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Biopartitioning micellar chromatography: An alternative high-throughput method for assessing the ecotoxicity of anilines and phenols

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Abstract

An investigation of the use of the chromatographic retention (log *k*) as an in vitro approach for modelling the toxicity to Fathead Minnows of anilines and phenols is developed. A data set of 65 compounds with available experimental toxicity data was used. Log *k* data at three pH values were used for the compounds classification and two groups or 'MODEs' were identified. For one 'MODE' a quantitative retention-activity relationship (QRAR) model was calculated. Finally, it was used to estimate the toxicity to Fathead minnows of anilines and phenols for which experimental data are not available. These estimations were compared to those obtained from another toxicity (to *Tetrahymena pyriformis*) data set and those estimated from a U.S. EPA QSAR approach (ECOSAR software) to decide on the toxicity level according to the Directive 3/21/EEC.

Keywords: Biopartitioning micellar chromatography; Ecotoxicity; Quantitative retention-activity relationships; Phenols; Anilines

1. Introduction

Environmental hazard and risk assessment of chemical substances requires comprehensive information on the exposure, fate and ecotoxicology of the contaminants; however, complete data sets are rarely available. One reason for these deficiencies is that testing capacities are limited, which impedes the thorough experimental investigation of all the existing and new chemicals. To fill at least some of the data gaps, mathematical modelling techniques are used to provide sufficiently accurate substitutes. The models can be used to estimate the parameters related to the fate and effects of chemicals and hence to identify contaminants of special environmental concern and to obtain a ranking of potentially hazardous pollutants. In this way, the priority compounds can then be subjected to detailed testing and the limited resources for experimental investigations can be directed effectively to the chemicals that are most likely to have an environmental impact [1].

Attention in mathematical modelling techniques also arises from their application as absolute alternatives to animal experiments, in the interests of time-effectiveness, cost-effectiveness and animal welfare. Alternative methods assist the policy of the "Three Rs" (replacement, reduction and refinement of the use of laboratory animals) and several regulatory organisations have been established to investigate and promote alternative methods [2,3].

Chemical modelling techniques are based on the premise that the structure of a compound determines all its properties. The study of the type of chemical structure of a foreign substance which will interact to a living system and produce a well-defined biological endpoint is commonly referred to as (quantitative) structure-activity relationships (Q)SARs [4]. The use of (Q)SARs for toxicity estimation of new chemicals [5,6] or to regulatory toxicological assessment [2,3] is increasing, especially in aquatic toxicology.

Alternatively to (Q)SAR models (quantitative) retention-activity relationships (Q)RARs, represent other kind of modelling techniques, in which chromatographic retention parameters are used as descriptor and/or predictor variables of a given biological response of chemicals. QRAR models using retention factors (log *k*) obtained using conventional RP-HPLC [7–9], micellar liquid chromatography (MLC) [10] and biopartitioning micellar chromatography (BMC) [11–13] have been reported.

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In general (Q)SARs must be developed for chemical substances that interact with a target site by the same mechanism of action (MOA) [14]. The information on MOA of compounds is used to define the called response applicability domain of QSAR models (the response space where models can provide reliable predictions) [15]. The assessment of a compound's likely MOA is critical for a correct QSAR selection; incorrect MOA-based QSAR selections can result in 10- to 1000-fold errors in toxicity predictions [16].

The aim of this study is to derive (Q)RAR models based on the BMC chromatographic retention (log *k*) to predict the toxicity to Fathead minnows of 65 anilines and phenols. In order to develop an independent methodology, the role of the chromatographic classification instead of the conventional mechanism of toxic action classification of these compounds is studied. Finally, toxicity levels according to the Directive 93/21/EEC were assigned to those compounds with no available experimental data.

2. Experimental

2.1. Instrumental

An Agilent 1100 chromatograph with a quaternary pump and an UV–vis detector (variable wavelength detector) was employed. It is equipped with a column thermostat with 9 μL extra-column volume for preheating mobile phase prior to the column and an autosampler with a 20 μL loop. All the assays were carried out at 25 °C. Data acquisition and processing were performed by means of an HP Vectra XM computer (Amsterdam, The Netherlands) equipped with an HP-Chemstation software (A.07.01 [682] ©HP 1999).

Two Kromasil C_{18} columns (5 μm, 150 mm × 4.6 mm i.d.; Scharlab S.L., Barcelona, Spain) and (5 μm, 50 mm × 4.6 mm i.d.; Scharlab) were used. The mobile phase flow rate was 1.0 or 1.5 mL min^{-1} for the 150 mm and 50 mm column length, respectively. The detection was performed in UV at 254 nm for acetanilide, antipyrine and propiophenone (reference compounds), and 240 nm for phenols and anilines.

2.2. Reagents and standards

Micellar mobile phases were prepared by dissolving polyoxyethylene(23)lauryl ether (Brij35, Fluka, Buchs SG, Switzerland) in aqueous solution of 0.025 M phosphate buffer and 0.025 M citrate buffer to get a final surfactant concentration of 0.04 M. The buffer solutions were prepared with sodium dihydrogen phosphate (analytical reagent, Panreac, Barcelona, Spain) and trisodium citrate (analytical reagent, Guinama, Valencia, Spain). The pH was potentiometrically adjusted by addition of either sodium hydroxide (97%, purissimum, Panreac) or hydrochloric acid (for analysis, Merck, Darmstadt, Germany) aqueous solutions to get the final pH values 5.50, 7.35 and 7.90. Ionic strength of the mobile phase was adjusted at 0.25 M by addition of the appropriate amount of sodium chloride (analytical reagent, Panreac).

Compounds used in this study were obtained from different sources. Standards of the reference compounds acetanilide and antipyrine were obtained from Fluka and propiophenone from Aldrich (St. Louis, Missouri, USA). The compounds 2,6-dichlorophenol, 2,5-dinitrophenol, 3-hydroxyphenol, 3-methoxyphenol, 4-methoxyphenol, 4-phenoxyphenol, 4-*tert*-butylphenol, 2,3,6-trimethylphenol, 2,4,6-trimethylphenol, pentafluoroaniline, 2-chloro-4-methylaniline, 4-butylaniline and 4-octylaniline were obtained from Aldrich; 4-chlorophenol, 2,6-di(*tert*)butyl-4-methylphenol, 4-ethylphenol, 2-phenylphenol, *N*-methylaniline, *N*,*N*-dimethylaniline and 3,4-dichloroaniline from Fluka; 3,5-dichlorophenol, 2,6-dinitrophenol, nonylphenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol, 2,3,5-trichlorophenol, 2,3,6-trichlorophenol, 2-chloroaniline, 2,3,4-trichloroaniline and 2,3,5,6-tetrachloroaniline from Riedel-de Haën (Seelze, Germany); 3,4,5-trichlorophenol from Supelco (Bellefonte, Pennsylvania, USA); 2,4,6-trichlorophenol, 2-methylphenol, 4,6-dinitro-2-methylphenol, pentachlorophenol, 2,4-dinitrophenol, 2,4-dichlorophenol, phenol, 4-nitrophenol, 2-nitrophenol, 2,4-dimethylphenol, 4-chloro-3-methylphenol, 4-methylphenol, 2-chlorophenol, aniline, 4-nitroaniline, 2,6-dimethoxyphenol, 3-nitrophenol, pentabromophenol, 4-propylphenol, 4-*tert*-pentylphenol, 2,4,6-tribromophenol, 2,4,5-trichlorophenol, 2,4,6-triiodophenol, 4-methylaniline, 2,4-dinitroaniline, 4-ethylaniline, 2-chloro-4-nitroaniline, 4-ethoxy-2-nitroaniline, 4-hexyloxyaniline, 2,6-diisopropylaniline, 2,6-diisopropylphenol, 2,6-dichloro-4-nitroaniline and 4-chloroaniline from Acros Organics (Geel, Belgium) and 2,3,4,5-tetrachlorophenol from Dr. Ehrenstorfer (Augsburg, Germany).

Stock standard solution of every compound was prepared by dissolving 10 mg of each compound in 10 mL of acetonitrile or methanol. Working solutions were prepared by dilution of the stock standard solutions using the mobile phase solution. The solutions were stored under refrigeration at 5 °C. As reference solutions, two binary mixtures (acetanilide–propiophenone and antipyrine–acetanilide) were prepared.

Barnstead E-pure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

2.3. BMC measurements

The retention factor of reference compounds were obtained according to the IUPAC approach [17], based on the extra-column time, t_{ext}, correction:

$$k = \frac{t_R^g - t_M^g}{t_M^g - t_{ext}} \quad (1)$$

where t_M^g is the gross hold up times and t_R^g is the gross retention time. The retention factor (k) of anilines and phenols was estimated according to a recent approach [18].

$$k = \frac{k_2(t_R^g - t_{R1}^g) + k_1(t_{R2}^g - t_R^g)}{t_{R2}^g - t_{R1}^g} \quad (2)$$

where t^{g}_{R1} and t^{g}_{R2} are the gross retention times of two references molecules and k_1 and k_2 are their retention factors, while t^{g}_{R} is the gross retention time of test chemicals. This approach provides internal consistency among k estimations of compounds in a long-term sense [18]. Highly retained chemicals ($k>15$) were measured using 50 mm column length, 1.5 mL min^{-1} flow rate and acetanilide–propiophenone reference mixture conditions. Weakly retained chemicals ($k<15$) were measured using 150 mm column length, 1 mL min^{-1} flow rate and antipyrine–acetanilide reference mixture conditions. Injections of reference mixture were carried out at regular intervals along the injections of the test chemicals, whose k-estimates were obtained using the retention information for the precedent and posterior reference mixture injections.

2.4. Biological activity (Fathead minnows)

Thirty phenols and 20 anilines for which experimental toxicity (LC_{50}) values to Fathead minnows (*Poecilia reticulata*) were available [19] were used. The original LC_{50} data (mmol L^{-1} units) of compounds were transformed into mg L^{-1} ones (used in Directive 93/21/EEC [20]) by multiplying them by their corresponding molecular weights. After that, the negative decadic logarithm of these values (pLC_{50}; usually used as dependent variable) was obtained. The correlation between pLC_{50} data of compounds (from LC_{50} data in mmol L^{-1}) and those from LC_{50} data in mg L^{-1}, was high ($r^2>0.98$), which suggests equivalent results (QSAR/QRAR model of similar quality) in both cases. For one aniline and 14 phenols LC_{50} data to Fathead minnows are not available.

3. Results and discussion

3.1. Chromatographic classification of compounds

The compounds data set, ordered by family (FAM = 1 for phenols, FAM = 2 for anilines) and by the assigned mechanism of action (MOA) [19] is shown in Table 1. In spite of the importance of MOAs, errors in their assignment would affect the model quality and then, the toxicity estimations. In an independent investigation on the use of log k to model the pH-dependence of the toxicity to Guppy of 19 phenols with available experimental toxicity-pH data (paper in preparation), log k data at three pH values were used for the classification of phenols and two groups were identified. This strategy was extended to the 65 available phenols and anilines. Fig. 1 shows the differences in log k at pH levels 5.5, 7.35 and 7.9 combined to map the phenols and anilines compounds in Table 1. Positive [log k(5.5) − log k(7.9)] values (x-axis in Fig. 1) reflects acidic compounds, such as phenols, while negative values reflects basic compounds, such as anilines. On the other hand, compounds located close to the zero x-axis, exhibit almost neutral behaviour in the pH range studied. As Fig. 1 indicates, ionizable phenols exhibit larger ionization degree than ionizable anilines, since the last are close to the zero x-axis.

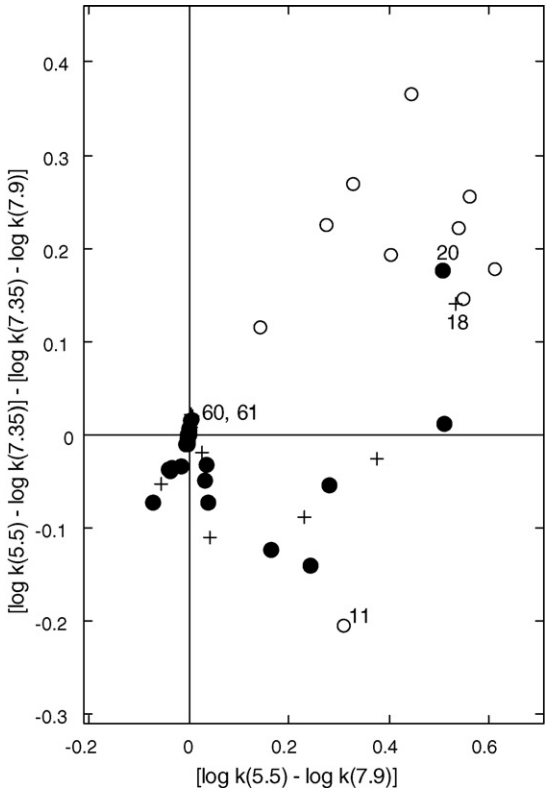

Fig. 1. Maps of compounds in Table 1 based on log k differences respect to pH (indicated in parenthesis). Symbols were used for MOA: (●) MOA = 1; (○) MOA = 2; (+) other MOAs. N is included for some compounds.

Moreover, the retention differences [log k(5.5) − log k(7.35)] − [log k(7.35) − log k(7.9)] (y-axis in Fig. 1) should discriminate the compounds by the shape of the log k-pH curve in the [5.5–7.9] pH range. This curve is a sigmoid centered on the pK_a value in BMC conditions, which does not necessarily coincide with the aqueous pK_a. For ionizable phenols and anilines, the 'retention difference' function varies depending on what zone of the sigmoid is involved in (i.e. the location and proximity respect to the pK_a value in BMC conditions). BMC mobile phases have been designed to reproduce the physiological ambient closer than water [11]; therefore, a classification based on aqueous pK_a values could be, a priori, more risky than that using the experimental log k data for this task. On the other hand, using this approach, the knowledge of pK_a is not a previous requisite to classify a new molecule.

As shown in Fig. 1, compounds with assigned MOA = 2 (except N = 11, 60 and 61) have markedly positive y-values. In contrast, compounds with assigned MOA = 1, 3 and 4 have close to zero or negative y-axis values (except N = 18 and 20). These observations could suggest that other criterion to classify the phenols and anilines, alternative to MOA, could be suitable for modelling purposes. According to the 'chromatographic retention-differences map' information in Fig. 1, we have introduced the variable 'MODE' (mode of classification based on retention-pH data) in Table 1. Thus, MODE = 1 was set to those compounds showing close to zero or negative y-axis values in Fig. 1, while MODE = 2 to those having markedly positive y-values.

Table 1
Categorical, retention and toxicity data of compounds

CAS	Compound	N[a]	FAM[b]	MOA[c]	MODE[d]	log k[e] (5.5)	log k[e] (7.35)	log k[e] (7.9)	pLC_{50}[f] (Fathead m.)	pLC_{50}[g] (ECOSAR)	pIG_{50}[h] (Tetrah. p.)	MW[i] g/mol
933-78-8	2,3,5-Trichlorophenol	1	1	1	1	1.809	1.757	1.564	-	−0.57	0.07	197.45
2416-94-6	2,3,6-Trimethylphenol	2	1	1	1	1.621	1.626	1.623	−0.91	−0.59	−1.85	136.20
95-95-4	2,4,5-Trichlorophenol	3	1	1	1	1.799	1.778	1.633	-	−0.57	−0.20	197.45
88-06-2	2,4,6-Trichlorophenol	4	1	1	1	1.738	1.477	1.227	−0.69	−0.57	−0.89	197.45
120-83-2	2,4-Dichlorophenol	5	1	1	1	1.665	1.673	1.632	−0.89	−0.89	−1.17	163.00
87-65-0	2,6-Dichlorophenol	6	1	1	1	1.574	1.460	1.292	-	−0.89	−1.48	163.00
95-57-8	2-Chlorophenol	7	1	1	1	1.487	1.485	1.450	−1.06	−1.18	−1.93	128.56
591-35-5	3,5-Dichlorophenol	8	1	1	1	1.748	1.765	1.709	-	−0.89	−0.64	163.00
106-48-9	4-Chlorophenol	9	1	1	1	1.529	1.529	1.529	−0.79	−1.18	−1.56	128.56
108-95-2	Phenol	10	1	1	1	1.248	1.245	1.246	−1.51	−1.44	−2.18	94.11
4901-51-3	2,3,4,5-Tetrachlorophenol	11	1	2	1	1.874	1.822	1.565	0.39	−0.24	0.34	231.89
58-90-2	2,3,4,6-Tetrachlorophenol	12	1	2	2	1.789	1.394	1.176	−0.01	−0.24	−0.16	231.89
935-95-5	2,3,5,6-Tetrachlorophenol	13	1	2	2	1.760	1.352	1.199	-	−0.24	−0.15	231.89
329-71-5	2,5-Dinitrophenol	14	1	2	2	1.422	1.017	0.977	−0.53	−1.01	−1.32	184.11
87-86-5	Pentachlorophenol	15	1	2	2	1.765	1.384	1.224	0.65	0.10	−0.38	266.34
554-84-7	3-Nitrophenol	16	1	3	1	1.451	1.448	1.426	-	−1.37	−1.63	139.11
100-02-7	4-Nitrophenol	17	1	3	1	1.449	1.378	1.218	−1.65	−1.37	−0.72	139.11
933-75-5	2,3,6-Trichlorophenol	18	1	4	2	1.686	1.349	1.152	-	−0.57	-	197.45
609-19-8	3,4,5-Trichlorophenol	19	1	4	1	1.783	1.817	1.741	-	−0.57	-	197.45
609-23-4	2,4,6-Triiodophenol	20	1	1	2	1.800	1.459	1.293	−0.08	0.01	0.00	471.80
527-60-6	2,4,6-Trimethylphenol	21	1	1	1	1.626	1.629	1.627	−1.11	−0.59	−1.85	136.20
105-67-9	2,4-Dimethylphenol	22	1	1	1	1.566	1.567	1.567	−1.22	−0.88	−2.02	122.17
91-10-1	2,6-Dimethoxiphenol	23	1	1	1	0.973	0.979	0.976	-	−1.87	−2.79	154.17
128-37-0	2,6-Di(*tert*)butil-4-methylphenol	24	1	1	1	2.351	2.351	2.351	0.44	0.36	−0.54	220.36
90-43-7	2-Phenylphenol	25	1	1	1	1.704	1.709	1.706	−0.79	−0.61	−1.14	170.21
95-48-7	2-Methylphenol	26	1	1	1	1.465	1.465	1.465	−1.15	−1.16	−2.32	108.14
150-19-6	3-Methoxyphenol	27	1	1	1	1.269	1.266	1.268	−1.87	−1.51	−2.42	124.14
59-50-7	4-Chloro-3-methylphenol	28	1	1	1	1.593	1.600	1.596	−0.74	−0.89	−1.35	142.59
123-07-9	4-Ethylphenol	29	1	1	1	1.548	1.552	1.550	-	−0.92	−1.89	122.17
831-82-3	4-Phenoxiphenol	30	1	1	1	1.679	1.683	1.681	−0.69	−0.47	−0.91	186.21
150-76-5	4-Methoxyphenol	31	1	1	1	1.162	1.158	1.160	−2.04	−1.51	−2.23	124.14
106-44-5	4-Mehtylphenol	32	1	1	1	1.409	1.411	1.410	-	−1.16	−2.21	108.14
645-56-7	4-Propylphenol	33	1	1	1	1.664	1.669	1.666	-	−0.66	−1.49	136.20
98-54-4	4-*Tert*-butylphenol	34	1	1	1	1.743	1.748	1.746	−0.71	−0.47	−1.27	150.22
80-46-6	4-*Tert*-pentylphenol	35	1	1	1	1.836	1.841	1.839	−0.41	−0.21	−0.99	164.25
104-40-5	Nonylphenol	36	1	1	1	2.180	2.186	2.183	0.85	0.95	0.13	220.36
108-46-3	3-Hydroxyphenol	37	1	1	1	1.054	1.044	1.05	-	−1.81	−2.69	110.11
118-79-6	2,4,6-Tribromophenol	38	1	2	2	1.764	1.417	1.216	−0.82	−0.34	−0.49	330.80
51-28-5	2,4-Dinitrophenol	39	1	2	2	1.241	0.943	0.913	−1.04	−1.01	−1.19	184.11
573-56-8	2,6-Dinitrophenol	40	1	2	2	0.921	0.793	0.779	−1.60	−1.01	−1.73	184.11
534-52-1	4,6-Dinitro-2-methylphenol	41	1	2	2	1.254	1.004	0.979	−0.24	−0.74	−0.58	198.14
608-71-9	Pentabromophenol	42	1	2	2	1.652	1.354	1.249	1.03	0.58	−0.03	488.59
88-75-5	2-Nitrophenol	43	1	3	1	1.376	1.200	0.999	−2.20	−1.37	−1.47	139.11
2078-54-8	2,6-Diisopropylphenol	44	1	4	1	1.990	1.986	1.988	-	−0.45	-	178.28
95-76-1	3,4-Dichloroaniline	45	2	0	1	1.568	1.563	1.566	−0.88	−1.41	-	162.02
121-69-7	*N*,*N*-Dimethylaniline	46	2	0	1	1.588	1.642	1.644	−1.81	−1.79	−1.80	121.18

634-67-3	2,3,4-Trichloroaniline	47	2	1	1	1.666	1.670	1.668	−0.56	−1.02	−0.94	196.46
24544-04-5	2,6-Diisopropylaniline	48	2	1	1	1.898	1.896	1.897	−1.18	−0.26	−1.47	177.29
615-65-6	2-Chloro-4-methylaniline	49	2	1	1	1.574	1.576	1.575	−1.56	−1.43	−1.97	141.60
95-51-2	2-Chloroaniline	50	2	1	1	1.459	1.459	1.459	−0.76	−1.79	−2.36	127.57
104-13-2	4-Butylaniline	51	2	1	1	1.706	1.713	1.710	−1.01	−0.84	−1.10	149.24
106-47-8	4-Chloroaniline	52	2	1	1	1.409	1.408	1.408	−1.50	−1.79	.	127.57
589-16-2	4-Ethylaniline	53	2	1	1	1.407	1.446	1.445	−1.86	−1.48	−2.05	121.18
616-86-4	4-Ethoxy-2-nitroaniline	54	2	1	1	1.527	1.526	1.527	−1.41	−1.30	−1.50	182.18
39905-57-2	4-Hexyloxyaniline	55	2	1	1	1.761	1.785	1.775	−0.48	−0.57	−0.91	193.29
106-49-0	4-Methylaniline	56	2	1	1	1.155	1.227	1.226	−2.20	−1.79	−2.08	107.16
16245-79-7	4-Octylaniline	57	2	1	1	2.041	2.043	2.042	0.92	0.47	0.12	205.35
62-53-3	Aniline	58	2	1	1	0.954	0.988	0.986	−2.02	−2.13	−1.86	93.13
100-61-8	*N*-Methylaniline	59	2	1	1	1.312	1.351	1.352	−2.00	−2.26	−1.97	107.16
3481-20-7	2,3,5,6-Tetrachloroaniline	60	2	2	1	1.834	1.832	1.833	0.57	−0.62	−0.47	230.91
97-02-9	2,4-Dinitroaniline	61	2	2	1	1.487	1.476	1.481	−1.17	−1.14	−1.54	183.12
121-87-9	2-Chloro-4-nitroaniline	62	2	3	1	1.506	1.496	1.501	−1.30	−1.63	−1.49	172.57
771-60-8	Pentafluoroaniline	63	2	3	1	1.674	1.668	1.67	−1.57	−1.68	−2.00	183.08
100-01-6	4-Nitroaniline	64	2	4	1	1.270	1.257	1.266	−2.10	−2.01	.	138.13
99-30-9	2,6-Dichloro-4-aniline	65	2	4	1	1.589	1.576	1.583	.	−1.23	.	207.02

[a] Compound identification number used along the text.
[b] Chemical family (1, phenols; 2, anilines).
[c] Assigned mechanism of toxic action (0, non-polar narcotic; 1, polar narcotic; 2, respiratory uncoupler; 3, soft-electrophile; 4, unknown). MOA values are taken from reference [19].
[d] Alternative mode of classification consistent with Fig. 1 observations (1 = close to MOA = 1; 2 = close to MOA = 2).
[e] Decadic logarithm of the retention factor in BMC at indicated (in parenthesis) mobile phase pHs.
[f] Negative decadic logarithm of the median lethal concentration (pLC_{50}) to Fathead minnows (*Pimephales promelas*). Data taken from reference [19]. LC_{50} values in mg L^{-1}.
[g] Negative decadic logarithm of the median lethal concentration (pLC_{50}) to Fish from ECOSAR software [22]. LC_{50} values in mg L^{-1}.
[h] Negative decadic logarithm of the median inhibitory growth concentration (pIG_{50}) to *Tetrahymena pyriformis*. Data taken from reference [19]. IG_{50} values in mg L^{-1}.
[i] Molecular weight of compounds. It was used to convert the original LC_{50} (mmol/L) into LC_{50} (mg/L).

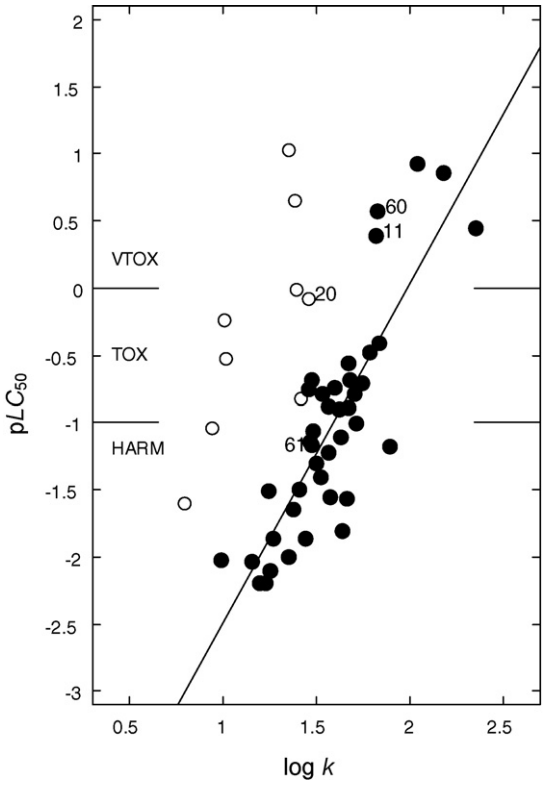

Fig. 2. $pLC_{50} - \log k$ relationships. Symbols were included for MODE: (●) MODE = 1; (◯) MODE = 2. *N* is included for the same compounds as in Fig. 1.

3.2. Retention–toxicity relationships

Fig. 2 shows the retention–toxicity relationship corresponding to data at pH 7.35. The compounds are labelled according to MODE. Fig. 2 also includes three toxicity levels assigned to the pLC_{50} values in fish, taking into account the Directive 93/21/EEC [20]. As can be observed, there is a linear trend for MODE = 1 compounds, while the MODE = 2 ones exhibit higher toxicity values. These results are related to the observations obtained from Fig. 1, thus, compounds *N* = 11, 60 and mainly 61 (with assigned MOA = 2) are closer to the MODE = 1 linear trend than to the MODE = 2 zone. The opposite can be stated for compound *N* = 20. This encourages us to consider the use of 'MODE' as an alternative criterion for classifying phenols and anilines. Therefore, the approach to develop quantitative retention-activity relationships (QRAR) could be to include MODE = 1, rather than MOA = 1, compounds to develop a retention–toxicity model. For instance, if 2,4,6-trichorophenol (*N* = 20) was predicted according to the MOA = 1 assignation a HARMFULL level will be predicted by the relationship in Fig. 2; in contrast the MODE = 2 classification suggests a higher toxicity level, as it occurs attending its experimental pLC_{50} value in Table 1 (−0.08; TOXIC/VERY TOXIC limit). Obviously, a perfect agreement between classification/modelling and experimental data (also subject to high variability) is not feasible and some compounds could present a different behaviour from the expected one and even become outliers. Such outliers are usually excluded to improve the model quality. However, we have decided to perform a QRAR model including all MODE = 1 compounds.

3.3. Model quality evaluation

The equations and statistics for the (Q)RAR models were adjusted to the format recommended by Sagrado and Cronin [21], however, some classical statistics (historically used to characterize QSAR models) have been also included. For MODE = 1 compounds the following equation (QRAR model) was found:

$$pLC_{50} = -5.02(\pm 1.28) + 2.52(\pm 0.83)\ \log k \quad (3)$$

No = 41*; *Nv* = 1; *Dp* = 71%; *Pp* = 67%; Mp = 69%

* None outlier was excluded (recommended presentation [21]).

RMSEC = 0.43, RMSECV = 0.45, $r^2 = 0.71$, $q^2 = 0.68$, $F = 97.3$, $p < 0.0001$, $s_e = 0.44$ (classical statistics [21]), where *No* refers to the number of objects (available retention–toxicity data pairs) used for regression, *Nv* refers to the number of predictor variables (here 1 descriptor; log *k*), *Dp* and *Pp* are diagnostic statistics (0–100% range) that reflect the descriptive and predictive power, respectively, of the model and *Mp* (=0.5 *Dp* + 0.5 *Pp*) represents the overall model quality [21]. The uncertainty of the *b*-coefficients, *U*(*b*), calculated from the jack-knife approach based on 6-subsets cross-validated results, is indicated in parenthesis.

Assuming the suggested limiting values (60% for *Dp* and *Pp* statistics) [21], this model falls into the QRAR category (for quantitative toxicity estimations). It should be noted that QRAR (as QSAR) models quality mainly depends on the uncertainty in toxicological data [11] (e.g. interquartile ranges larger than one pLC_{50} unit for the toxicological values reported, can be encountered in the bibliography). As Fig. 2 suggests, for MODE = 2 compounds a linear trend exists, which suggests the possibility of developing a model to make predictions. On the other hand, due to the low number of MODE = 2 compounds, a statistically consistent QRAR model is not feasible (e.g. *Dp* = 7.4%; *Pp* = 0%, under the limiting value of 60% [21]; consistent with the classical $r^2 = 0.46$ and $q^2 = 0.3$). Therefore, only qualitative toxicity estimations should be considered for these compounds.

Fig. 3 shows the fitted and cross-validated predicted pLC_{50} values from Eq. (3), which provides an idea of the predictive ability that can be expected when using the proposed QRAR model.

3.4. Model utilization

Eq. (3) can be used to estimate pLC_{50} to Fathead minnows of anilines and phenols after classification as MODE = 1 compounds, then obtaining log *k* data at different pH values. Table 2 shows the estimations for all compounds in Table 1 with no experimental pLC_{50} data. It should be noted that two of them (*N* = 13 and 18) are MODE = 2 compounds, so they cannot be estimated by Eq. (3). However, a rough estimation can be made for such compounds. As suggests Fig. 2, MODE = 2 compounds exhibit pLC_{50} values at least one unit higher than the predicted

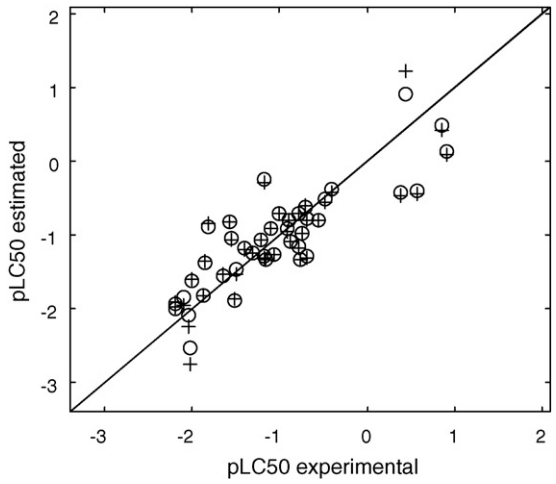

Fig. 3. Validation plots for pLC_{50} values showing the fitted (◯) and cross-validated (+) results corresponding to Eq. (3).

by Eq. (3), so since the estimated value for both compounds is −1.6, we can assume a pLC_{50} value larger than −0.6 mg/mL (see Table 2).

The lack of experimental data does not permit comparison (e.g. as in Fig. 3) of the predicted toxicity for compounds in Table 2. Alternatively, we can use other ways to predict the same values, at least to check the consistency of the results. Table 1 shows the values of toxicity (pIG_{50}) to the protozoan *Tetrahymena Pyriformis* [19] and those estimated from a US EPA QSAR approach (ECOSAR software) [22] to fish for these compounds. The use of alternative species different from fish to perform hazard classification of acute aquatic toxicity has been promoted by official organizations in order to reduce its number for in vivo test [23]. The pIG_{50} data in Table 1 show some degree of correlation with those available for pLC_{50} for Fathead minnows, which can be expressed as:

$$pLC_{50} = 0.31 + 0.93\,pIG_{50} \quad (4)$$

Eq. (4) exhibits lower quality than Eq. (3) (i.e. $r^2 = 0.65$; $Pp = 56\%$), however, their pLC_{50} estimations can be compared (see Table 2) with those from Eq. (3). Unfortunately, there are no pIG_{50} values for four of the compounds. On the other hand, ECOSAR offers direct estimations of pLC_{50} for fish from prefixed QSAR models as a function of the prefixed chemical class of the compound [23]. Most of the differences between the ECOSAR and experimental pLC_{50} values in Table 1 are in the ±0.5 range (although in some cases it is larger than 1). Table 2 also shows the predicted values from ECOSAR.

The differences between toxicity values estimated from Eqs. (3) and (4) and ECOSAR in Table 2 are lower than one pLC_{50} unit. This result suggests that a toxicity level (Directive 93/21/EEC) can be inferred from Table 2 results. Table 2 includes this estimation based on at least 2 coincident estimations. For five compounds the three criteria are coincident ($N = 8$, 16, 23, 32 and 37). In two cases, Eqs. (3) and (4) indicate the same toxicity level ($N = 6$ and 29) and in eight additional cases Eq. (3) and ECOSAR are coincident ($N = 1$, 3, 13, 18, 19, 33, 44 and 65); although for the two MODE = 2 compounds ($N = 13$ and 18) some caution is necessary. It should be noticed that in all cases, the decision on the toxicity level in Table 2 would be identical to that based just on Eq. (3) pLC_{50} estimations, which suggest the importance of the proposed QRAR model.

3.5. Final remarks

From a practical point of view, an advantage of QRAR-MODE strategy over QSAR-MOA (e.g. based on log *P* or log *D*, that includes the pK_a information, and other still non-harmonised descriptors), is that a single descriptor (log *k*) is enough for both classification and prediction tasks. Although at present QSAR descriptors can be estimated from software packages (with more or less reliability) the fact of combining several of them contributes to enhance the overall uncertainty; meanwhile log *k* data has a controlled low uncertainty [18]. Moreover, sometimes MOA is not available for the species of interest, so data from other sources have to be used. In addition, classification rates (e.g. from discriminant algorithms) are not always

Table 2
Categorical, predicted toxicity (with 95% confidence levels) and toxicity level (directive 93/21/EEC) data of test compounds

CAS	Compound	*N*	FAM	MOA	MODE	pLC_{50} (Eq. (3))	pLC_{50}(Eq. (4))	pLC_{50} (ECOSAR)	Toxicity level
933-78-8	2,3,5-Trichlorophenol	1	1	1	1	−0.59 ± 0.17	0.38 ± 0.4	−0.57	TOX
95-95-4	2,4,5-Trichlorophenol	3	1	1	1	−0.53 ± 0.17	0.13 ± 0.3	−0.57	TOX
87-65-0	2,6-Dichlorophenol	6	1	1	1	−1.34 ± 0.15	−1.07 ± 0.16	−0.89	HARM
591-35-5	3,5-Dichlorophenol	8	1	1	1	−0.57 ± 0.17	−0.28 ± 0.2	−0.89	TOX
935-95-5	2,3,5,6-Tetrachlorophenol	13	1	2	2	>−0.6	0.17 ± 0.3	−0.24	TOX
554-84-7	3-Nitrophenol	16	1	3	1	−1.37 ± 0.15	−1.21 ± 0.17	−1.37	HARM
933-75-5	2,3,6-Trichlorophenol	18	1	4	2	>−0.6	-	−0.57	TOX
609-19-8	3,4,5-Trichlorophenol	19	1	4	1	−0.43 ± 0.19	-	−0.57	TOX
91-10-1	2,6-Dimethoxyphenol	23	1	1	1	−2.5 ± 0.3	−2.29 ± 0.4	−1.87	HARM
123-07-9	4-Ethylphenol	29	1	1	1	−1.10 ± 0.14	−1.45 ± 0.19	−0.92	HARM
106-44-5	4-Methylpenol	32	1	1	1	−1.46 ± 0.16	−1.75 ± 0.2	−1.16	HARM
645-56-7	4-Propylphenol	33	1	1	1	−0.81 ± 0.15	−1.07 ± 0.16	−0.66	TOX
108-46-3	3-Hydroxyphenol	37	1	1	1	−2.4 ± 0.3	−2.19 ± 0.3	−1.81	HARM
2078-54-8	2,6-Diisopropylphenol	44	1	4	1	0.0 ± 0.3	-	−0.45	TOX
99-30-9	2,6-Dichloro-4-nitroaniline	65	2	4	1	−1.04 ± 0.14	-	−1.23	HARM

Details as in Table 1.

close to 100%, and in some cases (e.g. phenols), the impact of using log D or the combination of log P and pK_a as discriminant variables (or to built QSARs) is ambiguous [24–27].

In the present case, the $pLC_{50} - \log P$ relationship (plot not shown) partially merges MOA = 2 data into the rest, which could suggests the possibility of using a unique model for all compounds; however, for some MOA = 2 compounds the pLC_{50} estimations would result lower than the experimental ones. The $pLC_{50} - \log D$ relationship (plot not shown) was similar to that in Fig. 2, reflecting the fact that log k accounts for hydrophobicity and ionisation (as log D). However, for two MOA = 2 compounds (that appear between the MOA = 1 data) the 'qualitative' pLC_{50} estimations would result higher than the experimental ones. These facts could be risky in terms of toxicity assessment. All these aspects point out that the right approach should be the use of several strategies (QRAR-MODE, QSAR-MOA, etc.) as complementary (rather than competitive) tools to offer to regulatory agencies a reinforced toxicological assessment of chemicals.

The present approach, evaluated in BMC conditions, could also be possible using conventional chromatography (e.g. reverse phase liquid chromatography; RPLC), but also other more complex liquid chromatographic processes (e.g. immobilised artificial membranes, IAM, and immobilised biomembranes, IBC). Micellar mobile phases reproduce the physiological ambient more closely than aqueous ones [11]; so, a priori, BMC should exhibit higher applicability in QRAR than RPLC. On the other hand, BMC is simpler than IAM or IBC. The use of log k as single descriptor for both classification and prediction tasks is an idea that deserves more attention and study. Alternatively, log k could be used as descriptor to perform QSAR models using it with other molecular descriptors.

4. Conclusions

The toxicity–retention results for a set of 65 compounds are reasonably consistent, taken into account that experimental pLC_{50} data are not free from error and reference data does not exist in this kind of studies. A unique descriptor, log k, measured at different pH values is necessary to estimate pLC_{50} values. From log k data at three pH values, a new compound could be classified into MODE = 1 or MODE = 2 type (close to MOA classification, but with exceptions). In the first case, the appropriate QRAR model can be selected for prediction. In the second one, only a minimum toxicity expected limit can be qualitatively suggested.

The results of this investigation suggest that BMC chromatographic retention data allows fish toxicity modelling of phenols and anilines classified as MODE = 1. Moreover, BMC becomes a credible high-throughput tool for assessing the toxicity of organic compounds and an alternative to assist regulatory agencies in toxicological assessment of these substances. However, in this sense, our opinion is that is preferable to take decisions based on more than one criterion. Therefore, in order to reduce the number of fishes used in the assessment of acute aquatic toxicity, besides in vivo test using alternative species, the use of log k in BMC could become a simple, economic and high reproducible option.

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References

[1] M. Nendza, Structure-Activity Relationships in Environmental Sciences, first ed., Chapman & Hall, London (UK), 1998.
[2] M.T.D. Cronin, J.D. Walker, J.S. Jaworska, M.H.I. Comber, C.D. Watts, A.P. Worth, Environ. Health Perspec. 111 (2003) 1376.
[3] M.T.D. Cronin, J.S. Jaworska, J.D. Walker, M.H.I. Comber, C.D. Watts, A.P. Worth, Environ. Health Perspec. 111 (2003) 1391.
[4] R.L. Lipnick, in: G.M. Rand (Ed.), Fundamentals of Aquatic Toxicology, Taylor & Francis, Florida, 1995.
[5] T.W. Schultz, M.T.D. Cronin, J.D. Walker, A.O. Aptula, J. Mol. Struc. Theochem. 622 (2003) 1.
[6] T.W. Schultz, M.T.D. Cronin, T.I. Netzeva, J. Mol. Struct. Theochem. 622 (2003) 23.
[7] M.M. Hsieh, J.G. Dorsey, Anal. Chem. 67 (1995) 48.
[8] Z. Chilmonczyk, H. Ksycińska, I. Poleć, J Chromatogr. B 720 (720) (1998) 65.
[9] L.P. Szabó, J. Guczi, W. Kördel, A. Zsolany, V. Major, P. Keresztes, Chemosphere 39 (1999) 431.
[10] E.D. Breyer, J.K. Strasters, M.G. Khaledi, Anal. Chem. 63 (1991) 828.
[11] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, M.J. Medina-Hernández, R.M. Villanueva-Camañas, S. Sagrado, J. Chromatogr. B 814 (2005) 115.
[12] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, R.M. Villanueva-Camañas, M.J. Medina-Hernández, S. Sagrado, J. Chromatogr. A 1063 (2005) 153.
[13] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, M.J. Medina-Hernández, R.M. Villanueva-Camañas, S. Sagrado, TrAC Trends Anal. Chem. 25 (2006) 122.
[14] European Commission. Technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances and comission regulation (EC) No. 1488/94 on risk assessment for existing substances. Part III. Office for Official Publications of the European Communities, Luxembourg (1996). [http://ecb.jrc.it/Documents/TECHNICAL_GUIDANCE_DOCUMENT/EDITION_1/tgdpart3.pdf - search=%22tgdpart3.pdf%22].
[15] T.I. Netzeva, A.P. Worth, T. Aldenberg, R. Benigni, M.T.D. Cronin, P. Gramatica, J.S. Jaworska, S. Kahn, G. Klopman, C.A. Marchant, G. Myatt, N. Nikolova-Jeliazkova, G.Y. Patlewicz, R. Perkins, D.W. Roberts, T.W. Schultz, D.T. Stanton, J.J.M. van de Sandt, W. Tong, G. Veith, C. Yang, ATLA 33 (2005) 1.
[16] S.P. Bradbury, SAR QSAR Environ. Res. 2 (1994) 89.
[17] J.A. García-Domínguez, J.C. Díez-Masa, Pure Appl. Chem. 73 (2001) 969.
[18] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, R.M. Villanueva-Camañas, M.J. Medina-Hernández, S. Sagrado, J. Chromatogr. A 1094 (2005) 24.
[19] S. Ren, P.D. Frymier, T.W. Schultz, Ecotox. Environ. Safe. 55 (2003) 86.
[20] Commission of the European Communities 1993. Official Journal L 110, EEC Directive 93/21/EEC.
[21] S. Sagrado, M.T.D. Cronin, J. Chem. Inf. Model. 46 (2006) 1523.
[22] U.S. Environmental Protection Agency. Ecological Structure Activity Relationships (ECOSAR). [http://www.epa.gov/opptintr/newchems/tools/21ecosar.htm].
[23] T. Hartung, ECVAM. Institute for Health & Consumer Protection. Joint Research Centre. European Commission. Statement on the scientific validity of the upper threshold concentration (UTC) step-down approach—a new testing strategy to reduce the use of fish in acute aquatic toxicity testing.

Ispra (2006). [http://ecvam.jrc.it/ft_doc/ESAC statement UTC step down approach 20060515.pdf].
[24] H. Köneman, A. Musch, Toxicology 19 (1981) 223.
[25] J. Saarikoski, M. Viluksela, Ecotoxicol. Environ. Safe. 6 (1982) 501.
[26] M.T.D. Cronin, Y.H. Zhao, R.L. Yu, Environ. Toxicol. 15 (2000) 140.
[27] G. Schüürmann, A.O. Aptula, R. Kühne, R.U. Ebert, Chem. Res. Toxicol. 16 (2003) 947.