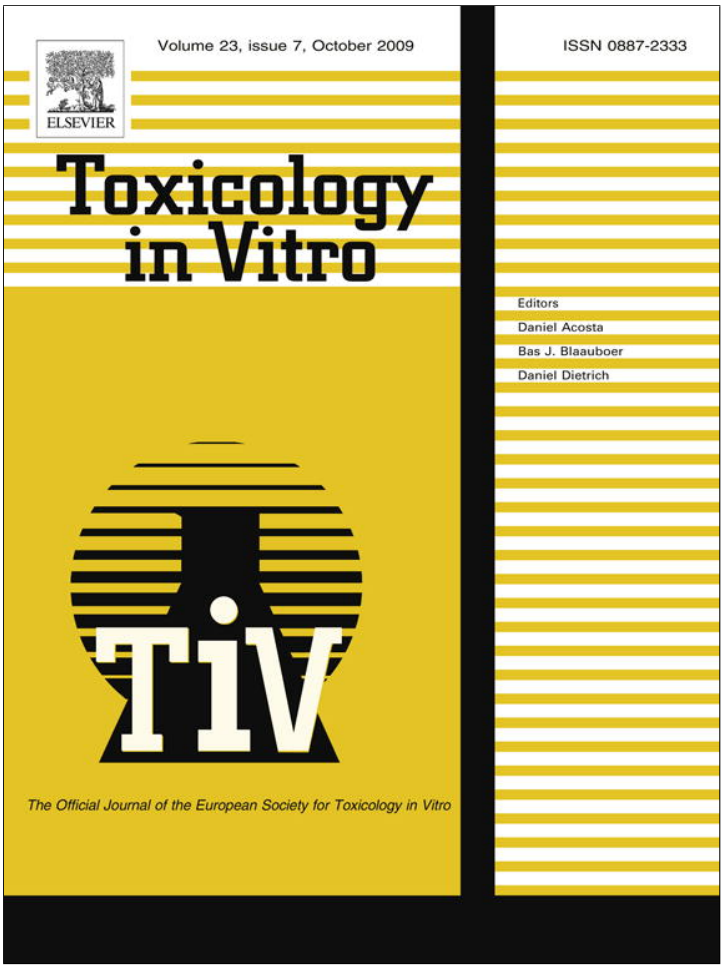

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The influence of modes of action and physicochemical properties of chemicals on the correlation between *in vitro* and acute fish toxicity data

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ABSTRACT

New EU legislation is providing an impetus for research aimed at replacing acute fish toxicity testing with *in vitro* alternatives. In line with such research, the objective of this study was to determine what factors influence the correlation between *in vitro* and fish toxicity data. Basal cytotoxicity (IC_{50}) and acute toxicity data from fathead minnow (LC_{50}) of 82 industrial organic chemicals were obtained from the Halle Registry of Cytotoxicity and the US EPA Fathead Minnow Database. A good correlation between IC_{50} with LC_{50} data was found (r 0.84). Yet, IC_{50} data were less sensitive than LC_{50} data by an order of magnitude. Using multiple regression analysis, the octanol–water partition coefficient (K_{OW}) and the Henry's Law Constant (H) were found to significantly explain the low absolute sensitivity. The mode of action (MOA) of the chemical was found to significantly explain the general variation in the log IC_{50}/log LC_{50} regression line. These results support the notion that (a) the bioavailability of hydrophobic (high K_{OW}) and volatile (high H) chemicals is significantly lower in *in vitro* assays than in the fish bioassay and (b) multiple cell types and endpoints should be included to mimic the modes of action possible in the whole organism.

1. Introduction

Basic ecotoxicity testing of chemicals commonly involves conducting the fish acute toxicity test (OECD test guideline 203, 1992). This test requires a substantial number of fish as at least ten fish are exposed per concentration of test compound and a minimum of five different concentrations are tested in addition to a control. Moreover, the number of fish used for ecotoxicity testing is expected to increase by another 4.4 million in the next decade with the introduction of the EU's new chemicals legislation, Registration, Evaluation and Authorisation of Chemicals (REACH; EC 1907/2006) (Castaño et al., 2003; European Commission, 2006). These developments give rise to serious ethical concerns for acute fish toxicity testing also because the test suffers from a number of scientific and economical limitations. The test is labour and equipment intensive (OECD test guideline 203, 1992; Walker, 1997). The endpoint, death of 50% of fish within 96 h of exposure (LC_{50}), is an integrative but crude endpoint. It does not differentiate between mechanisms of toxicity or detect subtle, longer term effects (Castaño et al., 2003; Schirmer, 2006). The search for non-animal alternatives to acute toxicity testing with fish is therefore timely.

In vitro cell assays are potential alternatives to the fish acute toxicity test (Ahne, 1985; Dayeh et al., 2002; Castaño et al., 2003; Segner, 2004; Bols et al., 2005). A large number of test compounds can be exposed to cells and analyzed in a high throughput system using multi-well plate readers. No (in the case of cell lines) or few (in the case of primary cell cultures) animals are used, little test substance is needed and little toxic waste is produced. Furthermore, *in vitro* studies can address aspects of toxic mechanisms underlying a general toxic response (Castaño et al., 2003; Schirmer, 2006).

Studies that compare *in vitro* data and acute fish toxicity values repeatedly noted differences in the relative and absolute correlation (reviewed most recently in Schirmer (2006)). In relative terms, strong correlations between median effect or inhibition concentrations (EC_{50}s or IC_{50}s) from vertebrate cell lines and LC_{50}s from fish exist (the correlation coefficient, r, is usually higher than 0.8).

Abbreviations: EPI, Estimation Program Interface modules; FATS, Fish Acute Toxicity Syndromes; H, Henry's Law Constant; IC_{50}, median inhibitory/effect concentration for a test compound *in vitro*; K_{OW}, octanol–water partition coefficient; LC_{50}, median effect concentration for a test compound in a fish bioassay; MOA, mode of action; OECD, Organisation for Economic Co-operation and Development; (Q)SAR, (quantitative) structure–activity relationship; REACH, Registration, Evaluation and Authorisation of Chemicals; US EPA, Environmental Protection Agency of the United States of America.

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These cell line studies generally measure basal cytotoxicity, as determined by loss of membrane integrity. Reasonably good correlations have been found between different methods to measure basal cytotoxicity (Lenz et al., 1993; Saito et al., 1994; Castaño et al., 1996), between different fish cell lines (Saito and Shigeoka, 1994; Zahn et al., 1996; Gagné and Blaise, 1998; Caminada et al., 2006) and between fish cell lines and mammalian cell lines (Lilius et al., 1994; Clemedson et al., 1998; Castaño and Gómez-Lechón, 2005). These correlations support the basal cytotoxicity concept of Ekwall (1995), the idea that the majority of chemicals exert their acute toxicity through interference with basal cellular functions and that this results in similar effect levels in all cell types.

In absolute terms, basal cytotoxicity effect concentrations were on average one or two orders of magnitude higher (i.e., less sensitive) than corresponding fish acute toxicity values (Babich and Borenfreund, 1987; Saito et al., 1991; Lilius et al., 1995; Castaño et al., 1996; Gülden and Seibert, 2005). Thus, higher nominal concentrations of chemicals are required in cell cultures than in the fish bioassay to detect a toxic response. Schirmer (2006) summarizes two proposed hypotheses for this sensitivity difference. Firstly, a single culture with basal cytotoxicity as an endpoint cannot detect toxicities involving specific metabolic pathways and target sites it does not mimic. As an example, Saito et al. (1991) report a poor prediction of organophosphate pesticide toxicity by gold fish scale cells (GFS) and attribute this to the inability of GFS cells to mimic acetylcholine esterase inhibition, the primary mechanism of toxicity for these pesticides. Secondly, the bioavailability of the test compound is lower in *in vitro* systems than in fish bioassays. A number of competing processes, such as binding to serum protein, may lower the bioavailable fraction of the test compound *in vitro*. Gülden and Seibert (2005) found that the absolute sensitivity increased when IC_{50}s, based on the estimated unbound concentration of a number of pesticides in a Balb/c 3T3 cytotoxicity assay, were used to predict LC_{50}s of three species of fish.

Toxicity models based on chemical structures and physicochemical properties (e.g., structure–activity relationships, SARs) may prove useful in understanding the correlation between IC_{50} and LC_{50} for fish acute toxicity. Russom et al. (1997) and Verhaar et al. (1992), for example, developed SARs and rule based expert systems categorizing industrial organic chemicals according to their mode of action (MOA), a general type of interaction of a chemical with the organism causing toxicity (Escher and Hermens, 2002). Specifically, Russom et al. (1997) assigned industrial organic chemicals in the fathead minnow database to eight classes of MOA and a certainty level according to thorough analysis of dose response- and behavioural-assessments, joint toxic action studies, Fish Acute Toxicity Syndromes (FATS) and literature. They further associated each MOA class with sub-structural fragments of chemicals in a set of rules (Könemann, 1981; Veith et al., 1983; Karcher and Karabunarliev, 1996; Freidig et al., 1999). By separating chemicals according to their MOA, models of this type can be used to investigate the aforementioned hypothesis that a low absolute sensitivity is due to the poor mimicry of specific MOAs by basal cytotoxicity assays. The physicochemical properties used as descriptors in SARs, such as the octanol–water partition coefficient (K_{OW}) describing the partition behaviour of chemicals to cell lipid, can also be used to investigate cell and non-cellular sorption processes *in vitro*. By doing so, one could investigate for what chemicals the hypothesis that the sensitivity difference *in vitro* and *in vivo* is due to differences in non-specific binding or loss of a chemical (i.e., loss of bioavailability) applies.

Indeed, the aim of this study was to systematically determine the role of MOA and bioavailability of test chemicals on the relative and absolute sensitivity of *in vitro* assays to predict acute fish toxicity. Previous *in vitro–in vivo* extrapolations for fish acute toxicity generally investigated few chemical classes and a small number of chemicals. Moreover, the application of chemical descriptors to understand the extrapolation has been limited. Therefore, in this study, mean literature values of IC_{50} from the Halle Registry of Cytotoxicity were correlated with acute fathead minnow (*Pimephales promelas*) LC_{50} values from the US EPA Fathead Minnow Database for a large number of organic chemicals (82) spanning across various chemical and MOA classes. The statistical influence of modelled MOA (as defined by Russom et al. (1997) and simplified according to Verhaar et al. (1992)), log K_{OW}, and log Henry's Law Constant (H), the latter being a proxy for the evaporation potential of chemicals, on the IC_{50}/LC_{50} regression line was assessed using multiple regression analysis. The results of the analysis were used as a basis for a discussion on possible *in vitro* testing strategies for acute fish toxicity. In fact, the same data set was used in a related study to develop a list of 60 reference chemicals to gather mechanistic (i.e., experimental) evidence for the influence of MOA, K_{OW}, H and the performance of alternatives to the fish test including vertebrate cell lines and fish embryos (Schirmer et al., 2008).

2. Materials and methods

2.1. Data collection

This paper is based on literature values of *in vitro* basal cytotoxicity and *in vivo* acute toxicity data for fish. Acute toxicity test results for fathead minnow (*P. promelas*) were obtained from the Fathead Minnow Database compiled by the Mid-Continent Ecology Division of the US EPA's National Health and Environmental Effects Research Laboratory (Duluth, MN; http://www.epa.gov/med/Prods_Pubs/fathead_minnow.htm; Russom et al., 1997). The database is a subset of the ECOTOX database (http://cfpub.epa.gov/ecotox) and is regarded as one of the most reliable fish LC_{50} databases available (Cronin and Schulz, 2003). It stores acute median lethal concentrations (LC_{50}) for 617 industrial organic compounds together with their chemical structures (SMILES notations) and a classification of the modes of action (MOA) as described by Russom et al. (1997). The LC_{50}s are all based on flow-through exposures for 96 h with analytically determined water concentrations. The geometric mean of LC_{50} was used when more than one value was reported. LC_{50} data were originally given in mg/L and were transformed into mmol/L (mM) using the molar masses of the chemicals tested.

For cytotoxicity values of chemicals, the Halle Registry of Cytotoxicity was used as it is considered the most comprehensive, currently available *in vitro* database (Registry of Cytotoxicity version 3, Halle, 2003). The database is a collation of published literature values of effective (inhibitory) concentrations (IC_{50}) of 540 chemicals. IC_{50} values are obtained from poorly-metabolizing mammalian cell lines or primary cells which have been exposed for more than 16 h to the chemical in generally serum containing medium and in plastic microtitre plates. Cytotoxicity was determined as cell viability measured using membrane integrity, metabolic, differentiation or proliferation markers. At least two IC_{50} values, obtained from separate experiments, were ascribed for each chemical and the geometric mean of IC_{50}s was used in the data analysis.

2.2. Variable calculations

Chemicals which were both present in the Fathead Minnow Database and in the Registry of Cytotoxicity were used in this study. To determine log octanol–water partition coefficients (log K_{OW}) and log Henry's Law Constants (H; bond estimation method; atm m^3/mol) for each chemical, the Estimation Program Interface modules (EPI Suite version 3.12; http://www.epa.gov/oppt/exposure/docs/episuitedl.html), developed by the Syracuse research

Corporation on behalf of the US EPA, were used. It should be noted that the physicochemical properties calculated by EPI Suite are prone to modeling deficiencies for a number of chemicals (Goss et al., 2008). For this study, these deficiencies are considered to be negligible because the study's focus is not directly mechanistic and uniformity was considered more important. Once, however, a better mechanistic understanding of the relationships between physicochemical properties and *in vitro–in vivo* extrapolation power found in this study is experimentally assessed in follow-up studies, improved methodologies for measuring or modeling physicochemical parameters should be considered (Schirmer et al., 2008).

For simplification, the MOA for each chemical, assigned using the Russom et al. (1997) scheme, was adapted to the scheme recommended by Verhaar et al. (1992). The chemicals were subdivided into five classes in this study: classes 1 and 2 included inert chemicals causing baseline toxicity via non-polar and polar narcosis respectively, class 3 included reactive chemicals that unselectively react with biological molecules, class 4 included the specifically acting chemicals (toxicity due to specific interaction with receptor molecules), and class 5 included chemicals whose toxicity was mixed or unknown according to Russom et al. (1997).

2.3. Statistical analyses

The statistical Package for Social Scientists (SPSS) version 13 for Windows (SPSS, Chicago, IL) was used to perform linear, curvilinear and multivariate stepwise regression analyses per MOA class and all chemicals together. Log IC_{50}, log K_{OW} and log H were used as independent variables and log LC_{50} (mM) and the log difference between LC_{50} and IC_{50} (mM) (i.e., log LC_{50} − log IC_{50}) were used as dependent variables. Summary statistics (number, mean, mode, standard deviation and skewness) for each continuous variable were used to examine the normality of the data. The Pearson's Product Moment Correlation Coefficient was calculated for each combination of variables to initially assess the linear relationship between the variables. Scatter plots and curve-estimating regression analyses between the different variables were used to assess linearity and curvilinearity. The strength of the R^2 value was used to determine whether a linear or quadratic relationship is assumed. Analysis of variance was used to determine whether groups of variables differ from each other. All significance levels (p-values) were calculated to 0.05.

3. Results

Eighty-two chemicals were found in both the Halle Registry of Cytotoxicity and the Fathead Minnow Database. They spanned across a large range of toxicities (LC_{50}/IC_{50} 0.02/0.12 μM for rotenone to 912/933 mM for methanol–rhodamine B), log K_{OW} (−6.12 for sodium azide to 7.43 for permethrin), and log HCl (−22.41log atm m^3/mol for amphetamine sulfate to 0.23log atm m^3/mol for hexane). All logged continuous variables were normally distributed.

The majority (60%) of compounds were classified as acting through narcosis, which is higher than the estimated fraction of narcosis type compounds among the high production volume chemicals (Verhaar et al., 1994). The bias of the data set towards narcosis is simply related to the fact that experimental work (both *in vitro* and *in vivo*) does not cover the full range in modes of action. Of these narcotics, 69% (34) and 22% (11) were classified as non-polar and polar narcotics, respectively. The remaining four narcotic compounds were considered to act through mixed or unknown narcosis. Eighteen percent of the compounds were classified as acting through specific toxicities, including respiratory blockers like rotenone, uncouplers like pentachlorophenol, neurotoxicants like caffeine, neurodepressors like phenobarbital and compounds acting on the acetyl choline receptor like malathion. Nine percent were classified as reactive compounds. The remaining 13% were classified as having an unknown or mixed mode of action. All 82 compounds and their properties are presented as Supplementary data.

A good correlation (i.e., relative sensitivity) was found between the IC_{50}s and LC_{50}s of the 82 chemicals analysed in this study (Fig. 1). From regression model between log IC_{50} and log LC_{50}, the following significant linear relationship with an R^2 of 0.70 was found (n = 82, F = 191.12, p < 0.05):

$$\text{Log}\,LC_{50}\ (\text{mM}) = 1.11\ (\pm 0.08)\text{log}\,IC_{50}\ (\text{mM}) - 0.82\ (\pm 0.11). \quad (1)$$

Nevertheless, four outliers from the regression line were identified: permethrin, allyl alcohol, hexamethylenetetramine and 2-aminoethanol. Moreover, as the deviation of the regression line from the 1:1 line and the slope of 1.11 (±0.08) in Eq. (1) indicate, IC_{50} values were on average one order of magnitude lower than corresponding LC_{50} values and LC_{50}s corresponded better with IC_{50} values of less toxic compounds than more toxic compounds (i.e., the difference between LC_{50} and IC_{50} was smaller for less toxic chemicals).

When only narcotic compounds are used in a linear regression model between log IC_{50} for basal cytotoxicity and log LC_{50} for fathead minnow, the correlation improves to an R^2 of 0.80 and the regression equation becomes (n = 49, F = 187.97, p < 0.05, Fig. 2):

$$\text{Log}\,LC_{50}\ (\text{mM}) = 1.11\ (\pm 0.08)\text{log}\,IC_{50}\ (\text{mM}) - 0.81\ (\pm 0.12). \quad (2)$$

This improvement of the regression with narcotic compounds suggests that acute fish toxicity of narcotic compounds is better predicted by *in vitro* assays than other modes of action examined in this study. However, the absolute sensitivity of *in vitro* assays remains unchanged, i.e., lower than the fish bioassay, and the toxicity

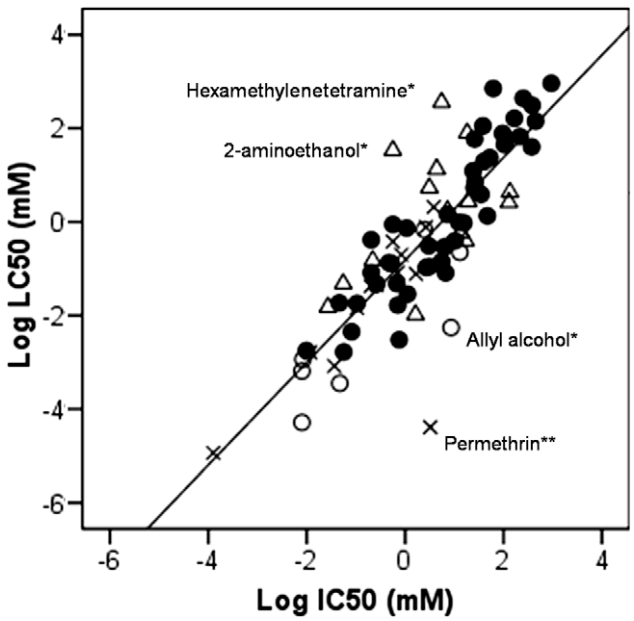

Fig. 1. Scatter plot of log IC_{50} against log LC_{50} for acute toxicity of 82 industrial organic chemicals to fathead minnow. Points are separated by their mode of action: narcosis (●), reactive (○), specifically acting (×) and mixed or unsure mode of actions (△). The dotted line represents the $y = x$ line and the black line is the regression line drawn through all data points. Labeled points are outliers by 2 (∗) or 3 (∗∗) standard deviations from the regression line.

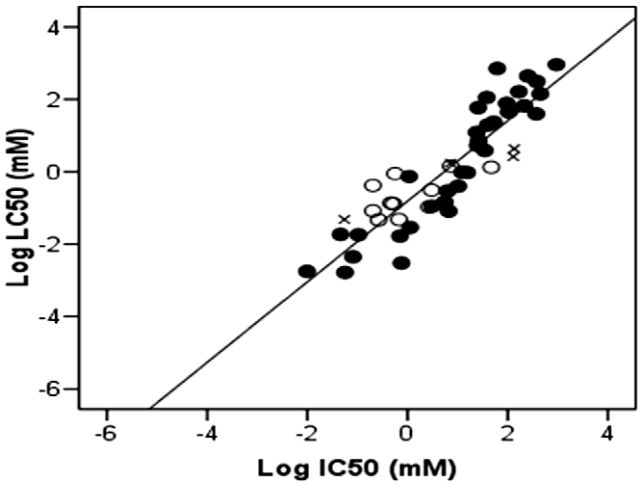

Fig. 2. Scatter plot of log IC_{50} against log LC_{50} for acute toxicity to fathead minnow of all 49 narcotic compounds. Points are separated by their type of narcosis: non-polar (●), polar (○) and unknown types of narcosis (×; according to Russom et al. (1997)). The dotted line represents the $y = x$ line and the black line is the regression line drawn through all data points.

of less toxic compounds remain better predicted than more toxic compounds. Like in Fig. 1, the slope is still 1.11 (±0.08) and regression line is below the 1:1 line.

In Fig. 2, narcotic compounds were further divided into non-polar narcotics, associated with lethargy, and polar narcotics, associated with hyperactivity in Fish Acute Toxicity Syndrome (FATS) studies (Netzeva et al., 2008). Yet, no discernible differences were observed in the prediction of LC_{50}s by cytotoxicity assays when separating polar and non-polar narcotics. It should be noted, however, that the number of polar narcotics in this study is few, making comparisons between narcosis types difficult. Therefore, it is difficult to verify whether non-polar and polar narcosis are two different mechanisms and whether cell assays mimic one type of narcosis better than another (Vaes et al., 1998; Escher and Hermens, 2002; Roberts and Costello, 2003).

Scatter plots of log K_{OW} against log LC_{50} and log IC_{50} of all narcotic compounds in Fig. 3 reveal significant negative linear relationships ($n = 49$, R^2 0.89 and 0.68, $F = 372.62$ and 98.20, respectively, $p < 0.05$):

$$\log LC_{50}\ (\text{mM}) = -0.94\ (\pm 0.05)\log K_{OW} + 1.57\ (\pm 0.11). \quad (3)$$

$$\log IC_{50}\ (\text{mM}) = -0.66\ (\pm 0.07)\log K_{OW} + 1.85\ (\pm 0.15). \quad (4)$$

The regression coefficients of Eq. (3) fall within the same range as linear QSAR models using log K_{OW} to predict acute guppy and fathead minnow toxicity for narcotic compounds (Könemann, 1981; Veith et al., 1983). The linear relationship between log K_{OW} and cytotoxicity (Eq. (4)), however, is weaker than for acute fish toxicity.

To quantify the correlation between a chemical's log K_{OW} and the underestimation of fish acute toxicity by *in vitro* assays, the difference between log LC_{50} and log IC_{50} was calculated for narcotic compounds and plotted as a function of log K_{OW} (Fig. 4). Only narcosis compounds have been used to derive this correlation in order to separate the effect of MOA from physicochemical properties possibly related to the bioavailability of a chemical, such as log K_{OW}. A significant linear relationship was found ($R^2 = 0.38$, $n = 49$, $F = 28.81$, $p < 0.05$):

$$\log LC_{50} - \log IC_{50}\ (\text{mM}) = -0.26\ (\pm 0.05)\log K_{OW} - 0.30\ (\pm 0.11). \quad (5)$$

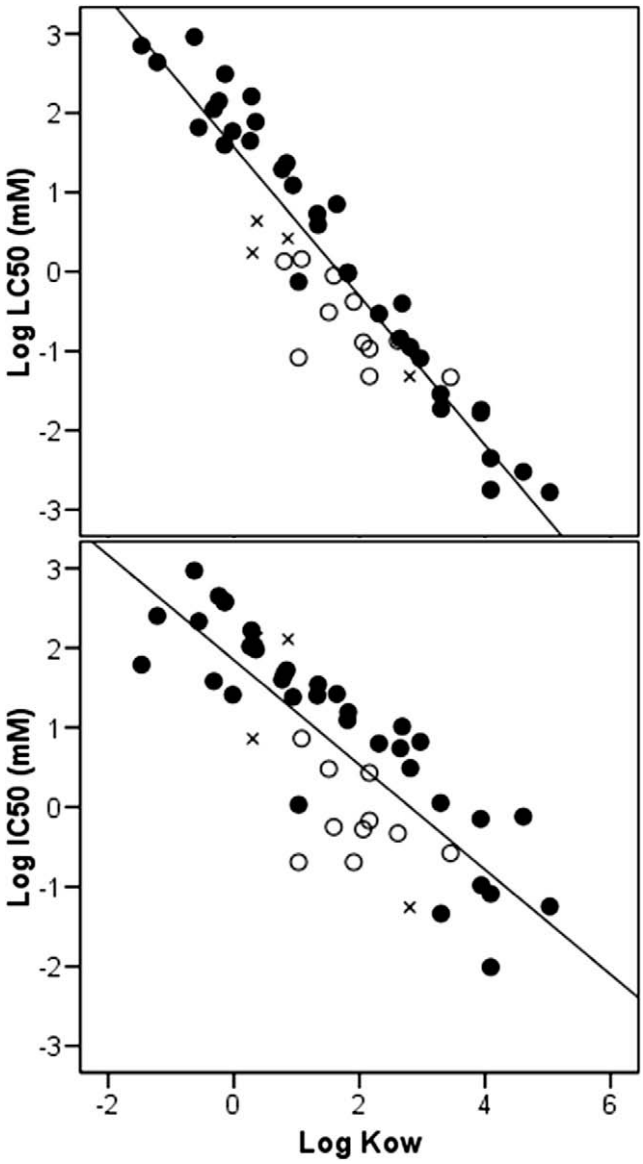

Fig. 3. Scatter plots of log K_{OW} against log LC_{50} (A) and log IC_{50} (B) of narcotic compounds. Points are separated by their type of narcosis: non-polar (●), polar (○) and unknown types of narcosis (×; according to Russom et al. (1997)). The black line is the regression line drawn through all data points.

This relationship explains the observation that the LC_{50} of compounds with a low toxicity is better predicted *in vitro* than the highly toxic compounds (Fig. 2). Likewise it can explain why a weaker correlation and slope is found between log K_{OW} and log IC_{50} than between log K_{OW} and log LC_{50} (Fig. 3). Highly toxic narcotics tend to have a high K_{OW}, and high K_{OW} compounds are less well-predicted *in vitro* (Fig. 3).

The difference between log IC_{50} and LC_{50} is also greater the higher the log H (i.e., volatility) of the compound is (Fig. 5). This is illustrated by the significantly linear relationships found between the difference in log LC_{50} and log IC_{50} and log H of narcotic chemicals ($n = 49$, $R^2 = 0.25$, $F = 15.82$, $p < 0.05$):

$$\log LC_{50} - \log IC_{50}\ (\text{mM}) = -0.17\ (\pm 0.04)\log H\ (\text{atm m}^3/\text{mol}) - 1.70\ (\pm 0.26). \quad (6)$$

The effect of log H is considered separate from the effect of log K_{OW} because log H and log K_{OW} are not correlated with one another ($r = 0.07$, $n = 49$, $F = 0.25$, $p = 0.62$, see Supplementary data for figure).

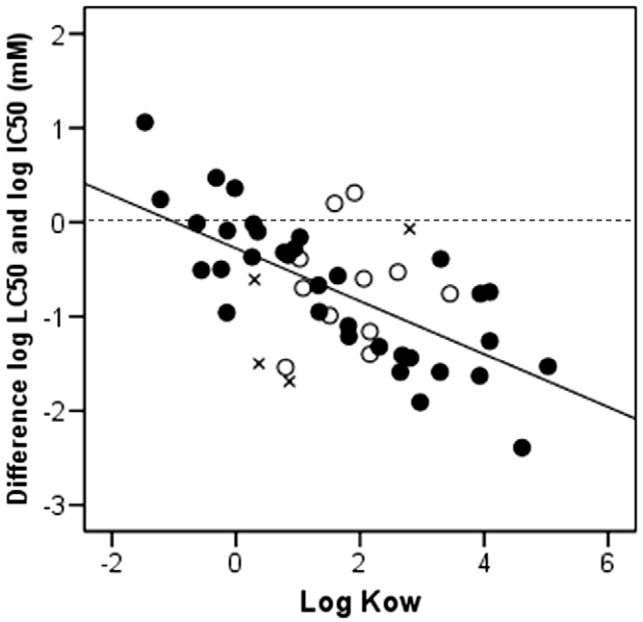

Fig. 4. Scatter plot of log K_{OW} against the difference between log LC_{50} and log IC_{50} (i.e., log LC_{50} − log IC_{50}) for narcotic compounds. Points are separated by their type of narcosis: non-polar (●), polar (○) and unknown types of narcosis (×; according to Russom et al. (1997)). There is no difference between *in vitro* and *in vivo* derived toxicity values for chemicals on the dotted line. For all points under this line, *in vitro* assays underpredicted acute toxicity to fathead minnow. The regression between log K_{OW} and the difference between LC_{50} and IC_{50} is represented by the black line.

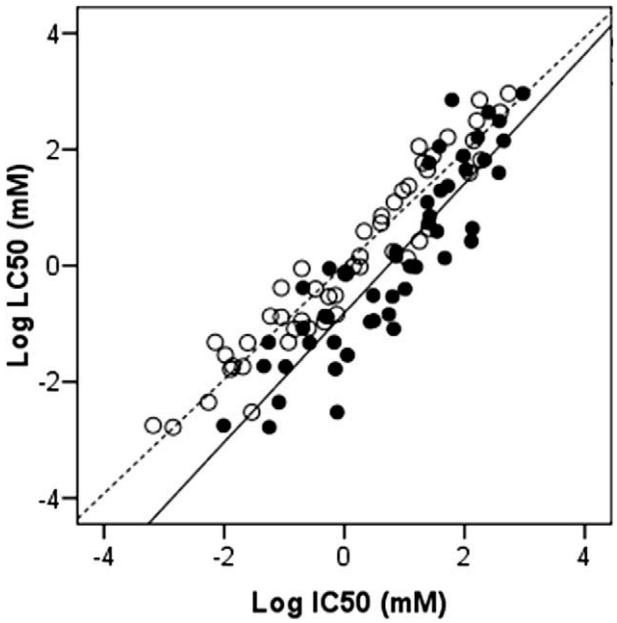

Fig. 6. Scatter plot of log IC_{50} against log LC_{50} for acute toxicity to fathead minnow of all 49 narcotic compounds. Original IC_{50}s are indicated by (●). Corrected IC_{50}s, corrected by subtracting the predicted difference between LC_{50} and IC_{50}, based on the chemical's log K_{OW} and log H, from the original IC_{50}, are indicated by (○). The thick dotted line represents the $y = x$ line, the black line below the $y = x$ line is the regression line drawn through the original IC_{50} and LC_{50}, and the thin dotted line on the $y = x$ line is the regression line drawn through the corrected IC_{50} and LC_{50}.

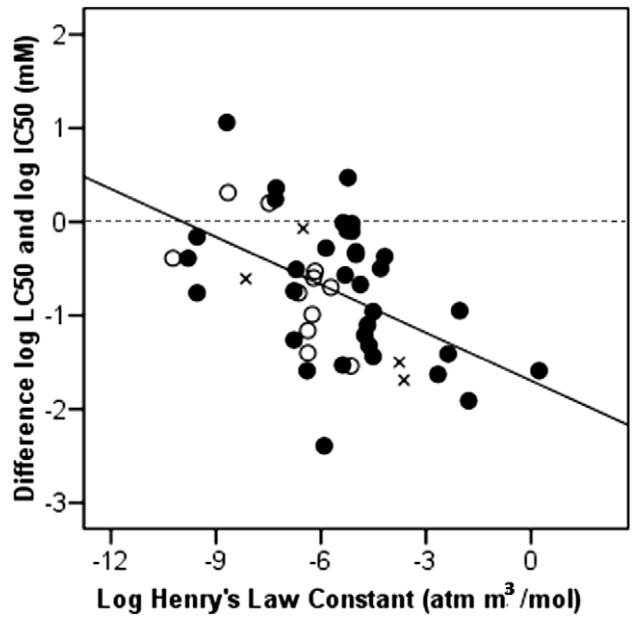

Fig. 5. Scatter plot of log H against the difference between log LC_{50} and log IC_{50} (i.e., log LC_{50} − log IC_{50}) for narcotic compounds. Points are separated by their type of narcosis: non-polar (●), polar (○) and unknown types of narcosis (×; according to Russom et al. (1997)). There is no difference between *in vitro* and *in vivo* derived toxicity values for chemicals on the dotted line. For all points under this line, *in vitro* assays underpredicted acute toxicity to fathead minnow. The regression between log H and the difference between LC_{50} and IC_{50} is represented by the black line.

The correlation between log K_{OW}, log H and the difference between log LC_{50} and log IC_{50} can be used to predict when a narcotic compound's IC_{50} is significantly higher than its LC_{50} and to subsequently correct for this underestimation. To exemplify this point, log K_{OW} and log H were used in a stepwise multiple regression analysis to predict the difference between log IC_{50} and log LC_{50} of narcotic compounds. The addition of each physicochemical property significantly increased the prediction power of the regression (R^2 increased from 0.38 to 0.58, $F_{change} = 21.58$, $n = 49$ and $p_{change} < 0.05$ when log H was added in addition to log K_{OW} in the regression model). The predicted difference between log IC_{50} and log LC_{50} of the model,

$$\begin{aligned}\log LC_{50} - \log IC_{50}\ (\mathrm{mM}) = &-0.24\ (\pm 0.04)\log K_{OW} \\ &- 0.15\ (\pm 0.03)\log H\ (\mathrm{atm\ m^3/mol}) \\ &- 1.20\ (\pm 0.22),\end{aligned} \tag{7}$$

was subsequently used to correct log IC_{50}. Fig. 6 illustrates how the difference in sensitivity between log IC_{50} and log LC_{50} disappears when IC_{50}s are corrected and the correlation further improves to an R^2 of 0.91 ($n = 49$, $F = 16496.57$, $p < 0.05$):

$$\begin{aligned}\log LC_{50}\ (\mathrm{mM}) = &\ 0.96\ (\pm 0.43)\ \text{'corrected'}\ \log IC_{50}\ (\mathrm{mM}) \\ &+ 0.00\ (\pm 0.07).\end{aligned} \tag{8}$$

4. Discussion

The goal of this study was to explore the hypotheses that MOA and physicochemical properties have significant influence on the correlation between *in vitro* and *in vivo* acute fish toxicity data. Chemicals were selected based on their presence in both the Halle Registry of Cytotoxicity and the US EPA Fathead Minnow Database, yielding 82 chemicals with a wide range of toxicities and physicochemical features.

A good relative agreement between the *in vitro* and *in vivo* acute fish toxicity was found. Yet, the *in vitro* data were less sensitive in absolute terms by an order of magnitude. These results compare well with correlations found in experimental literature for fish

acute toxicity summarized most recently in Schirmer (2006). The high correlation is remarkable in that a large variety of organic compounds are grouped together and the IC_{50}s from the Halle Registry of Cytotoxicity are not based on a standard protocol. Numerous different mammalian cell lines, basal cytotoxicity endpoints and laboratories are represented in the Halle Registry of Cytotoxicity. By nature, this data will be subject to considerable variation and consequently be of poorer statistical fit than would be expected with data obtained more uniformly (Cronin and Schulz, 2003; Coecke et al., 2005).

This study also supports literature findings that *in vitro* assays are considerably less sensitive than the fish bioassay (Babich and Borenfreund, 1987; Saito et al., 1991; Lilius et al., 1995; Castaño et al., 1996; Gülden and Seibert, 2005) (Fig. 1). This study shows that MOA significantly explains the relative sensitivity difference between *in vitro* and *in vivo* toxicity data (i.e., the general variation). When separating compounds by their MOA, the outliers to the log IC_{50}/log LC_{50} regression line are all compounds with specific modes of toxicity that are not mimicked in basal cytotoxicity assays. Permethrin, an outlier by three standard deviations, is used as a synthetic pyrethroid insecticide and is a strong neurotoxicant to fish (Rice et al., 1997; Ray, 2004). This mode of action is not mimicked by basal cytotoxicity assays, which would explain why the IC_{50} for permethrin is much higher than its LC_{50}. The toxicity of allyl alcohol is also significantly underpredicted by *in vitro* assays (two standard deviations from the regression line). Allyl alcohol is a hepatoxin (Atzori et al., 1989; Auerbach et al., 2008). Its bioactivation to the more toxic acrolein is not mimicked in basal cytotoxicity assays as these cells generally have poor metabolic capabilities (Halle, 2003). Similar reasoning applies to the outliers, hexamethylenetetramine and 2-aminoethanol. They are metabolized *in vivo* to less toxic compounds (JECFA, 1974; Klain et al., 1985).

With regard to MOA, this study therefore supports the notion that for *in vitro* assays to replace the fish acute toxicity assay, a battery of *in vitro* assays is required to mimic the various modes of action observed in fish (Gülden and Seibert, 2005; Schirmer, 2006; Knauer et al., 2007). Indeed, basal cytotoxic concentrations are certainly acutely toxic, but acute toxicity may occur at lower than basal cytotoxic concentrations. Cytotoxic potency can only indicate the minimal acute toxic potency of a chemical. A better correspondence between *in vitro* and *in vivo* data can only be achieved if basal cytotoxicity testing is combined with *in vitro* tests which cover metabolism-mediated and cell type-specific cytotoxicity and impairment of cell-specific functions. Hypothetically, when using a battery of *in vitro* tests, it could be possible to identify modes and mechanisms of toxicity and thus determine the appropriate IC_{50} value (i.e., that of the most appropriate or sensitive cell type and toxicity endpoint) to use in an *in vitro–in vivo* acute toxicity extrapolation. Two examples of such MOA based test batteries are those developed by Costa et al. (2007) and Escher et al. (2005). They are not specifically designed to mimic acute fish toxicity, however. To develop *in vitro* test batteries specifically for acute fish toxicity, (Q)SARs and computer based expert systems, as used in this study, can form the basis for their design. They can categorize industrial organic chemicals according to their likely MOA. An *in vitro* test battery using non-metabolizing cells, cells with active phase I enzymes and cells with phases I and II enzymes may likewise shed light into possible metabolic pathways and the toxicity of metabolites of test chemicals (Mingoia et al., 2007). When allyl alcohol, for example, is tested in both a metabolically competent and incompetent cell line for basal cytotoxicity, higher toxicity is likely observed in the former. This would indicate that at least one of the metabolites of the chemical is more acutely toxic than the parent compound and more comparable to the effect observed in the metabolically competent fish.

MOA does not explain the absolute sensitivity difference observed between *in vitro* and *in vivo* toxicity data in this study. When considering only narcotic compounds, whose toxicity should be mimicked *in vitro*, the absolute sensitivity of *in vitro* assays remains unchanged, i.e., lower than the fish bioassay (Fig. 2). This underestimation is greater for hydrophobic (high log K_{OW}) and volatile (high log H) compounds, revealing that the absolute sensitivity of *in vitro* assays is significantly influenced by the chemical's physicochemical properties. The relationship between these physicochemical properties and the difference between IC_{50} and LC_{50} can be explained by a difference in free, unbound concentrations of test compounds related to K_{OW} and H. Only the free concentration is considered to exert a response in cells and organisms (Herve et al., 1994; Seydel and Schaper, 1981). A number of recent studies have found that chemicals can significantly bind to extracellular matrices *in vitro* such as serum protein and well plate plastic and subsequently reduce the free concentration (Schirmer et al., 1997; Gellert and Stommel, 1999; Hestermann et al., 2000; Gülden et al., 2002; Seibert et al., 2002; Heringa et al., 2004). Studies have also found that the extent of this binding is positively related to the log K_{OW} of compounds (deBruyn and Gobas, 2007). In turn, these reductions in free concentrations due to increased non-specific binding have been shown to significantly increase the IC_{50} *in vitro* (Hestermann et al., 2000; Gülden and Seibert, 2005; Gülden et al., 2002; Seibert et al., 2002; Heringa et al., 2004). Binding to extracellular protein and aquarium walls is considered less extensive in the fish bioassay as it is based on flow-through exposures with analytically determined concentrations in water free of extracellular protein (OECD, 1992). Thus, greater non-specific binding *in vitro* compared to the *in vivo* test would explain the sensitivity difference between *in vitro* and *in vivo* toxicity of hydrophobic substances (high K_{OW}).

Analogously, studies by Thellen et al. (1989), Halling-Sorensen et al. (1996), Mayer et al. (2000), and Riedl and Altenburger (2007) have found effect concentrations for volatile substances to be higher in algal test systems conducted in open flasks than with sealed airtight flasks. The problem of test compound evaporation may be aggravated in not-airtight, static microtitre plates, commonly used in cell assays, as the surface area to volume ratio is high and the evaporated substances may not only dissipate but also evoke effects in adjacent wells (Eisentraeger et al., 2003). Evaporation is better accounted for in the *in vivo* fish test due to the flow-through exposure and analytically determined concentrations. Therefore, a difference in the evaporation of test compounds possibly explains the sensitivity difference between *in vitro* and *in vivo* toxicity of volatile substances (high H).

This study suggests that log K_{OW} and log H can be used to estimate when the free concentration is significantly different from the nominal concentration *in vitro*. Estimated free concentrations can in turn be used to calculate IC_{50}s more comparable to LC_{50}s calculated in acute fish assays, as exemplified by Eqs. (7) and (8). It should be noted that Eq. (7) is not directly mechanistically based. It is meant to illustrate the significance of physicochemical properties in improving the correlations. It can therefore serve as a basis for future mechanistically and experimentally based *in vitro* exposure models. More mechanistically based models in literature help support Eq. (7), at least qualitatively. For example, Gülden and Seibert (2003) proposed an equilibrium partition model to estimate free concentrations of organic chemicals *in vitro* based on log K_{OW} and serum protein binding parameters, which they derived experimentally. Using this algorithm, the authors were able to show that the extrapolation of *in vitro* Balb/c 3T3 cytotoxicity data correlated significantly better both in relative and absolute terms with fathead minnow acute toxicity of pesticides (Gülden and Seibert, 2005). This model, however, does not take into account evaporation or sorption to plastic of test compounds, which may be partic-

ularly significant when serum-free cell assays are used (Schirmer et al., 1997). Riedl and Altenburger (2007) developed a model to estimate the ratio between EC_{50}s from algal toxicity assays using microtiter plates and airtight glass containers. They used log K_{OW} to account for plastic sorption and log K_{AW} to account for evaporation. These models can serve as a basis for generating larger, more general models estimating the free concentration *in vitro*, such that sorption and evaporation can be accounted for *in vitro* and concentrations can be extrapolated to the fish acute toxicity assay. Indeed, these models, including the one proposed in this study, is currently used to design a mechanistic and systematic approach to extrapolate *in vitro* to *in vivo* concentrations of 60 chemicals selected on their log K_{OW} and log H (Schirmer et al., 2008).

Techniques currently used in pharmacology to measure directly or indirectly (using protein or plastic binding affinities) free concentrations can facilitate the development of *in vitro–in vivo* free concentration extrapolation models (Heringa et al., 2004; Seibert et al., 2002; Oravcová et al., 1996). Moreover, a number of *in vitro* techniques have been developed to minimize loss of compound through sorption or evaporation. These include the use of cell systems free of serum, the use of plate sealers, the use of cell suspension cultures in culture plates of non-binding material, and the use of solvent-free/continuous dosing techniques (Ackermann and Fent, 1998; Mori and Wakabayashi, 2000; Brown et al., 2001; Mori et al., 2002; Gerofke et al., 2004; Bopp et al., 2006; Schreiber et al., 2008).

In summary, these results support the hypotheses that the bioavailability of hydrophobic and volatile chemicals is lower in *in vitro* assays than in the fish test set-up and that a single *in vitro* assay with cell death as an endpoint fails to mimic all modes of toxicity possible *in vivo*. The sensitivity of *in vitro* toxicity tests to predict the *in vivo* situation may be improved if QSARs and/or a battery of *in vitro* assays testing for variety of endpoints are used to classify compounds according to their mode of action. Moreover, the free concentration is possibly a better measure of exposure in *in vitro* assays and may be modelled using a compound's K_{OW} and H.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2009.07.029. This includes the list of 82 chemicals used in this study and the scatter plot relating log K_{OW} with log H.

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