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QSAR Models for Predicting in Vivo Aquatic Toxicity of Chlorinated Alkanes to Fish

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Quantitative structure–activity relationship (QSAR) models are expected to play a crucial role in reducing the number of animals to be used for toxicity testing resulting from the adoption of the new European Union chemical control system called Registration, Evaluation, and Authorization of Chemicals (REACH). The objective of the present study was to generate in vitro acute toxicity data that could be used to develop a QSAR model to describe acute in vivo toxicity of chlorinated alkanes. Cytotoxicity of a series of chlorinated alkanes to Chinese hamster ovary (CHO) cells was observed at concentrations similar to those that have been shown previously to be toxic to fish. Strong correlations exist between the acute in vitro toxicity of the chlorinated alkanes and (i) hydrophobicity [modeled by the calculated log K_{ow} (octanol–water partition coefficient); $r^2 = 0.883$ and $r_{int}^2 = 0.854$] and (ii) in vivo acute toxicity to fish ($r^2 = 0.758$). A QSAR model has been developed to predict in vivo acute toxicity to fish, based on the in vitro data and even on in silico log K_{ow} data only. The developed QSAR model is applicable to chlorinated alkanes with up to 10 carbon atoms, up to eight chlorine atoms, and log K_{ow} values lying within the range from 1.71 to 5.70. Out of the 100204 compounds on the European Inventory of Existing Chemicals (EINECS), our QSAR model covers 77 (0.1%) of them. Our findings demonstrate that in vitro experiments and even in silico calculations can replace animal experiments in the prediction of the acute toxicity of chlorinated alkanes.

Introduction

The European Inventory of Existing Chemicals (EINECS)[1] contains over 100000 chemicals that are marketed within Europe for industrial or consumer needs. For most of these chemicals, there is insufficient (eco)toxicological information on their hazardous properties. To close these existing toxicity data gaps, the European Union parliament recently adopted a new chemical control system called Registration, Evaluation and Authorization of Chemicals (REACH) (*1*). One of the aims of REACH is to improve the protection of human health and the environment by requiring industry to provide toxicity information for the chemicals that they manufacture or distribute. There is currently an ongoing debate about the potentially large number of animals that have to be used for experimental toxicity testing as an outcome of REACH. Within REACH, however, there is a provision to use, among others, sufficiently validated computational prediction models based on quantitative structure–activity relationships (QSAR) to fill in the toxicity data gaps and thus save time and costs, reducing the number of experimental animals used. To increase the acceptability of QSAR models within REACH, guidelines for QSAR model development and validation proposed by the Organization for Economic Cooperation and Development (OECD) (*2*) are now widely accepted.

Chlorinated alkanes are an important group of chemicals on the EINECS list with widespread use, large production volumes, and thus a large potential for environmental pollution, and they are the focus of this article. Chlorinated *n*-alkanes are built from straight chains of carbon and hydrogen with varying numbers of hydrogen atoms replaced by chlorine atoms. The introduction of chlorine atoms into the hydrocarbon chain alters properties such as solubility, density, volatility, and toxicity (*3*). Some of these changes confer improvements that make the compounds useful commercially, but these changes can also make them more toxic. Chlorinated *n*-alkanes are broadly divided into two main groups depending on the number of carbon atoms present: lower chlorinated alkanes (LCA; C1–C9) and polychlorinated *n*-alkanes (PCA; C10–C30) (*4*). Mixtures of commercial PCAs, known as chlorinated paraffins, are divided into three groups: short-chain (C10–C13), medium-chain (C14–C17), and long-chain (C18–C30) paraffins with chlorine contents varying from 35 −70% by weight. The LCAs are widely used as industrial and household solvents, fumigants, and intermediates in chemi-

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[1] Abbreviations: CHO, Chinese hamster ovary, DMSO, dimethyl sulfoxide;, DMEM, Dulbecco modified Eagle's medium; EINECS, European inventory of existing chemicals; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; K_{ow}, octanol–water partition coefficient; LCA, lower chlorinated alkane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OECD, Organization for Economic Cooperation and Development; PBS, phosphate-buffered saline; PCA, polychlorinated alkane; PRESS, predicted sum of squares; QSAR, quantitative structure–activity relationship; REACH, Registration, Evaluation and Authorization of Chemicals; SMILES, simplified molecular input line entry system; SSD, sum of squared deviations.

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cal synthesis (*5*). The PCAs are often used as lubricating additives, adhesives, and flame retardants in rubber and textiles. The annual production volume of PCAs is greater than 300 kilotons (*4*). For the PCAs, toxicity is believed to decrease from the short to the long chain PCAs, due to a decrease in solubility (*4*). It has been suggested that the short-chain PCAs should be included in the list of persistent organic pollutants (*6*). They have a high bioaccumulation potential due to their high log K_{ow} (octanol–water partition coefficient) values, are persistent in the environment due to their resistance to degradation, and thus have a potential for long-range environmental transport. Short-chain PCAs are known to be highly toxic to aquatic organisms (*6*).

Despite their widespread use and presence, the amount of toxicological data on PCAs is rather limited. Because they are produced via free radical chlorination, a single PCA preparation can consist of many different congeners with a wide range of physicochemical properties (*4*). This presents problems in attempting to estimate the toxicity of these preparations as the toxicity of individual compounds cannot be identified. Using synthesized PCA congeners, Fisk and co-workers described their bioaccumulation in rainbow trout (*Onchorhynchus mykiss*) (*7*) and toxicity to Japanese medaka (*Oryzias latipes*) embryos (*4*).

For the LCAs, several studies describe their acute toxicity in literature. Crebelli and co-workers used electrophilicity descriptors to describe their aneugenic activity to the mold *Aspergillus nidulans* (*8*). The acute toxicity of LCAs to the protozoan *Tetrahymena pyriformis* (*9*), the marine bacterium *Photobacterium phosphoreum* in the Microtox test (*10*), the fathead minnow *Pimephales promelas* (*11*), the guppy *Poecilia reticulata* (*12*), and HeLa cells (*13*) was determined and related to the hydrophobicity of the compounds. However, in most of these studies, the number of chlorinated alkanes tested was either too small to be used for QSAR modeling or was for a small range of carbon chain lengths [e.g., C1 to C5 as in studies by Könemann (*12*)]. As far as we are aware, there exists no QSAR model to predict the in vivo acute toxicity of chlorinated alkanes. The objective of the present study was to generate in vitro acute toxicity data that could be used to develop a QSAR model to describe in vivo acute toxicity of chlorinated alkanes. Toxicity tests were performed for a large set of chlorinated alkanes across a wide range of hydrophobicity values and carbon chain lengths (C1–C10). The in vitro toxicity data were used to develop a validated QSAR model with defined applicability limits following OECD guidelines. The in vitro toxicity data were further compared to in vivo toxicity data for fish, and a prediction model for in vivo toxicity was developed using the in vitro data. Finally, an estimate was made of the number of EINECS compounds for which the QSAR model can make accurate predictions.

Materials and Methods

Materials. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and were at least 98% pure. Stocks of chlorinated alkanes were prepared in spectrophotometric grade dimethyl sulfoxide (DMSO) obtained from Acros Organics (Geel, Belgium). Phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), Dulbecco modified Eagle's medium (DMEM)/Ham's F12, fetal calf serum (FCS), and Trypsin-EDTA were supplied by Gibco-Invitrogen (Breda, The Netherlands). Chinese hamster ovary (CHO) wild-type cells were sourced from the American Type Culture Collection (Manassas, VA). Cell culture flasks (75 cm^2) were supplied by Corning Inc. (Corning, NY), and culture plates (24 and 96 wells) were provided by Greiner Bio-one (Alphen aan de Rijn, The Netherlands).

Cell Culture. The CHO cells were grown in 75 cm^2 culture flasks and maintained in a humidified incubator at 37 °C, 95% air/5% CO_2 in DMEM/F12 medium supplemented with 10% FCS. Once every three days, the cells were rinsed with HBSS, trypsinized, and then resuspended and cultivated in fresh culture medium. Cells from culture flasks with confluency of at least 90% were used for the cytotoxicity assay.

Cytotoxicity Assay. The cytotoxicity of the chlorinated alkanes to CHO cells was determined in triplicate in 96 well culture plates using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay following procedures described previously (*14*) with some modifications. Where appropriate, dilution of the test compounds was done in 24 well culture plates. Two methods of exposure were compared, direct and premix (the most commonly used exposure method during in vitro testing). For direct exposure, there was no prior dilution of the test compound in the dilution plate; the test compound solution in DMSO was added straight into the medium of the culture plate. For premix exposure, there was prior dilution of the test compound solution in DMSO using culture medium, and this culture medium containing the test compound at the desired concentration was added to the cells in the culture plate. For both methods, 100 μL of CHO cell suspension was seeded into each of the inner wells of the culture plate at a final concentration of 3×10^5 cells/mL, with 200 μL of HBSS added to the outer wells. The plate was incubated for 24 h to allow the cells to attach. For the direct exposure, 100 μL of culture medium with 10% FCS (37 °C) was then added to the inner wells followed by addition of 2 μL of each test compound solution at various concentrations. For the premix exposure, 50 times concentrated solutions of the test compounds in DMSO were diluted 50 times in culture medium with 10% FCS in the dilution plate, and then, 100 μL of these medium samples was transferred to the culture plate. For both methods in each independent experiment, (i) all concentrations were tested in 6-fold and (ii) two sets of controls were used, one with DMSO and another with culture medium without the test compounds or DMSO. Upon addition of the test compounds, the plates were shaken at 600 rpm on an orbital shaker (Incubator 1000, Hieroglyph Instruments, Germany) for 5 min, followed by 21 h of incubation in the humidified incubator at 37 °C. Then the MTT reagent was added to a final concentration of 0.5 mg/mL, and incubation continued for a further 3 h, for a total incubation time of 24 h. After this, the culture medium was removed with a vacuum pump and 100 μL of DMSO was added to lyse the cells. The plates were shaken for 5 min to dissolve the formazan crystals formed after reduction of MTT. Subsequently, two absorbance readings were recorded as follows: A_{562} for the color of the formazan crystals and A_{620} for cell debris and other nonspecific absorbance.

Calculation of EC_{50} Values. The A_{620} values were subtracted from the A_{562} values, and the result was expressed as a percentage of the response of the DMSO control. The EC_{50} values of the chlorinated alkanes were calculated using a Microsoft Excel plug-in, Life Sciences Workbench (LSW) Data Analysis Toolbox Version 1.1.1 (MDL Information Systems, CA) with the general sigmoidal curve with Hill slope ($a-d$) chosen as the best fit model.

Calculation of Theoretical Descriptors. Hydrophobicity of the chlorinated alkanes was modeled using log K_{ow}values calculated using the software CLogP version 4.0 (Biobyte, Claremont, CA) (*15*) as described previously (*16*). Briefly, the structure of each molecule was entered into CLogP as a simplified molecular input line entry system (SMILES) code. The SMILES codes were obtained from the SMILES-CAS database (Syracuse Research, Syracuse, NY). Solubility values were calculated using ACD/Laboratories version 8.14 for Solaris (Advanced Chemistry Development, Toronto, ON, Canada).

QSAR Modeling. The Statistical Package for Social Scientists (SPSS) version 13 for Windows (SPSS, Chicago, IL) was used to analyze the QSARs with log EC_{50} as the dependent variable and log K_{ow} as the independent variable. The quality of the QSAR model was characterized by the number of compounds used in the study (n), coefficient of determination (r^2), standard error of the estimate (s), variance ratio (F), the internally cross-validated coefficient of determination (r_{int}^2), and the externally validated

Table 1. Chlorinated Alkanes Present in the Training Set of the Present Study, Their Chemical Abstract Service (CAS) Numbers, Octanol/Water Partitioning Coefficients (K_{ow}), Water Solubility, and in Vitro and in Vivo EC_{50} Values[a]

					in vitro log EC_{50} (μM) MTT assay			in vivo log EC_{50} (μM)		
no.	compd name	CAS no.	log K_{ow}[b]	log H_2O solubility[c] at 25 °C (μM)	CHO cells,[d] 24 h	rat primary hepatocytes,[e] 2 h	HeLa cells,[f] 72 h	*Poecilia reticulata*,[g] 7 days	*Phosphobacterium phosphoreum*,[h] 5 min	*Pimephales promelas*,[i] 96 h
1	1,3-dichloropropane	142-28-9	1.71	4.11	2.99			2.87	3.02	3.06
2	1,2-dichloropropane	78-87-5	1.99	4.46	3.03			3.01		3.09
3	1,1,2-trichloroethane	79-00-5	2.05	4.42	3.04	3.40		2.85		2.79
4	1,2-dichlorobutane	616-21-7	2.52	3.87	2.61			2.39[k]		
5	1,1,2,2-tetrachloroethane	79-34-5	2.64	3.85	2.44	3.24		2.34		2.09
6	1-chloro-2, 2-dimethylpropane	753-89-9	2.79	3.76	2.30			2.11[k]		
7	carbon tetrachloride	56-23-5	2.88	3.72	2.40	2.89	1.00	2.20[k]		
8	1-chloro-2-methylbutane	616-13-7	2.92	3.54	2.30			2.11[k]		
9	1-chloropentane	543-59-9	3.05	3.34	2.24			2.05[k]	2.55	
10	1,6-dichlorohexane	2163-00-0	3.29	2.56	2.02			1.85[k]		
11	1-chlorohexane	544-10-5	3.58	2.76	2.24			2.05[k]		
12	1,1-dichloro-3, 3-dimethylbutane	6130-96-7	3.63	2.94	1.67			1.52[k]		
13	1-chloroheptane	629-06-1	4.11	2.18	1.73			1.58[k]		
14	1,8-dichlorooctane	2162-99-4	4.35	1.48	1.72			1.57[k]		
15	1-chlorooctane	111-85-3	4.64	1.61	1.43			1.30[k]		
16	1,9-dichlorononane	821-99-8	4.88	0.94	1.78			1.63[k]		
17	1-chlorononane	2473-01-0	5.17	1.04	1.35			1.23[k]		
18	1-chlorodecane	1002-69-3	5.70	0.52	1.44			1.31[k]		
19	1,10-dichlorodecane	2162-98-3	5.41	0.88	NTAS[j]					
20	1-chlorododecane	112-52-7	6.76	0.18	NTAS					
21	1-chlorotetradecane	2425-54-9	7.81	1.38	NTAS					

[a] The EC_{50} values in the present study were determined using an MTT test in CHO cells. [b] Calculated using ClogP version 4.0. [c] Calculated using ACD/Laboratories version 8.14 for Solaris. [d] Experimental toxicity data from the present study. [e] Experimental toxicity data from ref *26*. [f] Experimental toxicity data from ref *27*. [g] Experimental toxicity data from ref *12*. [h] Experimental toxicity data from ref *10*. [i] Experimental toxicity data from ref *11*. [j] Not toxic at saturation. [k] EC_{50} values predicted using eq 3.

coefficient of determination (r_{ext}^2). Internal validation of the QSAR model was performed using the leave-out-many cross-validation method, with 20% of the calibration compounds left out at each step as described previously (*16*). The external performance of the QSAR model was evaluated by testing five additional compounds that fit into the applicability domain of the model and then comparing the predicted and experimental toxicity values. The calculation of r_{ext}^2 was performed according to the formula:

$$r_{ext}^2 = 1 - \text{PRESS/SSD} \quad (1)$$

where PRESS (predicted sum of squares) is the sum of the squared differences between the predicted and the experimental toxicity values for each molecule in the validation set, and SSD is the sum of the squared deviations between the experimental toxicity values for each molecule in the validation set and the mean experimental toxicity values of the training set (*17*).

Results

Twenty-one LCAs were tested in the MTT assay with CHO cells, and these model compounds of the present study are listed in Table 1, together with their estimated log K_{ow}, water solubility, and the experimental EC_{50} values obtained. The MTT cytotoxicity results were obtained using the direct exposure method. When comparing the cytotoxicity of the same concentration of LCAs after premix and direct exposure of CHO cells, major differences were found. After direct exposure to increasing concentrations of 1-chlorononane, for example, a clear dose-dependent decrease in cell viability was observed (Figure 1). After premix exposure, however, none of the tested concentrations induced any cytotoxicity. Microscopic examination of the 96 well plate 10 min after exposure revealed no local cytotoxicity due to a possible temporary high concentration of DMSO when the direct exposure method was used. Because of the lack of a dose−response relationship with the premix method, further experiments were conducted using the direct exposure method to determine the EC_{50} values reported in Table 1.

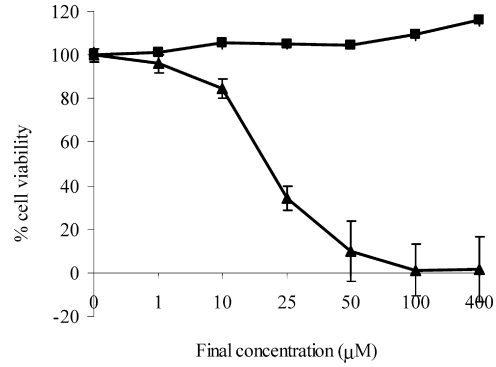

Figure 1. Comparison of the cytotoxicity of 1-chlorononane to CHO cells using direct (▲) and premix (■) methods of exposure in the MTT assay. The points on the graph represent the means ± standard errors of three experiments.

Influence of Chain Length and Extent of Chlorination on Toxicity. With an increase in chain length for single chlorinated compounds from 1-chloropentane, 1-chlorohexane, 1- chloroheptane, and 1-chlorooctane to 1-chlorononane, the cytotoxicity increased, with 1-chlorodecane showing a deviation (Table 1). For compounds with the same hydrocarbon backbone, an additional chlorine atom was associated with a decrease in toxicity. This is shown for example by 1,6-dichlorohexane vs 1-chlorohexane and 1,8-dichlorooctane vs 1-chlorooctane. The short-chain PCAs 1,10-dichlorodecane, 1-chlorododecane, and 1-chlorotetradecane did not show cytotoxicity up to the maximum soluble concentrations tested; therefore, they were excluded from the modeling process.

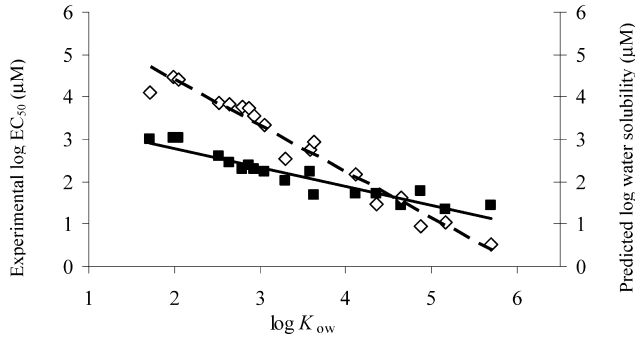

Figure 2. QSAR for the correlation between the decadic logarithm of the 24 h in vitro toxicity (EC_{50}) of chlorinated alkanes (C1–C10) to CHO cells in the MTT assay and the toxicity predicted based on log K_{ow} (■) shows a plot of log K_{ow} vs water solubility of the chlorinated alkanes ($n = 18$, $r^2 = 0.96$). The two plots cross where log $K_{ow} = 4.53$.

QSAR Modeling. An investigation of the relationship between log K_{ow} and the experimental toxicity data from the MTT test revealed a good correlation (Figure 2) and can be described by the following equation:

$$\log EC_{50}\ (\mu M) = -0.446(\log K_{ow}) + 3.685 \qquad (2)$$

where $n = 18$, $s = 0.193$, $F = 120$, $r^2 = 0.883$, and $r_{int}^2 = 0.854$.

An increase in log K_{ow} was associated with an increase in toxicity (Figure 2). However, with increasing log K_{ow} values, also the solubility decreased until above log $K_{ow} = 4.53$, where the EC_{50} becomes higher than the calculated water solubility (Figure 2). However, 1-chlorononane (log $K_{ow} = 5.17$) was still able to show toxicity above this cutoff value.

Applicability Domain of the QSAR Model. The range of compounds for which the model is valid (applicability domain) was determined by taking into account the minimum and maximum values of both the (i) carbon chain length (C1–C10), (ii) the chlorine atoms (Cl_1−Cl_8), and (iii) the log K_{ow} values (1.71–5.70) of the compounds included in the training set. For models within a one-dimensional descriptor space, the interpolation region is simply taken as the interval between the minimum and the maximum values of the training data set (*18*). The theoretical toxicity cutoff limit of log $K_{ow} = 4.53$ was not taken into account here as compounds with higher log K_{ow} values were able to show toxicity. Taking these criteria into consideration, our QSAR model is thus applicable to chlorinated alkanes with up to 10 carbon and eight chlorine atoms and log K_{ow} values between 1.71 and 5.70. These limits were used as selection criteria to extract compounds with similar properties from the EINECS list. Using procedures described previously (*16*), molecular formula, name, and SMILES codes of the EINECS compounds were used as filtering criteria in Microsoft Excel 2003 to extract 59 compounds satisfying the selection criteria. The 59 compounds and their predicted EC_{50} values are shown in Table 2. This implies that including the 18 compounds used to develop the MTT assay-based in vitro QSAR in CHO cells, our QSAR model covers 77 (∼0.1%) of the EINECS list compounds.

External Validation of QSAR Model. From Table 2, five compounds, with log K_{ow} values within the applicability domain of the training set, were selected for additional testing to externally validate our QSAR model. Suitable test concentrations were selected based on the predicted in vitro toxicity values, thereby skipping range-finding tests. The experimental toxicity values, shown in Table 2, correlated well with predicted values ($r_{ext}^2 = 0.741$).

In Vitro to in Vivo Correlation. In a final step, it was investigated whether the acute in vitro toxicity data generated in the present study could be used to build a model for making acute in vivo toxicity predictions for fish. Tables 1 and 2 show the acute in vivo toxicity data for the fish, *P. reticulata* (*12*), for the compounds that were also tested in the present study. A correlation of the in vitro and in vivo toxicity data is shown in Figure 3 and can be described by the following equation:

$$\text{in vivo log } LC_{50}\ (\mu m) = 0.926[\text{in vitro log } EC_{50}\ (\mu m)] - 0.023 \qquad (3)$$

where $n = 7$, $r^2 = 0.758$, $s = 0.230$, and $F = 16$.

This equation was used to make predictions for the compounds tested in vitro for which no acute in vivo fish toxicity data were available, and the results thus obtained are presented in Tables 1 and 2.

Discussion

During the development of QSAR models, it is usually recommended where possible to (i) use experimental data from the same laboratory to avoid interlaboratory variation (*19*) and (ii) use data sets where the ratio of number of test compounds to descriptors used for modeling is at least five (*20*). Both conditions have been satisfied in this study by generating toxicity data within the same laboratory for 26 compounds and developing a QSAR model based on one descriptor, log K_{ow}. The developed QSAR model also satisfies the five basic requirements for OECD guidelines for QSAR models: clearly defined end point, unambiguous algorithm, appropriate measures of goodness of fit, robustness and predictivity, a defined domain of applicability, and a mechanistic interpretation. First, the end points are clearly defined (24 h EC_{50} to CHO cells and 7 days LC_{50} to *P. reticulata*). Second, the methods for data collection and calculation of descriptors have been clearly described. Third, the QSAR model has been validated both internally and externally. Fourth, the applicability domain in terms of descriptor range and the actual list of compounds that fit into the domain have been provided. Fifth, hydrophobicity has been confirmed as an important parameter to describe the toxicity of the chlorinated alkanes. The strong correlation between toxicity and hydrophobicity found in the current study (Figure 2) supports a nonpolar narcotic mechanism of action for chlorinated alkanes described previously (*12, 21, 22*). Because hydrophobicity is important for the toxicity of chlorinated alkanes, it was essential to choose a suitable method of exposure. The direct method of exposure resulted in higher toxicity than the premix method (Figure 1). Hydrophobic compounds have been shown previously to readily adhere to plastic surfaces of culture plates (*23*). This situation can easily arise during the dilution step of the premix method where the medium containing the chlorinated alkanes is prepared in a premixing well before transfer to wells containing the cells. This provides an additional possibility for the chlorinated alkanes to adhere to the plastic surface of the well before the solution is actually transferred to the cells. During the direct exposure method, more of the compound is immediately available to the cells. However, one needs to mix the test compound thoroughly into the culture medium by carefully pipetting up and down several times to avoid any local cytotoxicity that can occur due to high concentrations of solvent or test compound. The presence or absence of local cytotoxicity should always be confirmed with microscopic observations. The in vitro toxicity

Table 2. List of EINECS Compounds That Fit into the Applicability Domain[a] of the QSAR Model Developed in This Study[b]

				in vitro log EC_{50} (μM) to CHO cells in MTT assay		in vivo log LC_{50} (μM) to the fish (*Poecilia reticulata*)[e]	
	compd name	CAS[c] no.	log K_{ow}[d]	experimental	predicted[e]	experimental[f]	predicted[g,h]
1	1,2,3-trichloropropane	96-18-4	1.98	3.12	2.80	2.45	2.87
2	1-chlorobutane	109-69-3	2.52	3.27	2.56	3.02	3.01
3	1,5-dichloropentane	628-76-2	2.77	2.82	2.45		2.59
4	pentachloroethane	76-01-7	3.63	2.17	2.07	1.87	1.99
5	1,1,1,2,2,2,2, 3-heptachloropropane	594-89-8	4.74	1.62	1.57		1.48
6	hexachloroethane	67-72-1	4.61		1.63		1.49
7	1,1-dichloroethane	75-34-3	1.78		2.89		2.66
8	1,3-dichloro-2,2-bis (chloromethyl)propane	3228-99-7	1.93		2.82		2.59
9	trichloromethane	67-66-3	1.95		2.81		2.58
10	1-chloropropane	540-54-5	1.99		2.80		2.57
11	2-chloropropane	75-29-6	1.99		2.80		2.57
12	1,3-dichlorobutane	1190-22-3	2.24		2.69		2.47
13	1,4-dichlorobutane	110-56-5	2.24		2.69		2.47
14	1,1-dichloropropane	78-99-9	2.31		2.66		2.44
15	2,2-dichloropropane	594-20-7	2.31		2.66		2.44
16	1,2,3-trichloro-2-methylpropane	1871-58-5	2.38		2.62		2.41
17	1,2-dichloro-2-methylpropane	594-37-6	2.39		2.62		2.40
18	1-chloro-2-methylpropane	513-36-0	2.39		2.62		2.40
19	2-chloro-2-methylpropane	507-20-0	2.39		2.62		2.40
20	1,1,1-trichloroethane	71-55-6	2.48		2.58		2.36
21	1,2,3,4-tetrachlorobutane	3405-32-1	2.50		2.57		2.36
22	2,3-dichlorobutane	7581-97-7	2.52		2.56		2.35
23	2-chlorobutane	78-86-4	2.52		2.56		2.35
24	1,1,3-trichlorobutane	13279-87-3	2.55		2.55		2.34
25	1,1,2,3-tetrachloropropane	18495-30-2	2.57		2.54		2.33
26	1,2,2,3-tetrachloropropane	13116-53-5	2.57		2.54		2.33
27	1,1,2-trichloropropane	598-77-6	2.58		2.53		2.32
28	1,2,2-trichloropropane	3175-23-3	2.58		2.53		2.32
29	1,3-dichloro-3-methylbutane	624-96-4	2.63		2.51		2.30
30	tetrachloroethane	25322-20-7	2.64		2.51		2.30
31	1,1,1,3-tetrachloropropane	1070-78-6	2.72		2.47		2.27
32	1,1-dichlorobutane	541-33-3	2.84		2.42		2.22
33	2,2-dichlorobutane	4279-22-5	2.84		2.42		2.22
34	1,1,3,3-tetrachlorobutane	39185-82-5	2.86		2.41		2.21
35	1-chloro-3-methylbutane	107-84-6	2.92		2.38		2.18
36	2-chloro-2-methylbutane	594-36-5	2.92		2.38		2.18
37	trichloropropane	25735-29-9	3.01		2.34		2.15
38	1,1,1,2-tetrachloroethane	630-20-6	3.03		2.33		2.14
39	2,3-dichloropentane	600-11-3	3.05		2.33		2.13
40	2-chloropentane	625-29-6	3.05		2.32		2.13
41	3-chloropentane	616-20-6	3.05		2.32		2.13
42	1,1,2,2,3-pentachloropropane	16714-68-4	3.17		2.27		2.08
43	1,1,1,3-tetrachlorobutane	13275-19-9	3.25		2.24		2.05
44	1-chloro-3,3-dimethylbutane	2855-08-5	3.32		2.20		2.02
45	1,1,1-trichlorobutane	13279-85-1	3.54		2.11		1.93
46	1,1,1,2-tetrachloropropane	812-03-3	3.56		2.10		1.92
47	1,1,1,3,3-pentachlorobutane	21981-33-9	3.57		2.10		1.92
48	2-chlorohexane	638-28-8	3.58		2.09		1.91
49	3-chlorohexane	2346-81-8	3.58		2.09		1.91
50	2-chloro-2,3,3-trimethylbutane	918-07-0	3.72		2.03		1.85
51	2,5-dichloro-2,5-dimethylhexane	6223-78-5	4.09		1.86		1.70
52	2-chloroheptane	1001-89-4	4.11		1.85		1.69
53	3-chloroheptane	999-52-0	4.11		1.85		1.69
54	1,1,1,3-tetrachloro-4-methylpentane	62103-09-7	4.18		1.82		1.66
55	1-chloro-2,2,4-trimethylpentane	2371-06-4	4.25		1.79		1.64
56	3-(chloromethyl)heptane	123-04-6	4.51		1.67		1.53
57	2-chlorooctane	628-61-5	4.64		1.62		1.47
58	3-chlorooctane	1117-79-9	4.64		1.62		1.47
59	4-chlorooctane	999-07-5	4.64		1.62		1.47

[a] Chlorinated alkanes with up to 10 carbon atoms and log K_{ow} values between 1.71 and 5.70. [b] This list excludes the 18 compounds in Table 1 that were used for the model development. The first five compounds were used as the external validation set. [c] Chemical Abstracts Service. [d] K_{ow} values calculated using ClogP version 4.0. [e] EC_{50} values predicted using eq 2. [f] EC_{50} values obtained from ref *12*. [g] EC_{50} values predicted using eq 3. [h] Compounds selected for external validation of eq 2.

of compounds with log $K_{ow} > 4.53$ (Figure 2) can be explained in three ways. First, the predicted solubility values of the chlorinated alkanes are for water only and the solvent used in this study, DMSO, is known to increase their solubility. Second, DMSO may increase the absorption of compounds across membranes (*23, 24*); thus, its use as a cosolvent could enhance their entry into the cells. Third, the test compounds can bind to the proteins or lipids in the FCS in the growth medium, thus increasing their solubility. Previous attempts to use hydrophobicity to explain the toxicity of chlorinated alkanes to bacteria in the Microtox test failed ($r^2 = 0.193$, $n = 18$) (*25*), possibly due to the

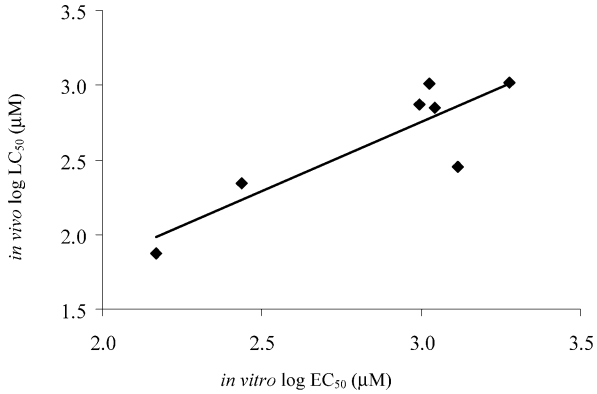

Figure 3. QSAR for the correlation between the decadic logarithm of the 24 h in vitro toxicity of chlorinated alkanes to CHO cells in the MTT assay and 7 days in vivo toxicity to fish (*Poecilia reticulata*).

short exposure time of the assay premix exposure as in the present study (Figure 1), and also the absence of serum in the medium that could increase the bioavailability of the test compounds.

Toxicity results for three of our training set compounds have been reported in other published studies and are comparable to our data. For example, there is close agreement between the cytotoxicity of 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, and carbon tetrachloride to CHO cells (current study) and to rat primary hepatocytes, both measured with the MTT assay (Table 1). The lower EC_{50} value of carbon tetrachloride to HeLa cells than to the CHO cells of the present study could be due to a longer exposure period (72 h as compared to 24 h in the present study). There is also close similarity between the EC_{50} values obtained in the current study to the concentrations that were toxic to the guppy and fathead minnow (Table 1). This similarity was extended to develop a prediction model for in vivo toxicity based on in vitro or in silico data. The good correlation (Figure 2, $r^2 = 0.883$) between the in silico-predicted log K_{ow} and the in vitro toxicity and the good correlation (Figure 3; $r^2 = 0.758$) obtained between in vitro and in vivo toxicity to fish support the possible use of QSAR approaches in the safety assessments within the framework of REACH, thereby reducing the use of experimental animals. Therefore, the results of the present study demonstrate that instead of performing toxicity testing of chlorinated alkanes (that fit into the applicability domain) on fish, one can carry out an in vitro CHO MTT test or even only calculate the log K_{ow} by available in silico models and use the QSAR models defined in the present study. On the basis of the QSAR models that we developed, one can use in vitro or even only in silico results to predict the in vivo toxicity to fish.

The experimental and predicted (Tables 1 and 2) in vivo toxicity data to fish can be used as a starting point for further risk assessment of the chlorinated alkanes. A toxicity ranking of the compounds will allow the identification of the most toxic and priority compounds. This will help to direct priorities for future testing to the most toxic compounds, thereby further refining and reducing the use of experimental animals.

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