is known about the ability of cell lines or fish embryos to predict the toxicity of specifically acting chemicals in fish. The current overemphasis on narcotic chemicals may therefore mask potential difficulties in the application of the alternative testing methods.

To overcome these deficiencies, we developed a list of 60 organic chemicals that we propose to test as part of the research project, CEllSens. This project aims to systematically gather mechanistic information on the performance of alternative testing methods. Specifically, the ability of fish cell lines and zebrafish embryos to detect specific modes of action, and the role of dosing and exposure schemes in the expression of toxicity of chemicals with a wide range of physico-chemical properties will be investigated. These investigations called for a carefully selected list of reference chemicals. The developed CEllSens chemical list has already sparked considerable interest by several stakeholder groups. Two examples are the OECD expert group on the fish embryo toxicity test (FET) and the Health and Environmental Science Institute (HESI) bioaccumulation working group. Their interest inspired us to recommend this list as a reference set of chemicals whose common use could help accelerate the development of non-animal alternatives in toxicology and ecotoxicology.

2. Materials and methods

2.1. Selection criteria for the CEllSens list of chemicals

The first decision in the development of the CEllSens chemical list was to focus the selection procedure on organic compounds. In contrast to inorganic chemicals, which are limited in their number and are rather well understood in terms of their toxicity, there are numerous old organic chemicals that will have to be tested anew under REACh and the development of new organic compounds is theoretically unlimited. The focus on organic compounds, however, also meant that complexes of inorganic and organic chemicals, such as metal-containing organic chemicals, were not considered for the development of the CEllSens chemical list.

Based on reports from other studies concerned with the improvement of alternative testing methods in toxicology, such as the Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) study (Clemedson et al., 1998a,b), a list of about 60 chemicals was judged appropriate for investigating the prediction power of both QSARs and experimental alternatives to the fish acute toxicity test. About half of these 60 chemicals should be treated with highest priority, especially for the experimental approaches, whereas the other half should be available as a back-up and possible extension according to the research question at hand.

Nine guiding principles were established to derive the list of organic chemicals from existing databases. These principles were as follows:

1. All chemicals should have been tested previously in the fish acute lethality test according to the OECD203 guideline, in order to avoid additional animal experiments.
2. Of these chemicals, the majority should also have been tested on cell lines or fish embryos.
3. Chemicals should be characterized by a wide range of $\log P$ and log HLC.
4. The values for $\log P$ and log HLC should be calculated based on the same algorithm for all chemicals to ensure comparability and transparency in the application of these physico-chemical parameters.
5. Chemicals should be characterized by a wide range of LC_{50} and IC_{50} values.
6. Chemicals should be included that show a large deviation (outliers) in effect concentrations in the fish acute toxicity test compared to tests with cell lines or fish embryos.
7. Chemicals should belong to classes of different modes of action according to Verhaar et al. (1992) and Russom et al. (1997).
8. In order to allow the influence of physico-chemical parameters of chemicals to be studied without the mode of action as an influence, a sub-set of about 10 chemicals of the non-polar narcotic mode of action type should be included.

9. For all chemicals, a relatively simple method for quantification by high-performance liquid chromatography or gas chromatography should be feasible.

2.2. Databases from which the CEllSens list of chemicals was derived

Three databases were found most comprehensive and appropriate with regard to the criteria laid out above. For fish acute lethality data, we selected the U.S. EPA fathead minnow database (http://www.epa.gov/med/Prods_Pubs/fathead_minnow.htm). This is a subset of the ECOTOX database (http://cfpub.epa.gov/ecotox/). The lethal concentrations (LC_{50}s) given in this database are all based on flow-through exposures for 96 h with analytically determined water concentrations. If more than one LC_{50} value is available per chemical, the database lists the geometric mean. In search for chemicals with specific modes of action, two additional chemicals, which were present in the ECOTOX database but not specifically in the fathead minnow database, were included in the CEllSens chemical list. These were lindane and parathion-ethyl. LC_{50} values for 96 h fathead minnow exposures in flow-through systems with measured exposure concentrations were available for both of these chemicals (Call et al., 1981 for lindane; Spacie et al., 1981 for parathion-ethyl).

Data for fish embryo toxicity were taken from the German Environmental Protection Agency (UBA) review assembled by Braunbeck and Lammer (2006). The UBA database contains lethal concentrations (LC_{50}s) for the 48 h zebrafish embryo test as proposed by Nagel (2002) and standardized under DIN 38415-6 (2001) and further developed in the OECD draft guideline on the fish embryo toxicity (FET) test (OECD, 2006). Otherwise available information with protocols deviating from the DIN norm or the OECD draft guideline (e.g. different exposure times, static vs. semi-static or flow-through exposure) are included as well.

For toxicity of chemicals to cell lines, the Halle Registry of Cytotoxicity is the most comprehensive currently available database (Halle, 2003). This database was kindly provided by Manfred Liebsch from the German Federal Institute for Risk Assessment (BfR), Centre for Alternative Methods to Animal Experiments-ZEBET. It contains a collection of effective (inhibitory) concentrations (IC_{50}s) of chemicals that cause an acute, i.e. rapidly developing, death of cells. Being based on a thorough literature search, it also contains information about basal cytotoxicity gathered in the MEIC study, which evaluated the relevance of *in vitro* toxicity tests to predict human acute systemic toxicity for 50 chemicals (Clemedson et al., 1998a,b). It should be noted that the compilation of cytotoxicity results of the Halle database does, however, not build on a standard protocol as for the fish acute lethality test and that many different cell lines (all mammalian) and means to detect cytotoxicity (protein content determination, Neutral Red uptake assay, lactate dehydrogenase assay, etc.) are represented in the database. The geometric mean of IC_{50} values was used if several IC_{50}s per chemicals were listed.

2.3. Preparing the CEllSens list of chemicals

Based on the above nine guiding principles and the three data sets, the chemicals were selected in a two-step approach. First, 34 chemicals for which all of the guiding principles applied were selected. In a second step, this list was extended to 60 chemicals, being careful to match the above-mentioned criteria as best as possible. Specifically, chemicals were selected to cover an even broader range of log *P*, log HLC, and LC_{50}/IC_{50}s and to have additional chemicals for each mode of toxic action. The approach involved the plotting of the fathead minnow LC_{50} values against the IC_{50} Halle Registry data or the embryo LC_{50} values according to their modes of action. Moreover, it comprised the plotting of the log *P* and log HLC values for all chemicals of each particular mode of action from the U.S. EPA fathead minnow database in order to allow the selection of chemicals with an even wider range of physico-chemical properties. The physico-chemical parameters, log *P* and log HLC, were uniformly calculated using EPI Suite (http://www.epa.gov/oppt/exposure/pubs/episuite.htm). Values for water solubility were taken from the PhysProp database (http://www.syrres.com/esc/physdemo.htm).

3. Results

3.1. Summary of information gathered from databases

At the time of our search, the U.S. EPA fathead minnow database listed LC_{50} fathead minnow acute lethality values for 617 chemicals (not including lindane and parathion-ethyl). The Halle Registry of Cytotoxicity contained IC_{50} values for 540 chemicals. For the fish embryo test using zebrafish, LC_{50} values for 198 chemicals were available. We found an overlap of the U.S. EPA fathead minnow database with, respectively, the Halle Registry of Cytotoxicity and the UBA fish embryo toxicity data collection for 85 and 40 chemicals. The Halle Registry of Cytotoxicity and the UBA fish embryo toxicity data collection contained 48 matching chemicals. A total of 19 chemicals were present in all three databases.

Table 1
Number of chemicals in U.S. EPA fathead minnow data base according to their different modes of action.

System used to define mode of action			
Russom et al. (1997) (according to U.S. EPA)		Verhaar et al. (1992)	
Mode of action	Number of chemicals	Mode of action	Number of chemicals
Narcosis I	241	Non-polar narcosis (NPN)	241
Narcosis II	38		
Narcosis I and II	13	Polar narcosis (PN)	77
Narcosis ester	26		
Reactives	97	Reactives	97
Acetylcholin esterase (ACHE)-inhibition	17		
Respiratory blocker	4		
Neurotoxicant	9	Specifically acting	48
Neurodepressor	6		
Uncoupler of ox. phosphorylation	12		
Unsure	154	Unsure	154

Table 2
Number of chemicals common to the three data sets for different modes of action.

Mode of action for the chemical	Number of chemicals in common			
	U.S. EPA* vs. Halle Registry	U.S. EPA* vs. UBA embryo toxicity	Halle Registry vs. UBA embryo toxicity	U.S. EPA* vs. Halle Registry vs. UBA embryo toxicity
Narcosis (NPN, PN)	49	21	12	13
Reactive	7	1	1	1
Specifically acting	17	6	5	4
Unsure	12	12	30	1
Total	85	40	48	19

Asterisk (*) refers to U.S. EPA fathead minnow database including lindane and parathion ethyl.

The U.S. EPA fathead minnow database assigns modes of action to each chemical according to the Russom scheme (1997). For simplification, we adapted this classification to the scheme recommended by Verhaar et al. (1992). Table 1 summarizes the classification of chemicals of the U.S. EPA fathead minnow database according to modes of action. Table 2 illustrates the assignment to the different modes of action for the chemicals present either in two or all three of the selected databases. From this comparison it becomes apparent that not only the number of overlapping chemicals is greatest between the U.S. EPA fathead minnow database and the Halle Registry of Cytotoxicity but also the reflection of different modes of action is broadest.

3.2. Selection of reference chemicals

As the U.S. EPA fathead minnow database and the Halle Registry of Cytotoxicity had the most overlap, these two databases were used as the primary source for reference chemical selection. In a first step, chemicals were selected from plots of fathead minnow LC_{50} values against the IC_{50} Halle Registry data according to their modes of action (see panel A in, respectively, Figs. 1–3) so as to cover a wide range of LC_{50}/IC_{50} values. As can be seen for specifically acting chemicals (Fig. 2A) and even more so for reactive chemicals (Fig. 3A), the choice of chemicals for which both *in vivo* and *in vitro* data were available was rather restricted. In a second step, log *P* and log HLC values for all chemicals of each particular mode of action from the U.S. EPA fathead minnow database were plotted (see panel B in, respectively, Figs. 1–3). The chemicals already selected in step one were highlighted and additional chemicals chosen according to the selection criteria listed above.

Outliers were selected on the basis of the difference between $\log LC_{50}$s of the U.S. EPA fathead minnow database and $\log IC_{50}$ of the Halle Registry of Cytotoxicity. Data for all 85 available chemicals (including unsure modes of actions, see Table 1) were included. An outlier was arbitrarily defined as a chemical those difference in $\log LC_{50}$ (fish) − $\log IC_{50}$ (cells) was at least two standard deviations apart from the mean *in vivo*/*in vitro* difference of the 85 chemicals, which was calculated to be −0.82 (Fig. 4). Five chemicals were identified in this way: 2-aminoethanol and hexamethylene tetramine (both unsure modes of action) appeared more toxic in the cell lines compared to fish; allyl alcohol (reactive chemical), lindane and permethrin (neurotoxicants) were more toxic in fish than in cell lines. Three of these chemicals, namely lindane, permethrin and allyl alcohol, had previously been selected (Figs. 2A and 3A).

To compare the chemicals selected above with the information available from the UBA embryo toxicity database, $\log LC_{50}$s from the UBA database were plotted against $\log LC_{50}$s from the U.S. EPA fathead minnow database (Fig. 5). Selected chemicals cover about six orders of magnitude in LC_{50} values in the embryo toxicity test. Based on the 40 chemicals that overlap between the U.S. EPA fathead minnow database and the UBA embryo toxicity database, two varied by more than two standard deviations from the mean of $\log LC_{50}$ (fish) − $\log LC_{50}$ (embryo) (which amounted to 0.07) and therefore qualify as outliers (Fig. 5, insert). The two chemicals were methyl anilines, which appeared significantly more toxic in the embryo compared to the acute fish test. Therefore one of them, *N*-

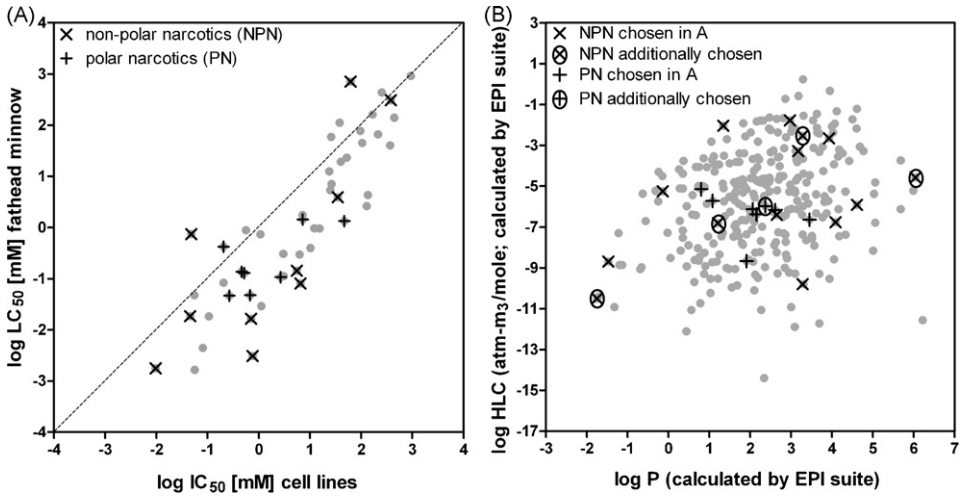

Fig. 1. Plot of chemicals acting through narcosis. All 49 narcotics for which both $\log LC_{50}$ (fathead minnow) and $\log IC_{50}$ (Halle Registry) data were available were correlated first (A). Each dot (●) represents one narcotic chemical. The chemicals chosen for the CEllSens list are marked with (×) for non-polar narcotics and (+) for polar narcotics. In a second step, all 318 narcotic chemicals of the U.S. EPA fathead minnow database were correlated with regard to their log *P* and log HLC, using values calculated by EPI Suite (B). Each dot (●) represents one narcotic chemical. Chemicals marked with (×) or (+) are those already chosen in (A). Based on their physico-chemical properties, four non-polar narcotics (⊗) and one polar narcotic (⊕) chemical were additionally chosen.

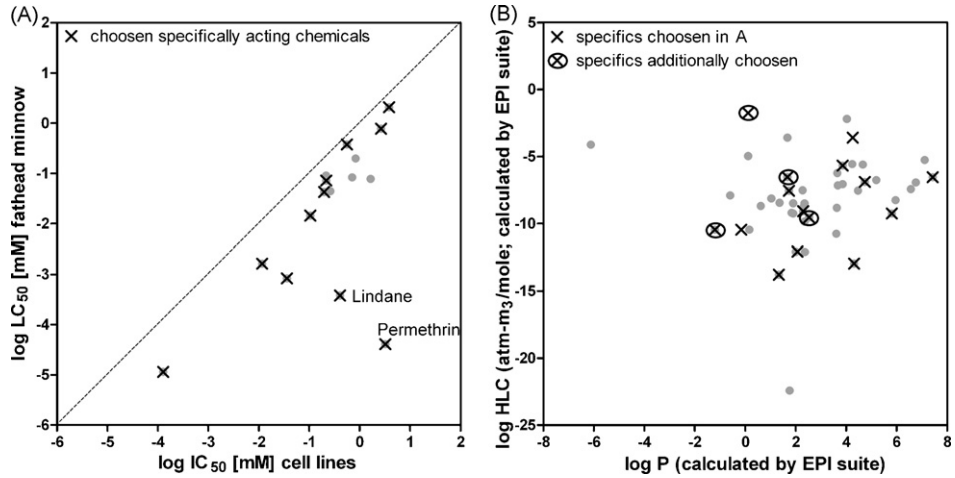

Fig. 2. Plot of specifically acting chemicals. The procedure followed is that described in Fig. 1. All 17 specifically acting chemicals for which both log LC_{50} (fathead minnow database) and log IC_{50} (Halle Registry) were available were correlated first (A). Each dot (●) represents one specifically acting chemical. The chemicals chosen for the CEllSens list are marked with (×). Care was taken to select at least one chemical from each type of specific toxicity (AChE-inhibitor: 2 chosen out of 3; respiratory blocker 1 out of 2; neurodepressor 2 out of 4; neurotoxicant 4 out of 5; and uncoupler 2 out of 2). In a second step, all 48 specifically acting chemicals of the fathead minnow database were correlated with regard to their log *P* and log HLC (B). Each dot (●) represents one specifically acting chemical. Chemicals marked with (×) are those already chosen in (A). Based on their physico-chemical properties, four chemicals (⊗) were picked additionally (two AChE-inhibitors, one neurotoxicant, one blocker of the respiratory chain). Note that Permethrin and Lindane were also identified as outliers, see Fig. 4.

methyl aniline, was added to the chemical list. Lindane, which was identified as an outlier in the fish vs. cell line comparison (see Fig. 4), was more than one but less than two standard deviations apart from the mean difference. It was more toxic in the fish than in the embryo, which was also the situation for the cell line data (Fig. 4).

Of the chemicals selected thus far, eleven were found in both the UBA embryo toxicity database and the Halle Registry of Cytotoxicity (Fig. 6). The direct comparison revealed two clusters: a cluster of narcotic chemicals and one of specifically acting and reactive chemicals. The narcotic chemicals are located close to the line of unity between the embryo and cell line data, whereas the specific and reactive chemicals appear more toxic in the embryos than in the cell lines. One chemical with an unsure mode of action (2-aminoethanol) seemed appreciably more toxic in cell lines compared to the embryos. This substance was also found to be more toxic to cell lines compared to fish (Fig. 4).

3.3. Assignment of positive control

We selected sodium dodecyl sulphate (SDS) as a positive control for four reasons. SDS exerts a low log *P* and log HCL value and is miscible in water (water solubility is 100 g/l). Because of these characteristics, it can be handled easily and hampering sorption to exposure vessels or evaporation can be excluded. SDS is classified as a membrane-damaging, i.e. narcotic, chemical. Therefore its toxicity should easily be detectable in any non-specific bioassay. Furthermore, SDS represents the group of anionic surfactants, which are widely used in many technological processes and are also of environmental concern (Cserháti et al., 2002). Finally, SDS was chosen as a reference chemical in the ACuteTox project, which focuses on a testing strategy to replace animal testing for human acute systemic toxicity (Clemedson et al., 2007). No embryo log LC_{50} or cell line log IC_{50}s were available in the respective databases. We

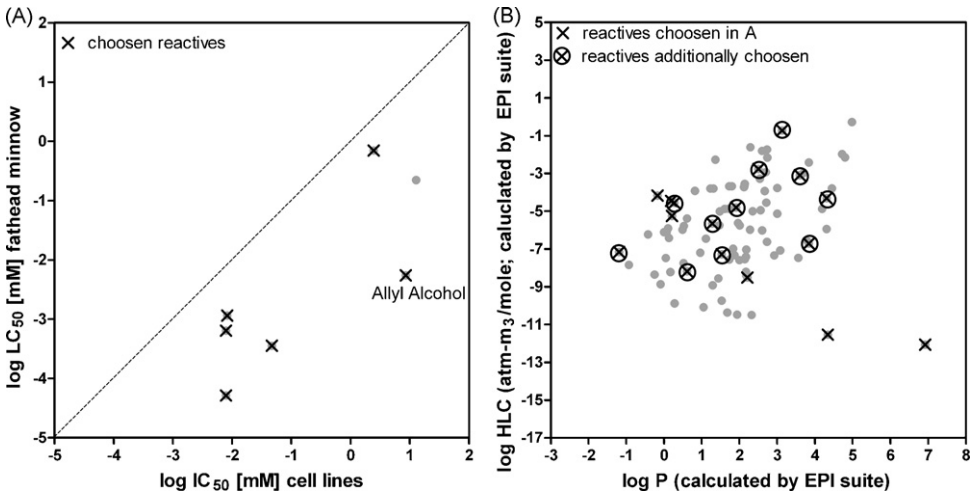

Fig. 3. Plot of reactive chemicals. The selection procedure followed is that described in Fig. 1. All seven reactive chemicals for which both log LC_{50} (fathead minnow database) and log IC_{50} (Halle Registry) were available were correlated first (A). Each dot represents one reactive chemical (●). The chemicals chosen for the CEllSens list are marked with (×) (6 out of 7). In a second step, all 97 reactive chemicals of the fathead minnow database were correlated with regard to their log *P* and log HLC (B). Each dot (●) represents one reactive chemical. Chemicals marked with (×) are those already chosen in (A). Additionally chosen chemicals are marked with (⊗). Note that allyl alcohol was also identified as outlier, see Fig. 4.

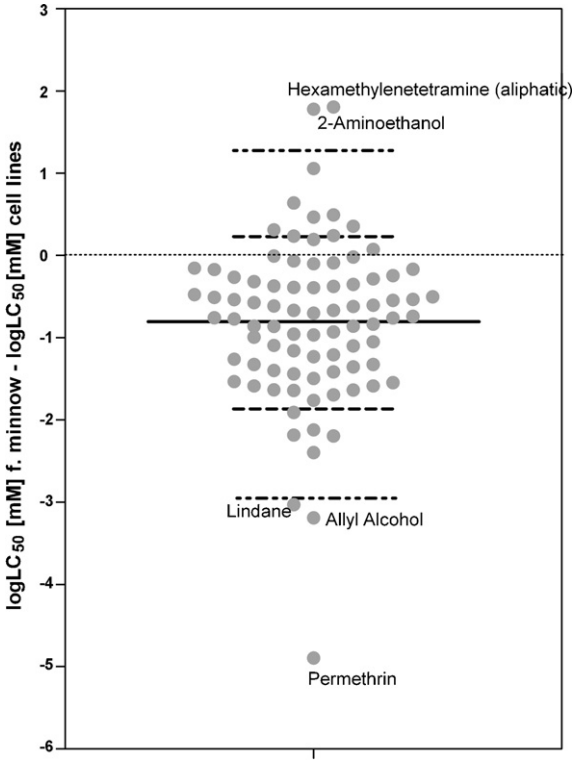

Fig. 4. Graphic representation for picking outliers. The difference between the log LC_{50} (fathead minnow) and the log IC_{50} (Halle Registry) was calculated and plotted for each of the 85 chemicals present in both the U.S. EPA fathead minnow database and the Halle Registry of Cytotoxicity. Each dot (●) represents one chemical. The horizontal arrangement of the dots helps to visualize the number of chemicals yielding similar log LC_{50}/log IC_{50} differences; there is no numerical *X*-axis. The solid horizontal line illustrates the mean log LC_{50}/log IC_{50} difference for all 85 chemicals with a value of −0.82, indicating that the cell lines are in average less sensitive than the fish. In case of unity between the log LC_{50} (fathead minnow) and the log IC_{50} (Halle Registry), dots should rest on the dotted horizontal line (difference *in vivo*/*in vitro* = 0). Dashed lines illustrate ±1 standard deviation from the mean log LC_{50}/log IC_{50} difference, whereas dashed-dotted lines represent ±2 standard deviations from the mean. Chemicals with a difference of at least two times the standard deviation were defined as an "Outlier" and are explicitly named: 2-aminoethanol (unsure mode of action) and hexamethylenetetramine (unsure) appeared more toxic in cell lines than in fish: allyl alcohol (reactive), lindane and permethrin appeared less sensitive in cell lines than in fish. Note that allyl alcohol had already been selected in Fig. 3A; lindane and permethrin had already been selected in Fig. 2A.

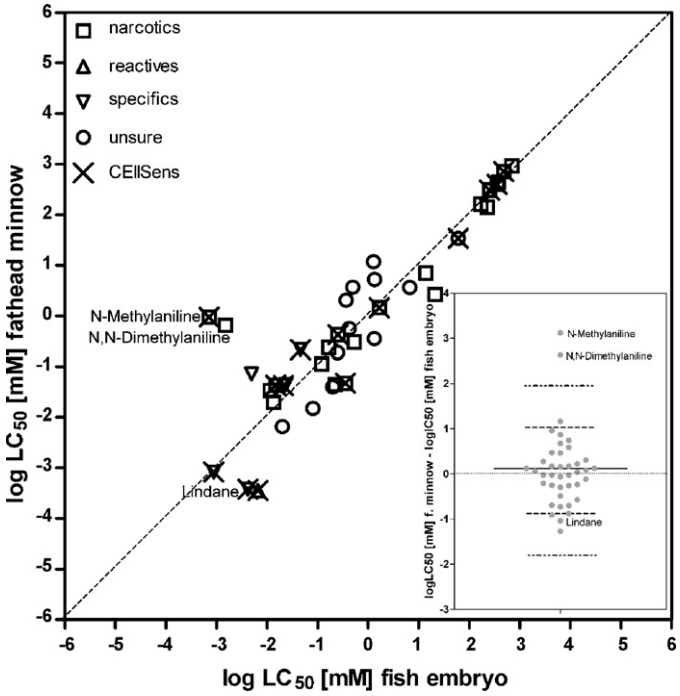

Fig. 5. Plot of U.S. EPA fathead minnow data vs. UBA zebrafish embryo toxicity data. All 40 chemicals for which both log LC_{50} (fathead minnow) and log LC_{50} (zebrafish embryo) were available were sorted according to their modes of action and correlated. Chemicals chosen for the CEllSens chemical list are marked with (×). Outliers were selected in the same manner as described in Fig. 4. Thus, the difference in log LC_{50} (fathead minnow) and log LC_{50} (zebrafish embryo) was calculated and plotted for each of the chemicals, with each chemical being respresented by one dot (●) (see insert). The solid horizontal line in the insert illustrates the mean log LC_{50} (fish)/log LC_{50} (embryo) difference for all 40 chemicals with a value of 0.07, indicating that the zebrafish embryo are on average slightly more sensitive than the fish. In case of unity between the log LC_{50}s for fathead minnow and zebrafish embryo, dots should rest on the dotted horizontal line (difference *in vivo*/*in vitro* = 0). Dashed lines illustrate ±1 standard deviation from the mean log LC_{50} (fish)/log LC_{50} (embryo) difference, whereas dashed-dotted lines represent ±2 standard deviations from the mean. Chemicals with a difference of at least two times the standard deviation (defined as an "Outlier") were *N*-methylaniline and *N*,*N*-dimethylaniline (both narcotics). The *N*-methyl aniline was therefore additionally added to the top 34 CEllSens reference chemical list. Lindane, which was an outlier for the cell lines, was more than one but less than two standard deviations apart from the mean for the embryos.

determined them for the embryo (according to DIN 38415-6) and a cell line from rainbow trout gill (RTgill-W1, according to Schirmer et al., 1997, 1998, using L15/ex as solvent and Neutral Red as indicator of cell viability) to be -1.71 ± 0.10 (logarithm of average of four independently obtained LC_{50} values [mM]) and -1.30 ± 0.09 (logarithm of average of six independently obtained IC_{50} values [mM]), respectively. For fathead minnow, log LC_{50}s were available from Conway et al. (1983) and from the MP Biochemicals Inc. material data safety sheet with values of respectively −1.63 (mM) and −1.11 (mM) (see Table 3).

3.4. Assignment of negative control

We did not specifically assign a negative control. According to the OECD 203 and the OECD draft guideline for the fish embryo toxicity (FET) test, LC_{50} values above 100 mg/l are considered non-toxic. If this rule is applied to the CEllSens chemical list, three chemicals can be regarded as non-toxic in all three models, namely fish, embryo, cell lines. These chemicals are ethanol, aniline and 2-hydroxy ethyl ether.

3.5. CEllSens chemical list

The final selection of chemicals is shown in Table 3. This list was designated the CEllSens chemical list and will be provided as Excel-sheet upon request.

4. Discussion

In this paper, we propose a list (CEllSens list) of 60 reference chemicals for systematic investigations on the ability of alternative testing strategies to predict acute toxicity to fish. Focusing on a common list of chemicals has helped to advance the development of alternative testing methods in the past. During the validation of the mouse fibroblast cell line 3T3-Neutral Red uptake phototoxicity test, which is now accepted under OECD (protocol 432, 2004a), 30 reference chemicals were tested blindly. They were chosen based on results from standardized photopatch testing in humans, covering all major classes of phototoxins (Spielmann et al., 1994). A second example is the MEIC study, where 50 chemicals with relevance to acute systemic toxicity to humans were studied using different mammalian cell lines and cytotoxicity tests. The favorable outcome of the MEIC study (70% of the test chemi-

Table 3
List of 60 CEllSens chemicals.

Reg. #	Name	CAS #	MOA*	log *P***	log HLC** (atm-m^3/mol)	log water solubility (mg/l)***	log LC_{50} (mM) fathead minnow	log IC_{50} (mM) Halle database	log LC_{50} (mM) embryo test (UBA)
TOP 34									
1	Ethanol	64,175	NPN	−0.14	−5.25	4.34	2.49	2.58	2.40
2	2,2,2-Trichloroethanol	115,208	NPN	1.21	−6.81	2.75	0.30		
3	Diethylphthalate	84,662	NPN	2.65	−6.40	0.69	−0.84	0.74	
4	Di-*n*-butylorthophthalate	84,742	NPN	4.61	−5.91	−1.40	−2.52	−0.12	
5	4-Decylaniline	37,529,309	NPN	6.04	−4.57	−2.84	−3.58		
6	Naphthalene	91,203	NPN	3.17	−3.28	−0.62	−1.32	−1.32 (a)	
7	1,2-Dichlorobenzene	95,501	NPN	3.28	−2.53	0.03	−1.19		
8	Dichloromethane	75,092	NPN	1.34	−2.04	2.18	0.59	1.54	
9	Tetrachloroethylene	127,184	NPN	2.97	−1.78	0.10	−1.09	0.82	
10	1,2,4-Trichlorobenzene	120,821	NPN	3.93	−2.66	−0.57	−1.78	−0.15	
11	Aniline	62,533	PN	1.08	−5.72	2.59	0.16	0.86	0.22
12	4-Chlorophenol	106,489	PN	2.16	−6.38	2.27	−1.32	−0.17	−0.45
13	2,4,6-Trichlorophenol	88,062	PN	3.45	−6.64	0.61	−1.33	−0.58	
14	3,4-Dichloroaniline	95,761	PN	2.37	−5.98	−0.25	−1,36		−1.83
15	*N*-Methylaniline	100,618	PN	1.62	−5.38	1.72	−0.03		−3.15
16	Allyl alcohol	107,186	Reactive	0.21	−5.25	4.24	−2.26	0.93	
17	Ethanal	75,070	Reactive	−0.17	−4.17	4.36	−0.16	0.39	
18	Acrolein	107,028	Reactive	0.19	−4.45	3.58	−3.45	−1.33	−2.18
19	2-Methyl-1,4-naphthoquinone	58,275	Reactive	2.21	−8.51	−0.03	−3.19	−2.10	
20	2,3-Dimethyl-1,3-butadiene	513,815	Reactive	3.13	−0.72	0.60	−1.08		
21	2,2′-Methylenebis(4-chlorophenol)	97,234	Reactive	4.34	−11.54	−0.95	−2.94	−2.08	
22	4-Fluoroaniline	371,404	Reactive	1.28	−5.65	2.00	−0.82		
23	2,2′-Methylenebis(3,4,6-trichlorophenol)	70,304	Reactive	6.92	−12.07	−0.46	−4.29	−2.08	
24	Malathion	121,755	Specific (ACHE)	2.29	−9.08	−0.36	−1.37	−0.7	−1.68
25	Disulfoton	298,044	Specific (ACHE)	3.86	−5.68	−1.23	−1.84	−0.98	
26	Rotenone	83,794	Specific (R. blocker)	4.31	−12.95	−3.29	−4.94	−3.90	
27	2,4-Dinitrophenol	51,285	Specific (uncoupler)	1.73	−7.56	1.18	−1.14	−0.67	−2.31
28	Pentachlorophenol	87,865	Specific (uncoupler)	4.74	−6.90	−1.26	−3.08	−1.44	−3.05
29	Permethrin	52,645,531	Specific (neurotox)	7.43	−6.54	−4.81	−4.39	0.51	
30	Lindane	58,899	Specific (neurotox)	1.67	−3.59	−1.60	−3.42 (b)	0.39	−2.38
31	Phenobarbital	50,066	Specific (neurodep)	1.33	−13.78	0.68	0.32	0.58	
32	Parathion-ethyl	56,382	Specific (neurotox)	1.67	−6.53	−1.42	−2.26 (c)	−1.03	
33	Hexamethylenetetramine (aliphatic)	100,970	UNSURE	−4.15 (2.46)	−0.79	3.51	2.55	0.74	
34	2-Aminoethanol	141,435	UNSURE	−1.61	−9.43	4.21	1.53	−0.25	1.78
Extended list									
35	Salicylanilide	87,172	NPN	3.30	−9.80	−0.59	−1.73	−1.34	
36	2-Hydroxyethylether	111,466	NPN	−1.47	−8.69	3.97	2.85	1.79	2.68
37	2,3,4,5-Tetrachlorophenol	4,901,513	NPN	4.09	−6.77	−0.91	−2.75	−2.01	
38	Triethylene glycol	112,276	NPN	−1.75	−10.50	3.82	2.60		2.55
39	4-Nitrophenol	100,027	PN	1.91	−8.66	1.92	−0.38	−0.69	−0.60
40	Pyridine	110,861	PN	0.80	−5.15	4.10	0.13	1.67	
41	*o*-Cresol	95,487	PN	2.06	−6.12	2.38	−0.89	−0.28	
42	2-Chlorophenol	95,578	PN	2.16	−6.38	2.78	−0.97	0.43	
43	2,4-Dimethylphenol	105,679	PN	2.61	−6.17	1.81	−0.87	−0.33	
44	1,1-Dimethylhydrazine	57,147	Reactive	−1.19	−7.16	4.22	−0.88		
45	1-Benzoylacetone	93,914	Reactive	0.61	−8.18	0.37	−2.17		
46	Phenyl disulfide	882,337	Reactive	4.31	−4.31	−1.56	−3.30		
47	1,3-Dibromopropane	109,648	Reactive	2.50	−2.76	0.93	−1.92		
48	A,A′-Dichloro-*p*-xylene	623,256	Reactive	3.60	−3.09	−1.00	−3.65		
49	2,2,2-Trifluoroethanol	75,896	Reactive	0.27	−4.55	4.00	0.08		
50	Xanthone	90,471	Reactive	3.84	−6.71	−1.64	1.73 (LC_{10})		
51	4-Nitrobenzaldehyde	555,168	Reactive	1.53	−7.28	1.19	−1.18		
52	*o*-Fluorobenzaldehyde	446,526	Reactive	1.91	−4.80	1.43	−1.96		

53	Azinphos-methyl	86,500	Specific (ACHE)	2.53	−9.54	−1.18	−3.70			
54	Oxamyl	23,135,220	Specific (ACHE)	−1.20	−10.45	3.11	−1.42			
55	Amobarbital	57,432	Specific (neurodep)	2.00	−12.07	0.36	−0.42	−0.25		
56	Kelthane (dicofol)	115,322	Specific (neurotox)	5.81	−9.25	−2.67	−2.79	−1.93		
57	Caffeine	58,082	Specific (neurotox)	0.16	−10.45	2.05	−0.11	0.42		
58	Chloroacetonitrile	107,142	Specific (R. blocker)	0.11	−4.97	3.12	−1.75			
59	4,6-Dinitro-o-cresol	534,521	Specific (uncoupler)	2.27	−7.52	−0.0003	−2.01		−1.34	
60	Diethyl sebacate	110,407	UNSURE	4.33	−5.27	−0.51	−1.98	0.21		
Positive control										
PC	Sodium dodecyl sulfate	151,213	PN	1.60	−6.74*** (est)	2.54	−1.63 (d), −1.11 (e)	−1.30 ± 0.09 (f)	−1.71 ± 0.10 (g)	

The chemicals were selected based on nine guidance criteria (see Section 2). The first 34 chemicals (TOP34) match all of these criteria. In a second step, the list was extended to 60 chemicals (extended list) to cover an even wider range of log *P*, log HLC, and LC_{50}/IC_{50} and to include additional chemicals for each mode of action. (a) From Schirmer et al. (1998). (b) From Call et al. (1981). (c) From Spacie et al. (1981). (d) From Conway et al. (1983); fathead minnow, followed EPA procedure. (e) From MP Biomedicals Inc., Material Safety data sheet (http://www.mpfinechemicals.corri); fathead minnow; 96 h, static setup. (f) From own work; determined with RTgill-W1 cells seeded into 24-well plates (150,000 ml^{-1}) and Neutral Red uptake assay. (g) From own work: conducted in 24-well plates with 1 embryo per well.

* MOA: mode of action; NPN: non-polar narcosis; PN: polar narcosis; ACHE: acetylcholin esterase.

** Calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm.

*** Taken from PhysProp database http://www.syrres.com/esc/physdemo.htm (data gained experimental; est: estimated).

Fig. 6. Plot of Halle Registry of Cytotoxicity data vs. UBA zebrafish embryo toxicity data. All 48 chemicals for which both log IC_{50} (Halle Registry) and log LC_{50} (zebrafish embryo) were available were correlated. Each chemical is represented by one dot (●). Chemicals selected for the CEllSens chemical reference list are marked with (×). In correlation to the line of unity between the log IC_{50} values from Halle Registry and the log LC_{50} embryo values (dashed diagonal line), two clusters of chemicals were identified (circled). One cluster was formed by narcotic chemicals, which were located close to the line of unity. The other cluster comprised specifically acting and reactive compounds, which were located above the line of unity (indicating greater sensitivity of the embryo compared to the cell lines). One chemical with an unsure mode of action (2-aminoethanol) was located well below the line of unity (indicating greater sensitivity of the cell lines).

cals could be correctly identified *in vitro* if correlated with lethal blood concentrations) has led to the ACuteTox project (Clemedson et al., 2007) whose aim is to replace animal testing for human acute systemic toxicity. The CEllSens approach differs from previous studies in three ways. We were able to base our selection on toxicity data already available for alternative methods in addition to the commonly required acute lethality data for fish. We, moreover, systematically accounted for physico-chemical properties and incorporated an about equal number of chemicals for each mode of toxic action. Finally, we considered ease of analytical detection in order to support the widespread use of the reference chemical list without analytics being a major hurdle.

The final list of chemicals contains many compounds that are included in the OECD list of high-production volume chemicals (OECD, 2004b). These are chemicals that are produced at levels greater than 1000 tons per year in at least one member country. Examples include ethanol, 3,4-dichloroaniline, acrolein, malathione. Lindane, 2-aminoethanol and hexamethylene tetramine, which were identified as outliers in the *in vivo* vs. embryo or cell line comparison, are also CEllSens chemicals identified as high-production volume chemicals. According to REACh, a production volume of more than 10 tons per year requires toxicity data for the acute fish lethality test. Therefore, the CEllSens list represents the type of organic industrial chemicals that are typically of environmental concern and required to be tested for their toxicity to fish very well. One should keep in mind, however, that the starting point for the CEllSens chemical list is acute toxicity to fish. Chemicals with prolonged or chronic modes of action, such as endocrine disrupting or carcinogenic potency, were not explicitly selected.

Preparing the CEllSens list of reference chemicals suggested several important directions of study in order to improve alternative testing methods. For fish acute toxicity data vs. cell lines, the latter appeared generally less sensitive than fish. This phenomenon has been noted repeatedly in the past (reviewed in Schirmer, 2006). This sensitivity issue and the role the mode of action and physico-chemical properties of the test chemicals play in explaining this phenomenon are currently being investigated and will be presented elsewhere (Kramer et al., in preparation). In the future, the CEllSens chemical list could be used to identify and possibly quantify the role of different processes leading to the *in vivo*/*in vitro* differences. Studies using continuous cell lines, for example, have generally focused on basal cytotoxicity as an endpoint and thus were not designed to monitor specific or reactive modes of action. The zebrafish embryo represents a full organism. Being organized in different, albeit developing, tissues and organs it allows different modes of action to proceed. However, lindane is an example of a chemical that appeared more toxic in fish than either in the cell lines or in the embryo. Geyer et al. (1994) showed that differences in lindane toxicity to different species of fish were abolished if LC_{50} values were normalized to the lipid content of each type of fish, indicating a lipid storage of lindane as a means of protection from lindane acute toxicity. A similar protective mechanism is unlikely to explain the difference between the fish, fish embryo and cell line data because a significantly higher lipid content, able to protect from lindane toxicity, cannot be expected in the embryos and cell lines. In embryos, the yolk may possibly serve as a reservoir. However, it mainly consists of proteins. To fully comprehend the differences in toxicity as seen for the three models under investigation, testing chemicals like lindane should be a priority because it would allow for the identification of parameters leading to the creation of an outlier.

The CEllSens reference chemical list was derived from the three most comprehensive databases available to date for *in vivo* fish acute lethality data, lethality of fish embryos and *in vitro* cytotoxicity. Despite this fact, one should keep the shortcomings of each of these databases in mind. For the U.S. EPA fathead minnow database, values are specific to one species of fish, the fathead minnow. Yet, differences in species sensitivity are to be expected (Geyer et al., 1994) and could, in addition to lipid content, be related to a species' tolerance with regard to salinity, oxygen and temperature. Differences in sensitivity for species are tolerated for the determination of acute fish lethality according to OECD 203, where seven different fish species are recommended for the acute fish lethality test. It would be of interest to deduce a level of tolerance for alternative testing strategies as well. However, comparison not only between conventional and alternative testing methods but also different species underlying these methods may complicate the derivation of acceptable levels of tolerance.

For the UBA database on zebrafish embryo toxicity, the range of chemicals studied thus far is still relatively limited. For the chemicals presented in Fig. 5, for example, only 2 out of 40 have a log*P* greater than four and 1 out of 40 has a water solubility below 10 mg/l. There is also uncertainty as to the barrier function of the chorion. Although only weak barrier effects of the chorion have so far been reported (Braunbeck et al., 2005), a role of the chorion in preventing uptake particularly of bulky compounds cannot entirely be excluded. The description of the test procedure is still evolving and owing to this, details of the performance of tests that led to published LC_{50} values are sometimes difficult to obtain. For example, a test volume is given in the DIN norm but the type of material (glass or plastics) is not specified. This however, may have a profound impact on the bioavailability of hydrophobic compounds. As well, the number of embryos per test and the number of independent biological replicates is often difficult to ascertain from the literature. Likewise, reported LC_{50} values are a mix from static (82%), static renewal (17%) and flow-through (1%) set-ups.

For the Halle Registry of Cytotoxicity, data from a wide variety of studies with different goals have been collected. Therefore, among the three databases, the Halle Registry contains data whose derivation was most diverse and least standardized. Moreover, focus of this database is on cell lines from mammals, not from fish. Although excellent correlations between mammalian and fish cell lines have been described for basal cytotoxicity (Castaño and Gómez-Lechnón, 2005; Clemedson et al., 1998a,b; Segner, 2004), the use of fish cell lines can offer significant advantages for correlating a wide variety of chemicals to the acute toxicity in fish (Bols et al., 2005; Schirmer, 2006). Fish cell lines are cultured at temperatures that closely reflect those found in the natural habitat of the fish. Temperature may not only be of physiological relevance but also impact on the behavior of chemicals, like their bioavailability. Moreover, fish cells can be cultured in the absence of serum. Serum impacts on bioavailability but also provides protective molecules that can mask a cytotoxic effect (Dayeh et al., 2005). Serum-free culture medium was successfully used to determine a rainbow trout gill cell line, RTgill-W1, IC_{50} value for naphthalene that closely resembled the LC_{50} for fish acute lethality (Table 3; Schirmer et al., 1998). This assay also detected 1 out of 16 paper mill samples that was toxic to rainbow trout (Dayeh et al., 2002). Therefore, the CEllSens project will systematically test the list of chemicals on well-characterized fish cell lines.

Taken together, the CEllSens list of 60 reference chemicals can serve as a basis for many different applications. Understanding *in vivo*/*in vitro* differences and similarities on a mechanistic level for chemicals with different properties and modes of toxic action is one that we currently tackle. A better mechanistic understanding of the differences should lead to improved testing protocols whose validity can likewise be tested using all or a sub-set of the reference chemical list. The reference list of chemicals can, moreover, be used to deduce effect parameters that are linked to the structure and physico-chemical properties of the chemicals, leading to quantitative structure–activity relationships (QSARs). Such analyses should go hand in hand with improved methodologies for measuring or modeling physico-chemical parameters. Goss et al. (2008), for example, recently described that EPI Suite, which was used herein to uniformly calculate log*P* and log HLC, cannot exactly model the three-dimensional structure of lindane (γ-hexachlorocyclohexane, HCH) and thus cannot distinguish log*P* and log HLC for isomeric structures. Established QSAR models could be blindly applied to the list of reference chemicals to validate the models. Finally, the information for each chemical on the list could be expanded in several different ways. One way would be to add further physico-chemical parameters, such as membrane–water partitioning coefficients. Another means of extending the list would be to include test results from other organisms or biochemical endpoints. Genome or proteome-wide expression analyses upon exposure of cells or organisms to selected chemicals could help identify mode-of-action specific biological response patterns (Voelker et al., 2007; Yang et al., 2007). In this way, the CEllSens chemical list aims to function as a catalyst that helps to direct research to and accelerate research on alternative methods for hazard identification in toxicology and ecotoxicology.

Acknowledgements

This work was supported by a CEFIC-LRI/DEFRA-UK research grant. We are grateful to the CEllSens liaison team for their support and advice.

References

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. In: Mohn, T.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 6. Elsevier Science, Amsterdam, Netherlands.

Braunbeck, T., Lammer, E., 2006. Background Paper on Fish Embryo Toxicity Assays (UBA Contract Number 203 85 422). German Federal Environment Agency, Dessau, Germany, p. 133.

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species—an update. ALTEX 22, 87–102.

Call, D.J., Brooke, L.T., Ahmad, N., Vaishnav, D.D., 1981. Aquatic Pollutant Hazard Assessments and Development of a Hazard Prediction Technology by Quantitative Structure–Activity Relationships; Second Quaterly Report, U.S. EPA Cooperative Agreement No. CR 809234-01-0, Ctr. for Lake Superior Environ. Stud., University of Wisconsin, Superior, WI 74p.

Castaño, A., Gómez-Lechnón, M.J., 2005. Comparison of basal cytotoxicity data between mammalian and fish cell lines: a literature survey. Toxicol. *in Vitro* 19, 695–705.

Clemedson, C., Barile, F.A., Ekwall, B., Gómez-Lechnón, M.J., Hall, T., Imai, K., Kahru, A., Logemann, P., Monaco, F., Ohno, T., Segner, H., Sjöström, M., Valentino, M., Walum, E., Wang, X., Ekwall, B., 1998a. MEIC evaluation of acute systemic toxicity. Part III. *In vitro* results from 16 additional methods used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 26, 93–129.

Clemedson, C., Andersson, M., Aoki, Y., Barile, F.A., Calleja, M.A., Castaño, A., Clothier, R.H., Dierickx, P., Ekwall, B., Ferro, M., Fiskesjö, G., Garza-Ocañas, L., Gómez-Lechnón, M.J., Gülden, M., Hall, T., Imai, K., Isomaa, B., Kahru, A., Kerszman, G., Kjellstrand, P., Kristen, U., Kunimoto, M., Kärenlampi, S., Lewan, L., Lilius, H., Loukianov, A., Monaco, F., Ohno, T., Persoone, G., Romert, L., Sawyer, T.W., Shrivastava, R., Segner, H., Seibert, H., Sjöström, M., Stammati, A., Tanaka, N., Thuvander, A., Torres-Alanis, Valentino, M., Wakuri, S., Walum, E., Wieslander, A., Wang, X., Zucco, F., Ekwall, B., 1998b. MEIC evaluation of acute systemic toxicity. Part IV. *In vitro* results from 67 toxicity assays used to test reference chemicals 31–50 and a comparative cytotoxicity analysis. ATLA 26, 131–183.

Clemedson, C., Kolman, A., Forsby, A., 2007. The integrated acute systemic toxicity project (ACuteTox) for the optimisation and validation of alternative in vitro tests. ATLA 35, 33–38.

Conway, R.A., Waggy, G.T., Spiegel, M.H., Berglund, R.L., 1983. Environmental fate and effects of ethylene oxide. Environ. Sci. Technol. 17, 107–112.

Cserháti, T., Forgáti, E., Oros, G., 2002. Biological activity and environmental impact of anionic surfactants. Environ. Int. 28, 337–348.

Dayeh, V.R., Lynn, D.H., Bols, N.C., 2005. Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicol. *in Vitro* 19, 399–410.

Dayeh, V.R., Schirmer, K., Bols, N.C., 2002. Development and application of fish cell line bioassays for evaluating the toxicity of industrial effluents. Water Res. 36, 3727–3738.

DIN 38415-6. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung-Suborganismische Testverfahren (Gruppe T)-Teil 6: Giftigkeit gegenüber Fischen; Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungsstufen (T6). Deutsches Institut für Normung e.V., Ausgabe 2001, 9.

Escher, B.I., Hermens, J.L.M., 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ. Sci. Technol. 36, 4201–4217.

EU Council Directive 86/609/EEC of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

Federal Law Gazette, 2005. Volume 2005 Part I No. 5, published in Bonn on January 25, 2005. Announcement of the amendment of the Wastewater Charges Act on January 18, 2005.

Geyer, H.J., Scheunert, I., Brüggemann, R., Matthies, M., Steinberg, C.E.W., Zitko, V., Kettrup, A., Garrison, W., 1994. The relevance of aquatic organisms' lipid content to the toxicity of lipophilic chemicals: toxicity of lindane to different fish species. Ecotoxicol. Environ. Saf. 28, 53–70.

Goss, K.-U., Arp, H.P.H., Bronner, G., Niederer, C., 2008. Partition behavior of hexachlorocyclohexane isomers. J. Chem. Eng. Data 53, 750–754.

Halle, W., 2003. The registry of cytotoxicity: toxicity testing in cell cultures to predict acute toxicity (LD_{50}) and to reduce testing in animals. ATLA 31, 89–198.

Nagel, R., 2002. DarT: the embryotest with the zebrafish *Danio rerio*—a general model in ecotoxicology and toxicology. ALTEX 19 (Suppl. 1/02), 38–48.

OECD 203, 1992. OECD Guideline for Testing of Chemicals. Test No. 203: Acute Fish Test.

OECD 432, 2004a. OECD Guideline for In Vitro 3T3 NRU Phototoxicity Test.

OECD, 2004b. The 2004 OECD List of High Production Volume Chemicals. Environment Directorate, OECD, Paris.

OECD, 2006. Draft Proposal for A New Guideline—Fish Embryo Toxicity (FET) Test. Draft Guideline. May 2006.

Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67, 2210–2220.

Russom, C.L., Bradbury, S.P., Broderius, S.J., Hammermeister, D.E., Drummond, R.A., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 16, 948–967.

Saito, H., Koyasu, J., Yoshida, K., Shigeoka, T., Koike, S., 1993. Cytotoxicity of 109 chemicals to goldfish GFS cells and relationships with 1-octanol/water partition coefficients. Chemosphere 26, 1015–1028.

Schirmer, K., 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. Toxicology 224, 163–183.

Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C., 1998. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicology 127, 129–141.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. *In Vitro* 11, 107–119.

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res Int. 15 (5), 394–404.

Segner, H., 2004. Cytotoxicity assays with fish cells as an alternative to the acute lethality test with fish. ATLA 32, 375–382.

Spacie, A., Vilkas, A.G., Doebbler, G.F., Kuc, W.J., Iwan, G.R., 1981. Acute and Chronic Parathion Toxicity to Fish and Invertebrates. EPA 600/3-81-047, U.S. EPA Washington DC 78p. US NTIS PB81-245862.

Spielmann, H., Lovell, W.W., Hölzle, E., Johnson, B.E., Maurer, T., Miranda, M., Pape, W.J.W., Sapora, O., Sladowski, D., 1994. In vitro phototoxicity testing. The report and recommendations of ECVAM workshop 2. ATLA 22, 314–348.

Vaes, W.H.M., Urrestarazu-Ramos, E., Verhaar, H., Hermens, J.L.M., 1998. Acute toxicity of nonpolar versus polar narcosis: is there a difference? Environ. Toxicol. Chem. 17, 1380–1384.

Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J.L.M., 1992. Classifying environmental pollutants. 1. Structure–activity relationships for prediction of aquatic toxicity. Chemosphere 25, 471–491.

Voelker, D., Veß, C., Tillmann, M., Nagel, R., Otto, G.W., Geisler, R., Schirmer, K., Scholz, S., 2007. Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. Aquat. Toxicol. 81, 355–364.

Yang, L., Kemadjou, J., Zinsmeister, C., Bauer, M., Legradi, J., Müller, F., Pankratz, J., Jaeke, J., Straehle, U., 2007. Genome Biol. 8, R227.