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Toxicity of 58 substituted anilines and phenols to algae *Pseudokirchneriella subcapitata* and bacteria *Vibrio fischeri*: Comparison with published data and QSARs

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ABSTRACT

A congeneric set of 58 substituted anilines and phenols was tested using the 72-h algal growth inhibition assay with *Pseudokirchneriella subcapitata* and 15-min *Vibrio fischeri* luminescence inhibition assay. The set contained molecules substituted with one, two or three groups chosen from -chloro, -methyl or -ethyl. For 48 compounds there was no REACH-compatible algal toxicity data available before. The experimentally obtained EC50 values (mg L^{-1}) for algae ranged from 1.43 (3,4,5-trichloroaniline) to 197 (phenol) and for *V. fischeri* from 0.37 (2,3,5-trichlorophenol) to 491 (aniline). Only five of the tested 58 chemicals showed inhibitory effect to algae at concentrations >100 mg L^{-1}, i.e. could be classified as "not harmful", 32 chemicals as "harmful" (10–100 mg L^{-1}) and 21 as "toxic" (1–10 mg L^{-1}). The occupied para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic. As a rule, the higher the number of substituents the higher the hydrophobicity and toxicity. However, in case of both assays, the compounds of similar hydrophobicity showed up to 30-fold different toxicities. There were also assay/organism dependent tendencies: phenols were more toxic than anilines in the *V. fischeri* assay but not in the algal test. The comparison of the experimental toxicity data to the data available from the literature as well as to QSAR predictions showed that toxicity of phenols to algae can be modeled based on hydrophobicity, whereas the toxicity of anilines to algae as well as toxicity of both anilines and phenols to *V. fischeri* depended on other characteristics in addition to logK_{ow}.

1. Introduction

REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals (EC, 2006) requires that all substances on the European market, which are manufactured or imported in quantities of 1 tonne or more per year have to be evaluated for hazardous effects to humans and environment by the year 2018. According to a recent evaluation the number of such chemicals is between 68 000 and 101 000 (Costanza and Hartung, 2009), exceeding the earlier estimate of 30 000 chemicals by the European Commision (Pedersen et al., 2003). This translates into expensive and ethically questionable toxicity testing unless alternative methods will be used (Hartung, 2009). For chemicals lacking experimental toxicity data the quantitative structure–activity relationships (QSARs) are expected to fill the gap (for a Review, see Netzeva et al., 2007). QSARs have been occasionally used in the regulatory assessment of chemicals in the EU but under REACH the use of QSARs is expected to increase remarkably. However, the existing QSAR models, including those proposed or developed for regulatory purposes (e.g., US EPA ECOSAR, Danish (Q)SAR Database) still require improvement (Reuschenbach et al., 2008). It has been recognized that their sometimes poor predictive performance is also due to scarce and inconsistent experimental toxicity data on which the models have been built.

According to REACH the basic ecotoxicological information requirements for substances manufactured or imported in quantities of 1–10 tonnes per year include short-term toxicity testing on crustaceans (preferred species *Daphnia,* OECD, 2004) and growth inhibition on aquatic plants (algae preferred, OECD, 2006). In addition, short-term toxicity testing on fish (OECD, 1992) is required in the next tonnage level (>10 tonnes per year). These three organism groups (crustaceans, algae, fish) represent different trophic levels of the aquatic food web, all of which have to be protected. The chemicals are classified according to the response of the most sensitive of these three species. However, REACH-compatible and reliable (eco)toxicity data can be found in few datasets, the biggest of which contains fathead minnow (*Pimephales promelas*) data on

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about 550 chemicals while the largest *Daphnia* dataset contains about 370 EC50 values. The situation is even worse for algal toxicity as there is no consistent dataset with more than 100 values (Netzeva et al., 2007). In addition, algal test results vary considerably due to the use of many different algal species and methods (see Section 3.2.). A number of studies have found algae more sensitive to chemicals than fish (Weyer et al., 2000; Hutchinson et al., 2003; Kahru and Dubourguier, 2010). This implies that reliable algal toxicity data may help to reduce the number of fish needed for regulatory toxicity testing (Jeram et al., 2005).

Another alternative to higher organisms in toxicity testing is the use of bacterial toxicity assays. The most widely used bacterium for the ecotoxicity analysis is naturally luminescent gram-negative marine bacterium *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*) for which toxicity data are available for more than 1000 chemicals (Kaiser and Palabrica, 1991). There are two ISO standards concerning the luminescence inhibition assay with *V. fischeri*: one for the water samples (ISO, 2007), and the most recent one for sediments, solid and colored samples (ISO, 2010). A number of comparisons of the *V. fischeri* test (e.g., Microtox™) with other toxicity bioassays have been done and significant correlations for many species, including fish, crustaceans and algae have been shown (Kaiser, 1998).

The aim of the current work was to obtain and critically analyze the toxicity data of a congeneric set of anilines and phenols to algae and bacteria in order to support the hazard classification and QSAR development for REACH. For that 58 substituted anilines and phenols were chosen and their toxicity tested with algae *Pseudokirchneriella subcapitata* and bacteria *V. fischeri*. The Algal OECD 201 assay was used due to its regulatory relevance and due to severe shortage of algal toxicity data. Bacteria *V. fischeri* were chosen for the comparison (decomposers *versus* primary producers) and due to the extensive *prior* use of *V. fischeri* data in QSAR modeling (Cronin and Schultz, 1997). In addition the data were compared to the available toxicity data from the literature and databases as well as to QSAR predictions.

Anilines and phenols are compounds of considerable industrial and commercial importance, which makes them important environmental pollutants (Keith and Telliard, 1979; Woo and Lai, 2004). Aniline (aminobenzene) and its derivates are introduced into the environment from many different fields of applications, such as the production of isocyanates, rubber processing chemicals, dyes and pigments, agricultural chemicals and pharmaceuticals (Rappoport, 2007). Phenol (hydroxybenzene) and its derivatives are released from industrial effluents such as those from the coal tar, gasoline, plastic, rubber proofing, disinfectant, pharmaceutical and steel industries and domestic wastewaters, agricultural runoff and chemical spills (Lin and Juang, 2009). However, for many substituted anilines and phenols there are no ecotoxicity data available. Moreover, all the 58 selected anilines and phenols have been pre-registered under REACH referring to European Union production or import quantities of 1 tonne or more per year. According to the European Chemical Substances Information System (ESIS, http://ecb.jrc.ec.europa.eu/esis/) database most of these chemicals have been used in quantities exceeding 10 tonnes per year.

2. Materials and methods

2.1. Chemicals

The 58 anilines and phenols chosen for this study were $\geqslant$95% pure (52 chemicals $\geqslant$98% pure). The chemicals are characterized in Table 1. For *V. fischeri* tests all stock-solutions were prepared in MilliQ-water and for the algae in the algal test medium. No co-solvents were used. If necessary, poorly soluble chemicals were dissolved by shaking the solutions overnight. The solutions were prepared in glass containers, sealed, stored in the dark at room temperature and tested for toxicity within 1–2 weeks. Although algal tests of phenols have been previously also performed in closed conditions (e.g. Chen and Lin, 2006) the volatility of aqueous solutions of our set of chemicals is not a concern in given conditions. The boiling points of phenol and aniline are 182 and 184 °C respectively, i.e. they evaporate at higher temperatures than water, the substituted anilines and phenols are even less volatile.

2.2. 72-h algal growth inhibition assay with P. subcapitata

In general, the OECD 201 algal growth inhibition test protocol (OECD, 2006) was followed. The algae were incubated in vials on a transparent shaking table that allowed simultaneous incubation of up to 136 samples. Algal biomass was measured by optical density at 682 nm directly from the incubation vials using a specially made vial holder for the spectrophotometer (Jenway 6300, Jenway Ltd., Essex, UK). This setting allowed to test 8–9 chemicals in one run in this otherwise laborious assay. In compliance with the OECD 201 guideline exponentially growing algal cultures were exposed to various concentrations of the test chemicals under controlled conditions whereas the concentration of algal cells in the control culture increased at least 16 times during 3 d. The algal biomass measurements were performed at least daily. The *P. subcapitata* stock culture for inoculation was taken from the commercial test system Algal Toxkit F (MicroBioTests Inc., Nazareth, Belgium). The number of the algal cells in the inoculum was determined by counting under microscope in the Neubauer haemocytometer and adjusted to yield 10 000 cells mL^{-1} in the sample after inoculation. The samples were incubated at 24 ± 1 °C for 72 h in 20-mL glass scintillation vials containing 9 mL of algal growth medium described in OECD 201 (2006). The vials were illuminated from below with Philips TL-D 38 W aquarelle fluorescent tubes. The pH of the medium was adjusted to 8.0 and did not change more than 0.5 units by the end of the test. All assays were run twice, all samples in duplicate with eight controls distributed evenly on the transparent table. A dilution series of aniline was included in all experiments as a positive control. In order to reduce the variability between the replicates the vials were single-use. The coefficient of variation of biomass density in replicate control cultures throughout the experiments did not exceed 5%. Each chemical was tested in either 6 or 7 concentrations, depending on previously available toxicity data from literature or preliminary experiments.

2.3. Acute bioluminescence inhibition assay with V. fischeri

The test (exposure time 30-s, 15-min and 30-min) was performed at room temperature (20 °C) in 96-well microplates following the Flash-assay protocol (ISO, 2010). The exact procedure is described in Mortimer et al. (2008) except the inhibition of bacterial bioluminescence was calculated as percentage of the unaffected control (2% NaCl). Reconstituted *V. fischeri* Reagent (Aboatox, Turku, Finland) was used for testing.

Chemicals and their dilutions were tested in 2% NaCl, at pH 6–7. Each chemical was tested in three different days, in 5–7 dilutions each in two replicates. The coefficient of variation of EC50 values obtained in different days did not exceed 20%. The luminescence was recorded with Microplate Luminometer Orion II (Berthold Detection Systems, Pforzheim, Germany), controlled by Simplicity Version 4.2 Software. Samples were not mixed during recording of the luminescence.

2.4. Statistical methods

The toxicity values (EC50) and their confidence intervals were determined from dose–response curves by the REGTOX software

Table 1
Characteristics of the studied chemicals.

No.	Chemical	Abbreviation	CAS no	Provider	Purity (%)	Molecular weight	Hydrophobicity ($\log K_{ow}$)[a]	Solubility (mg L^{-1})[a]
1	Aniline	A	62-53-3	Sigma–Aldrich	⩾99.5	93.1	0.90	36 000
2	2-chloroaniline	2-CA	95-51-2	Sigma–Aldrich	⩾99.5	127.6	1.90	8160
3	3-chloroaniline	3-CA	108-42-9	Sigma–Aldrich	99	127.6	1.88	5400
4	4-chloroaniline	4-CA	106-47-8	Sigma–Aldrich	98	127.6	1.83	3900
5	2,3-dichloroaniline	2,3-DCA	608-27-5	Sigma–Aldrich	99	162.0	2.82	262[b]
6	2,4-dichloroaniline	2,4-DCA	554-00-7	Sigma–Aldrich	99	162.0	2.78	620
7	2,5-dichloroaniline	2,5-DCA	95-82-9	Sigma–Aldrich	99	162.0	2.75	230[b]
8	2,6-dichloroaniline	2,6-DCA	608-31-1	Sigma–Aldrich	98	162.0	2.76	295[b]
9	3,4-dichloroaniline	3,4-DCA	95-76-1	Sigma–Aldrich	98	162.0	2.69	92
10	3,5-dichloroaniline	3,5-DCA	626-43-7	Acros-Organics	98	162.0	2.90	784
11	2,3,4-trichloroaniline	2,3,4-TCA	634-67-3	TCI Europe	>98	196.5	3.33	65[b]
12	2,4,5-trichloroaniline	2,4,5-TCA	636-30-6	Sigma–Aldrich	95	196.5	3.45	52[b]
13	2,4,6-trichloroaniline	2,4,6-TCA	634-93-5	Sigma–Aldrich	⩾98	196.5	3.52	40
14	3,4,5-trichloroaniline	3,4,5-TCA	634-91-3	Sigma–Aldrich	97	196.5	3.32	67[b]
15	2-methylaniline	2-MA	95-53-4	Fluka	⩾99.5	107.2	1.32	16 600
16	3-methylaniline	3-MA	108-44-1	Fluka	⩾99	107.2	1.40	15 000
17	4-methylaniline	4-MA	106-49-0	Fluka	⩾98	107.2	1.39	6500
18	2,3-dimethylaniline	2,3-DMA	87-59-2	Sigma–Aldrich	99	121.2	2.17[b]	5050[b]
19	2,4-dimethylaniline	2,4-DMA	95-68-1	Fluka	>98	121.2	1.68	6070[b]
20	2,5-dimethylaniline	2,5-DMA	95-78-3	Sigma–Aldrich	99	121.2	1.83	5600
21	2,6-dimethylaniline	2,6-DMA	87-62-7	Fluka	>98	121.2	1.84	8240
22	3,4-dimethylaniline	3,4-DMA	95-64-7	Fluka	⩾98	121.2	1.84	3800
23	3,5-dimethylaniline	3,5-DMA	108-69-0	Fluka	⩾97	121.2	2.17[b]	2050[b]
24	2,4,6-trimethylaniline	2,4,6-TMA	88-05-1	Sigma–Aldrich	98	135.2	2.72[b]	617[b]
25	2-ethylaniline	2-EA	578-54-1	Sigma–Aldrich	98	121.2	1.74	5320[b]
26	3-ethylaniline	3-EA	587-02-0	Sigma–Aldrich	98	121.2	2.11[b]	2320[b]
27	4-ethylaniline	4-EA	589-16-2	Fluka	>98	121.2	1.96	2110[b]
28	2,6-diethylaniline	2,6-DEA	579-66-8	Sigma–Aldrich	98	149.2	3.15[b]	670
29	Phenol	P	108-95-2	Merck	⩾95	94.1	1.46	82 800
30	2-chlorophenol	2-CP	95-57-8	Sigma–Aldrich	>99	128.6	2.15	11 300
31	3-chlorophenol	3-CP	108-43-0	Sigma–Aldrich	98	128.6	2.50	26 000
32	4-chlorophenol	4-CP	106-48-9	Sigma–Aldrich	>99	128.6	2.39	24 000
33	2,3-dichlorophenol	2,3-DCP	576-24-9	Sigma–Aldrich	98	163.0	2.84	3600
34	2,4-dichlorophenol	2,4-DCP	120-83-2	Sigma–Aldrich	99	163.0	3.06	4500
35	2,5-dichlorophenol	2,5-DCP	583-78-8	Sigma–Aldrich	99.7	163.0	3.06	2000
36	2,6-dichlorophenol	2,6-DCP	87-65-0	Sigma–Aldrich	99	163.0	2.75	1900
37	3,4-dichlorophenol	3,4-DCP	95-77-2	Sigma–Aldrich	99	163.0	3.33	9260
38	3,5-dichlorophenol	3,5-DCP	591-35-5	Sigma–Aldrich	97	163.0	3.62	5380
39	2,3,4-trichlorophenol	2,3,4-TCP	15950-66-0	Sigma–Aldrich	99	197.4	3.80	98[b]
40	2,3,5-trichlorophenol	2,3,5-TCP	933-78-8	Sigma–Aldrich	99.2	197.4	3.84	90[b]
41	2,3,6-trichlorophenol	2,3,6-TCP	933-75-5	Sigma–Aldrich	99.6	197.4	3.77	450
42	2,4,5-trichlorophenol	2,4,5-TCP	95-95-4	Sigma–Aldrich	99.6	197.4	3.72	1200
43	2,4,6-trichlorophenol	2,4,6-TCP	88-06-2	Sigma–Aldrich	98	197.4	3.69	800
44	2-methylphenol	2-MP	95-48-7	Merck	>99	108.1	1.95	25 900
45	3-methylphenol	3-MP	108-39-4	Merck	>99	108.1	1.96	22 700
46	4-methylphenol	4-MP	106-44-5	Merck	>98	108.1	1.94	21 500
47	2,3-dimethylphenol	2,3-DMP	526-75-0	Fluka	>99	122.2	2.48	4570
48	2,4-dimethylphenol	2,4-DMP	105-67-9	Fluka	>97	122.2	2.30	7870
49	2,5-dimethylphenol	2,5-DMP	95-87-4	Sigma–Aldrich	>99	122.2	2.33	3540
50	2,6-dimethylphenol	2,6-DMP	576-26-1	Sigma–Aldrich	>99	122.2	2.36	6050
51	3,4-dimethylphenol	3,4-DMP	95-65-8	Fluka	⩾98	122.2	2.23	4760
52	3,5-dimethylphenol	3,5-DMP	108-68-9	Sigma–Aldrich	⩾99	122.2	2.35	4880
53	2,3,5-trimethylphenol	2,3,5-TMP	697-82-5	Sigma–Aldrich	99	136.2	3.15[b]	762
54	2,3,6-trimethylphenol	2,3,6-TMP	2416-94-6	Sigma–Aldrich	95	136.2	2.67	1580
55	2,4,6-trimethylphenol	2,4,6-TMP	527-60-6	Sigma–Aldrich	97	136.2	2.73	1200
56	2-ethylphenol	2-EP	90-00-6	Sigma–Aldrich	99	122.2	2.47	5340
57	3-ethylphenol	3-EP	620-17-7	Sigma–Aldrich	98.9	122.2	2.40	11 300[b]
58	4-ethylphenol	4-EP	123-07-9	Sigma–Aldrich	99	122.2	2.58	4900

[a] Data from the SRC PhysProp Database (http://www.srcinc.com/what-we-do/databaseforms.aspx?id=386).
[b] Calculated values.

for Microsoft Excel (Vindimian, 2009) using the Log-normal model. Prism 5 (GraphPad Software Inc. www.graphpad.com) was used for calculations of algal growth rate and statistical significance of correlations. In order to evaluate the fit of QSAR predictions to experimental data, a method suggested by Golbraikh and Tropsha (2002) was used. When observed values are compared to predicted values not only linear correlation but also an exact fit is required and the linear regression should thus have a zero intercept (an intercept other than zero would mean the prediction needs adjustment and is therefore less accurate). The following parameters were calculated: linear correlation coefficient R^2 between observed and predicted values; correlation coefficients (R_0^2) and slopes (K) of linear regressions when intercept was set to zero. In the latter case the predicted *versus* observed and observed *versus* predicted correlation coefficients and slopes are different and designated as R_0^2, K and $R_0'^2$, K' respectively. The prediction is considered acceptable when (Golbraikh et al., 2003):

$$R^2 > 0.6 \quad (1)$$

Table 2
Toxicity (EC50, mg L^{-1}) of 58 substituted anilines and phenols to *Pseudokirchneriella subcapitata* and *Vibrio fischeri*.

No.	Chemical[a]	*P. subcapitata*			*V. fischeri*		
		72-h EC50[b] (mg L^{-1})	95% confidence Interval		15-min EC50[b] (mg L^{-1})	95% confidence Interval	
1	A	54.2	49.5	59.4	491	462	533
2	2-CA	39.1	36.6	43.5	42.8	39.0	47.1
3	3-CA	26.9	26.1	27.5	64.3	62.9	68.9
4	4-CA	3.55	2.31	5.50	15.5	15.2	17.3
5	2,3-DCA	6.75	5.22	7.22	14.2	14.0	14.8
6	2,4-DCA	3.96	3.32	4.27	16.6	16.1	17.5
7	2,5-DCA	16.5	11.7	25.2	16.7	15.4	18.3
8	2,6-DCA	23.2	22.5	26.6	13.0	12.6	13.8
9	3,4-DCA	2.50	1.97	2.99	4.28	4.23	4.71
10	3,5-DCA	4.39	3.71	5.17	35.8	34.3	37.8
11	2,3,4-TCA	3.55	3.16	3.98	10.5	9.25	12.2
12	2,4,5-TCA	3.14	1.88	5.65	7.92	7.14	8.87
13	2,4,6-TCA	4.94	4.74	5.57	>15	–	–
14	3,4,5-TCA	1.43	1.03	1.81	11.7	11.3	12.1
15	2-MA	109	99.6	113	187	179	201
16	3-MA	26.9	21.8	31.9	91.1	88.3	94.4
17	4-MA	42.7	28.8	50.3	41.6	40.3	47.2
18	2,3-DMA	30.8	16.8	35.2	117	104	133
19	2,4-DMA	39.4	34.7	46.3	77.7	72.4	88.0
20	2,5-DMA	70.6	66.7	78.5	66.8	66.1	72.1
21	2,6-DMA	107	105	109	77.8	73.9	84.0
22	3,4-DMA	7.34	5.35	10.1	6.98	6.62	7.66
23	3,5-DMA	27.8	26.1	28.8	71.8	62.9	85.4
24	2,4,6-TMA	20.3	19.3	25.1	89.8	87.7	94.7
25	2-EA	49.2	43.2	52.3	57.4	56.1	59.9
26	3-EA	14.2	10.9	17.9	41.4	40.7	44.0
27	4-EA	8.82	5.28	11.1	1.48	1.35	1.65
28	2,6-DEA	41.5	37.7	44.5	5.53	4.99	6.23
29	P	197	172	209	165	153	185
30	2-CP	51.8	42.8	66.2	69.5	62.8	77.0
31	3-CP	11.5	10.9	13.0	32.3	29.5	34.7
32	4-CP	31.4	29.3	33.5	9.71	9.08	10.6
33	2,3-DCP	10.9	10.1	11.5	13.4	12.5	13.6
34	2,4-DCP	8.13	2.00	15.8	7.14	6.51	7.50
35	2,5-DCP	3.68	2.37	5.21	10.3	9.54	10.7
36	2,6-DCP	16.1	10.7	18.1	16.5	15.3	17.3
37	3,4-DCP	2.19	1.85	2.50	3.60	3.27	3.87
38	3,5-DCP	2.10	1.86	2.82	2.66	2.60	2.70
39	2,3,4-TCP	4.16	3.66	4.69	0.90	0.85	0.91
40	2,3,5-TCP	2.26	1.99	2.67	0.37	0.35	0.38
41	2,3,6-TCP	8.05	7.46	10.2	3.30	3.07	3.41
42	2,4,5-TCP	7.57	5.93	7.99	0.56	0.53	0.57
43	2,4,6-TCP	5.64	4.87	7.02	3.61	3.38	3.72
44	2-MP	127	122	130	38.7	35.6	42.6
45	3-MP	145	141	150	36.1	34.8	39.9
46	4-MP	57.6	45.8	72.6	4.73	4.56	5.06
47	2,3-DMP	48.1	41.7	56.2	11.0	9.84	12.43
48	2,4-DMP	19.3	11.3	25.4	4.91	4.77	5.12
49	2,5-DMP	32.5	28.8	37.1	27.5	26.8	30.1
50	2,6-DMP	41.6	34.0	43.9	54.5	49.2	60.7
51	3,4-DMP	32.0	24.4	42.0	3.12	3.02	3.27
52	3,5-DMP	27.2	26.0	29.3	42.1	41.3	44.3
53	2,3,5-TMP	13.5	13.3	15.0	20.8	19.9	23.0
54	2,3,6-TMP	14.2	13.2	15.8	17.1	16.5	17.4
55	2,4,6-TMP	9.64	8.60	11.1	35.6	35.2	36.6
56	2-EP	31.4	30.5	33.9	39.7	36.1	43.3
57	3-EP	40.3	35.1	44.1	6.97	6.46	7.60
58	4-EP	21.9	19.1	28.3	0.43	0.40	0.48

[a] Abbreviations are explained in Table 1.
[b] The presented toxicity values are based on nominal initial exposure concentrations in a static test.

$$\frac{R^2 - R_0^2}{R^2} < 0.1 \text{ and } 0.85 \leqslant K \leqslant 1.15 \quad (2)$$

or

$$\frac{R^2 - R_0'^2}{R^2} < 0.1 \text{ and } 0.85 \leqslant K' \leqslant 1.15 \quad (3)$$

and

$$|R_0^2 - R_0'^2| \leqslant 0.3 \quad (4)$$

2.5. Previously published toxicity data

In order to compare the experimental data to previously existing toxicity data for the studied anilines and phenols relevant values were obtained from the US EPA ECOTOX (http://cfpub.epa.gov/ecotox/) database and from published papers. US EPA ECOTOX database search for algal toxicity data was performed in December 2010 using the Advanced Database Query and results (LC50, LD50, EC50, ED50, IC50, ID50) were downloaded as a Microsoft Excel

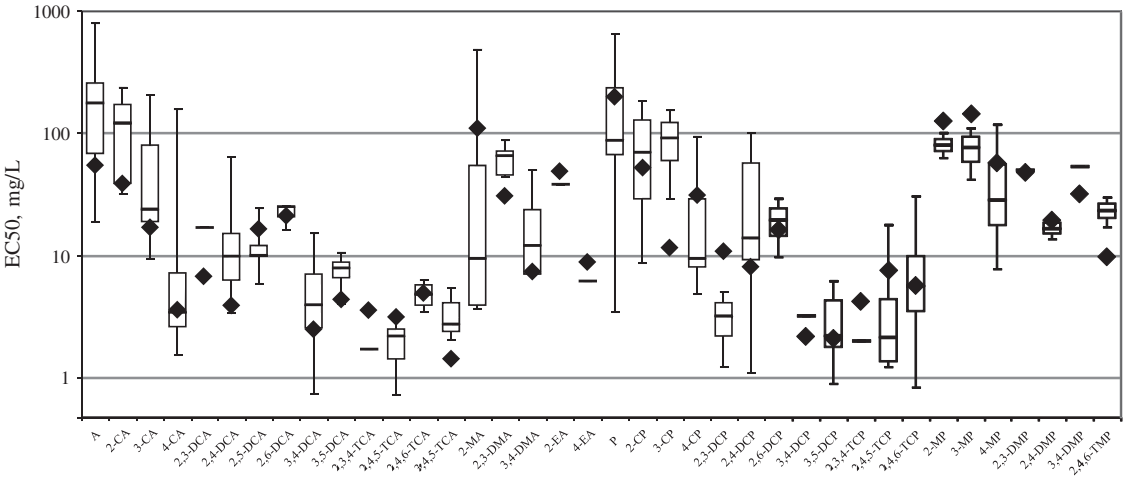

Fig. 1. Variation of *Chlorophyta* toxicity data EC50 (mg L^{-1}) (exposure time 1–4 d, median values) from US EPA ECOTOX database and published papers (see Table S1) on a boxplot. Experimental toxicity data from this study for *Pseudokirchneriella subcapitata* from Table 2 are shown as filled symbols. Abbreviations of the chemical names are explained in Table 1.

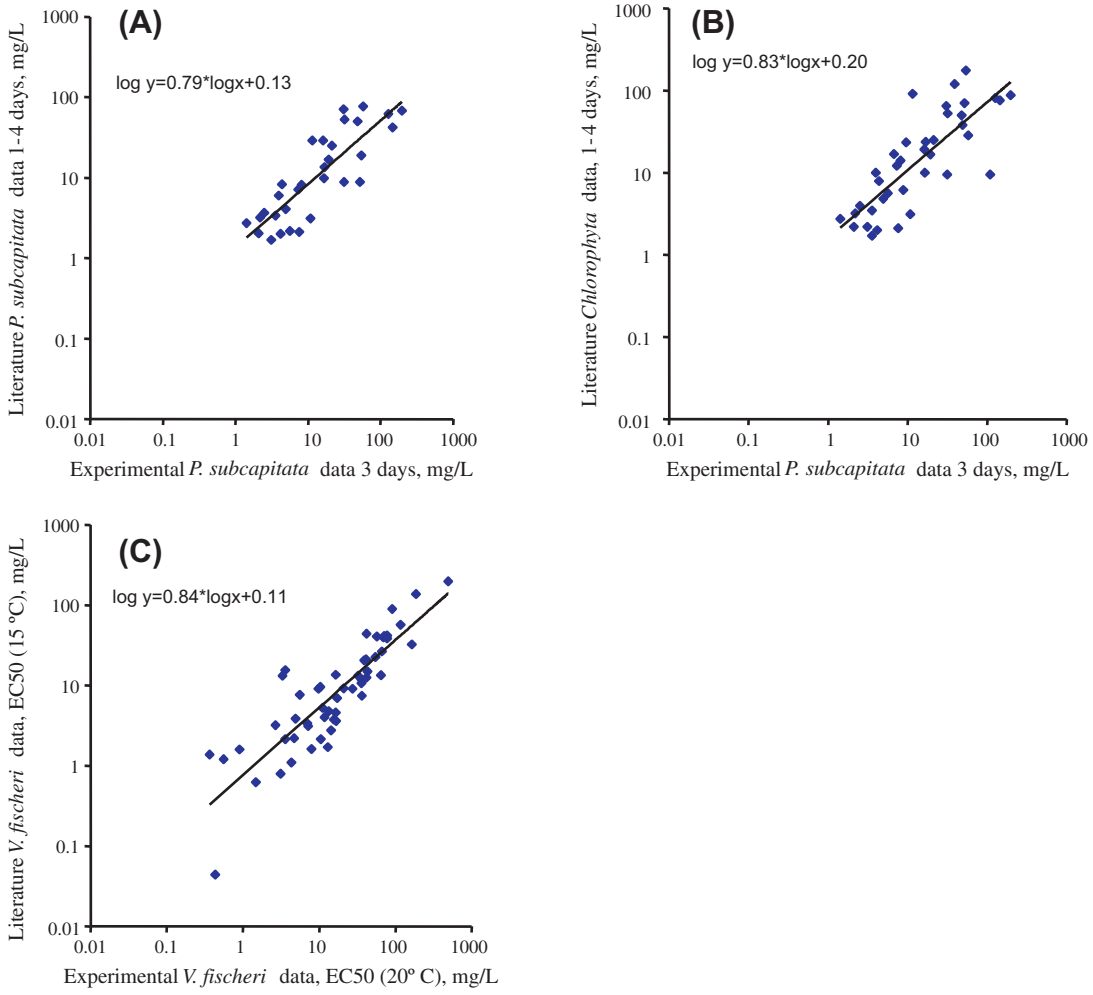

Fig. 2. Literature *versus* experimental data **A** – *Pseudokirchneriella subcapitata* toxicity data (1–4 d, median values) plotted against experimental data, $**R^2 = 0.71$, $p < 0.01$; **B** – *Chlorophyta* toxicity data (1–4 d, median values) plotted against experimental data, $R^2 = 0.64$, $n = 38$, $p < 0.01$, **C** – *Vibrio fischeri* toxicity data (15 min, 15 °C, median values) plotted against experimental data (15 min, 20 °C), $R^2 = 0.74$, $n = 54$, $p < 0.01$. Experimental data were taken from Table 2.

spreadsheet. Literature search of published toxicity data was carried out using Google Scholar, Science Direct and ISI Web of Knowledge. Additional toxicity data on algae and bacteria, which were not present in the US EPA ECOTOX database were collected from the literature (Table S1). The published data on *V. fischeri* luminescence inhibition assay were mainly obtained from the book

"Ecotoxicity of Chemicals to *Photobacterium phosphoreum*" by Kaiser and Devillers (1994).

3. Results and discussion

3.1. Experimental toxicity data

Experimentally determined EC50 values of *P. subcapitata* 72-h growth inhibition, as well as *V. fischeri* 15-min luminescence inhibition for the 28 anilines and 30 phenols are listed in Table 2. The algal EC50 values (mg L^{-1}) ranged from 1.43 (3,4,5-TCA) to 197 (phenol) and bacterial EC50 values from 0.37 (2,3,5-TCP) to 491 (aniline). Thus, the toxicities to algae spanned two orders of magnitude and to bacteria three orders of magnitude. The toxicity of the studied compounds was dependent on the type (chloro-, methyl-, ethyl-), number (mono-, di-, tri-) and position (ortho-, meta-, para-) of the substituents. The chloro-substituted molecules were generally more toxic than alkyl-substituted ones. Among mono-substituted substances the substituent in the para-position tended to increase toxicity whereas most of the ortho-substituted congeners were the least toxic. Similarly, the para-substituent tended to increase the toxicity of di-substituted molecules, especially when combined with the meta-substituent (i.e., 3,4-disubstituted). As a rule, the higher the number of substituents the higher the hydrophobicity and toxicity (Fig. 3). There were also assay-dependent tendencies: phenols were more toxic than anilines in the *V. fischeri* bioluminescence inhibition assay but not in the algal growth inhibition assay.

3.2. Experimental versus published data

Concerning published toxicity data on algae (see Section 2.5, Table S3) there were only 19 values for 10 compounds with strictly the same test conditions available, i.e. for the *P. subcapitata* 72 h growth inhibition test. When other exposure durations between 1 and 4 d were included, 118 data points for 31 substances were found. When all *Chlorophyta* 1–4 d toxicity data were included, altogether 228 data points for 38 substances were obtained (as defined in the ECOTOX database, the *Chlorophyta* included *Pseudokirchneriella*, *Chlamydomonas*, *Chlorella*, *Scenedesmus* and *Chlorococcales*). The variability of these *Chlorophyta* data are illustrated in Fig. 1. The presented toxicity values are based on nominal initial exposure concentrations in a static test. There was wide variation in the toxicity values reported for the same substances/species in different publications, in some cases spanning several orders of magnitude. However, the median values were generally in reasonable agreement with our experimental values (filled symbols in Fig. 1). Our experimental data are compared to median

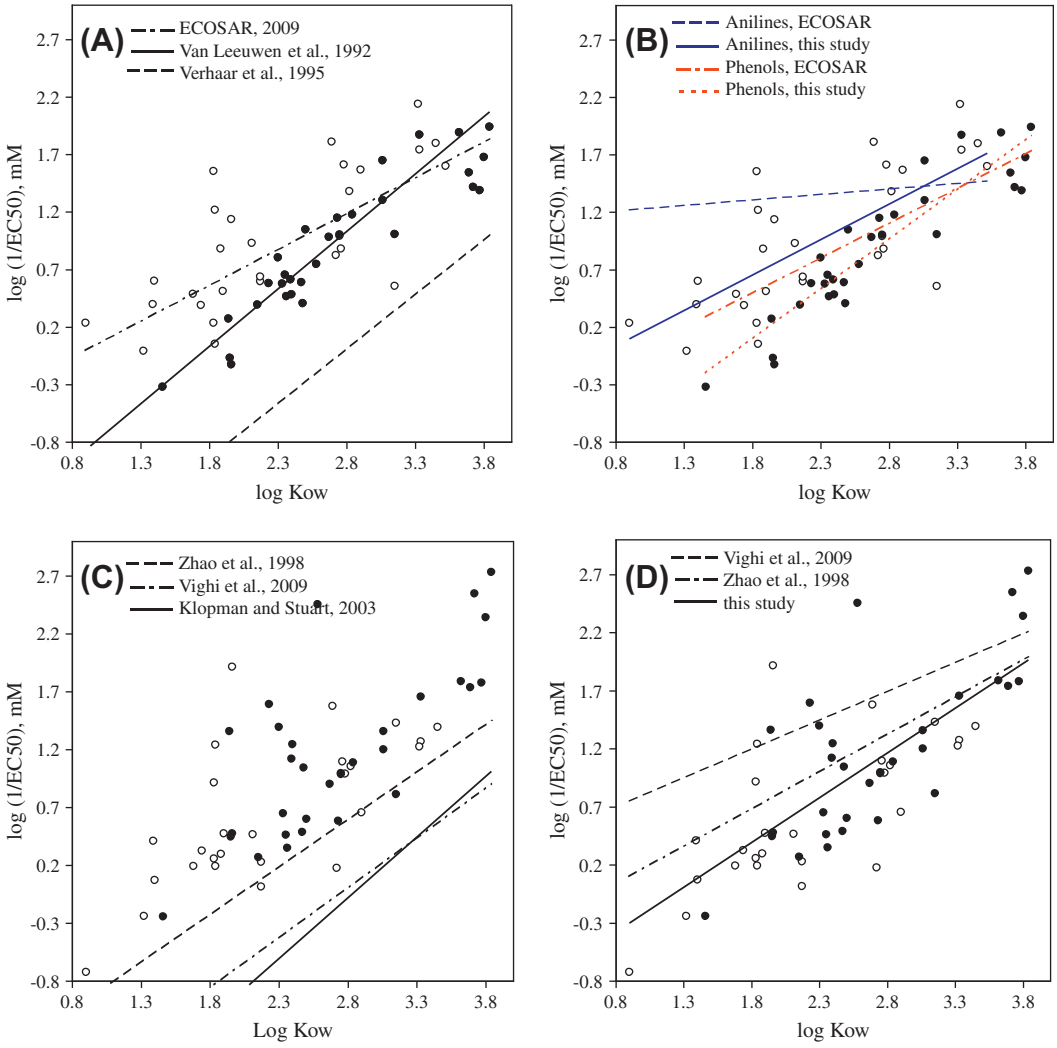

Fig. 3. Comparison of the experimental toxicity data with QSAR predictions. A and B – *Pseudokirchneriella subcapitata* (72-h EC50, mM), C and D – *Vibrio fischeri* (15-min EC50, mM), filled symbols depict phenols and open symbols anilines, equations for the lines are presented in Table 3.

Table 3
logK_{ow} based linear regression equations compared to experimental toxicity data of the studied 58 anilines and phenols. See also Fig. 3.

Organism/test endpoint	Chemical class	Equation	Training set size	R^2 reported for the model	Predicted *versus* observed R^2	R_0^2	K	$R_0'^2$	K'	Figure	Reference
Algae, growth inhibition (EC50, mM)											
Green algae (4d)	Nonpolar narotics	log (1/EC50) = 0.627 * logK_{ow} − 0.569	51	0.60	–	–	–	–	–	Fig. 3A	ECOSAR (2009)
Chlorella vulgaris (5d)	Nonpolar narotics	log (1/EC50) = 0.954 * logK_{ow} − 2.66	34	0.92	–	–	–	–	–	Fig. 3A	Verhaar et al. (1995)
Pseudokirchneriella subcapitata (3–4d)	Nonpolar narotics	log (1/EC50) = 1.00 * logK_{ow} − 1.77	10	0.93	–	–	–	–	–	Fig. 3A	Van Leeuwen et al. (1992)
Pseudokirchneriella subcapitata (3d)	Anilines and phenols	log (1/EC50) = 0.652 * logK_{ow} − 0.712	58	0.60	0.60	0.60	1.00	0.40	0.88		This study
Pseudokirchneriella subcapitata (3d)	Anilines	log (1/EC50) = 0.870 * logK_{ow} − 1.47	28	0.55	0.55	0.51	1.15	0.48	0.75	Fig. 3B	This study
Pseudokirchneriella subcapitata (3d)	Phenols	log 1/EC50 = 0.617 * logK_{ow} − 0.459	30	0.85	0.85	0.75	0.91	0.42	1.02	Fig. 3B	This study
Green algae (4d)	Anilines	log (1/EC50) = 0.095 * logK_{ow} + 1.14	4	0.18	0.55	0.11	0.72	−99.91	1.05	Fig. 3B	ECOSAR (2009)
Green algae (4d)	Phenols	log (1/EC50) = 0.609 * logK_{ow} − 0.599	40	0.67	0.85	0.73	0.92	0.29	1.00	Fig. 3B	ECOSAR (2009)
Algae	Not reported	EC50 Multicase model	476	n.r.	0.17	−0.03	0.65	−0.17	1.08		Danish (Q)SAR Database
Bacteria, luminescence inhibition (EC50, mM)											
Vibrio fischeri (15-min, 20 °C)	Nonpolar narotics	log (1/EC50) = 0.824 * logK_{ow} − 1.71	33	0.85	–	–	–	–	–	Fig. 3C	Zhao et al. (1998)
Vibrio fischeri (5-min, 15 °C)	Nonpolar narotics	log (1/EC50) = 1.05 * logK_{ow} − 3.02	179	0.94	–	–	–	–	–	Fig. 3C	Klopman and Stuart (2003)
Vibrio fischeri (15-min, 15 °C)	Nonpolar narotics	log (1/EC50) = 0.856 * logK_{ow} − 2.42	25	0.84	–	–	–	–	–	Fig. 3C	Vighi et al. (2009)
Vibrio fischeri (15-min, 20 °C)	Anilines and phenols	log (1/EC50) = 0.771 * logK_{ow} − 0.996	57	0.56	0.56	0.56	1.00	0.34	0.84	Fig. 3D	This study
Vibrio fischeri (15-min, 20 °C)	Anilines	log (1/EC50) = 0.619 * logK_{ow} − 0.727	27	0.47	0.47	0.46	0.87	0.26	0.87		This study
Vibrio fischeri (15-min, 20 °C)	Phenols	log (1/EC50) = 0.848 * logK_{ow} − 1.14	30	0.55	0.55	0.55	1.07	0.24	0.82		This study
Vibrio fischeri (15-min, 20 °C)	Polar narcotics	log (1/EC50) = 0.645 * logK_{ow} − 0.48	10	0.84	0.56	0.51	0.87	−0.34	0.94	Fig. 3D	Zhao et al. (1998)
Vibrio fischeri (15-min, 15 °C)	Polar narcotics	log (1/EC50) = 0.497 * logK_{ow} + 0.304	24	0.81	0.56	0.36	0.65	−4.01	1.18	Fig. 3D	Vighi et al. (2009)

Notes:
R^2 – correlation coefficient of linear regression.
R_0^2 – correlation coefficient of predicted *versus* observed linear regression when intercept is set to zero.
K – slope of predicted *versus* observed linear regression when intercept is set to zero.
$R_0'^2$ – correlation coefficient of observed *versus* predicted linear regression when intercept is set to zero.
K' – slope of observed *versus* predicted linear regression when intercept is set to zero.
"–" – not relevant.
n.r. – not reported.

values from the above-described datasets (i.e. for *P. subcapitata* and *Chlorophyta*) in Fig. 2A and B. Expectedly, the data for the same species correlated more closely with our experimental values (log–log $R^2 = 0.71$, $n = 31$) compared to data for *Chlorophyta* (log–log $R^2 = 0.64$, $n = 38$) but both correlations are significant ($p < 0.01$).

In the case of bacteria there were more toxicity data on anilines and phenols available in the literature (Table S3): for the conventional *V. fischeri* bioluminescence inhibition assay (Microtox) there were 111 data points for 54 substances with 15-min exposure time and 15 °C. Again, the median values of the published toxicity data correlated well with our experimental EC50 values on *V. fischeri* performed on 96-well microplates at 20 °C (log–log $R^2 = 0.74$, $n = 54$, $p < 0.01$; Fig. 2C).

3.3. Experimental data compared to QSAR-predicted toxicities

A valid QSAR model should be based on and used for compounds that act through a common or very similar mode of action (Verhaar et al., 1996). The most common method to group chemicals according to the mode of action is the Verhaar scheme that distinguishes four classes based on structural features of the molecules: class 1 – inert chemicals or non-polar narcotics; class 2 – less inert chemicals or polar narcotics; class 3 – reactive chemicals; and class 4 – specifically acting chemicals (Verhaar et al., 1992). The toxicity of the chemicals in classes 1 and 2 is known to be proportional to hydrophobicity (octanol/water partitioning coefficient, K_{ow}). Using toxicity data for a number of species, the class 2 chemicals have been shown to be 5–10 times more toxic than class 1 chemicals with the same K_{ow} (Vaal et al., 1997). This increased toxicity is often called "excess toxicity" as compared to the "baseline toxicity" of class 1 chemicals. All the 58 chosen chemicals belong to Verhaar class 2. The logK_{ow} values of the 58 compounds vary from 0.9 to 3.8 (Table 1), which is useful for logK_{ow} based QSAR modeling as the quality of the model usually increases with increasing logK_{ow} range (Dearden et al., 2009). As a next step in the analysis, experimental data were compared to existing QSAR predictions for algae and bacteria (Table 3, Fig. 3). The US EPA ECOlogical Structure Activity Relationships (ECOSAR, http://www.epa.gov/oppt/newchems/tools/21ecosar.htm) software is a QSAR tool that predicts the toxicity of industrial chemicals to aquatic organisms such as fish, aquatic invertebrates and algae. The classification of chemicals according to the ECOSAR is also included in the OECD QSAR software. In EU, the European Chemicals Bureau and the Danish EPA have jointly produced an internet-accessible version of the Danish (Q)SAR Database which can be used to retrieve predictions of *P. subcapitata* toxicity. In addition, QSAR equations can be found in the EU guidance documents (ECB, 2003; ECHA, 2008) as well as in scientific papers.

3.3.1. Algae: models versus experimental data

In Fig. 3A the experimental algal toxicity data (Table 2) were compared to three baseline QSARs (Van Leeuwen et al., 1992; Verhaar et al., 1995; ECOSAR, 2009). Note that the toxicity values are in the form of log $1/EC_{50}$ (mM). Theoretically all these three baselines describing the toxicity of class 1 chemicals should be similar and the toxicity of our set of chemicals (class 2) should be 5–10 times higher. However, Fig. 3A shows that only the QSAR suggested by Verhaar et al. (1995) and not the other two equations were in agreement with this concept. In comparison to this lowest baseline the toxicity of the anilines and phenols was up to 300-fold higher. Interestingly, the QSAR by Verhaar is not based on *P. subcapitata* but *Chlorella vulgaris* toxicity data (on 34 chemicals; $n = 34$). The other QSAR equations were built on toxicity data of *P. subcapitata*, ($n = 10$; Technical Guidance Document on Risk Assessment: TGD ECB, 2003) or several species including *P. subcapitata*, ($n = 51$; ECOSAR).

Concerning class 2 chemicals the TGD lacks an algal toxicity QSAR for and the Danish (Q)SAR database does not contain detailed information on the models, stating that it uses a Multicase model based on a training set of 476 chemicals (ECB, 2005). Apparently, this model uses other descriptors in addition to logK_{ow}, but still failed to predict our experimental data (predicted *versus* observed $R^2 = 0.166$, see Table 3). Comparison of our experimental toxicity data to the ECOSAR equations is shown in Fig. 3B. Note that ECOSAR provides different equations for anilines and phenols. While neither of the QSARs was acceptable according to strict validation criteria (see Section 2.4; Golbraikh et al., 2003) the observed toxicity of phenols was much closer to the prediction (Fig 3B, Table 3). The ECOSAR model for phenols is built on 40 chemicals whereas the aniline equation is derived from just 4 data points and does not correlate with hydrophobicity. Likewise, our own log 1/EC50 *versus* logK_{ow} regression line for phenols showed better fit than the one for anilines or the whole set of chemicals (Fig. 3B, Table 3). This is a consequence of much wider variation in the aniline data, with up to 30-fold difference in EC50 values for anilines with the same hydrophobicity. Similar results were obtained by analyzing the toxicity values for *P. subcapitata* by Chen et al. (2007). 17 chemicals that overlapped between the two studies, 12 anilines and 5 phenols, were compared. Analogously to our data, there was poor correlation between toxicity and logK_{ow} of anilines ($R^2 = 0.26$, $p > 0.1$) but a good correlation between the toxicity of the phenols and logK_{ow} ($R^2 = 0.85$, $p < 0.03$, data not shown).

3.3.2. Bacteria: models versus experimental data

Although toxicity of chemicals to bacteria is not taken into account in ecotoxicity assessment for regulatory purposes, a number of QSARs for the toxicity of different chemical groups and mixtures to *V. fischeri* can be found in the literature (Lessigiarska et al., 2005) (Table S2). The *V. fischeri* experimental data (Table 2) were compared to baseline equations as well as equations for polar narcotic chemicals (Table 3, Fig. 3C and D). The experimental values (log 1/EC50) were higher than all three baselines (Zhao et al., 1998; Klopman and Stuart, 2003; Vighi et al., 2009) and thus in accordance with the concept of excess toxicity of class 2 chemicals. Still, the toxicity of the tested anilines and phenols to *V. fischeri* was not well explained by hydrophobicity as evidenced by the distribution of values on Fig. 3C. Differently from algae, when the data for anilines and phenols were studied separately the correlations did not improve (Table 3). In addition, the comparison of our data with different class 2 QSARs showed that the best fit was observed for the equation based on toxicity data obtained at 20 °C (Zhao et al., 1998). Also, our work on the toxicities of aniline and phenol to *V. fischeri* at different temperatures has shown 2-fold decrease in toxicity at 20 °C compared to 15 °C in all incubation time-points (5, 15 and 30 min; unpublished data). This should be taken into account when comparing *V. fischeri* toxicity data.

3.4. Classification based on environmental hazard

Classification and labeling involves an evaluation of the intrinsic hazard of a chemical and communication of that hazard *via* the label. This evaluation must be made as set out in the new Classification, Labelling and Packaging Regulation (CLP; EC, 2008) for any substance or mixture/preparation manufactured or imported for the EU. Currently there are more than 7000 hazardous substances listed in the Annex VI to the CLP Regulation (previously Annex I to Directive 67/548/EEC; EC, 1967), however, the number of hazardous chemicals used in EU market is much bigger. By January 3rd 2011, European Chemicals Agency, ECHA (http://echa.europa.eu/) had received 3114 835 notifications of 24 529 substances for the Classification and Labeling Inventory. Comparing the classification and labeling of the selected 58 anilines and phenols it

Table 4
Classification of the studied chemicals.

No.	Chemical[a]	Production volume according to ESIS[b]	Classified under Annex I of directive 67/548/EEC[c]	Classification according to Annex VI of Directive 67/548/EEC[d]			
				Algae[e]	Bacteria[f]	ECOSAR, green algae[g]	Danish (Q)SAR database (multicase)[h]
1	A	HPV	+ (N)	Harmful	Not harmful	Toxic	Harmful
2	2-CA	HPV	—	Harmful	Harmful	Toxic	Harmful
3	3-CA	LPV	—	Harmful	Harmful	Toxic	Harmful
4	4-CA	LPV	+ (N)	Toxic	Harmful	Toxic	Harmful
5	2,3-DCA	#	—	Toxic	Harmful	Toxic	Harmful
6	2,4-DCA	LPV	—	Toxic	Harmful	Toxic	Toxic
7	2,5-DCA	LPV	—	Harmful	Harmful	Toxic	Toxic
8	2,6-DCA	LPV	—	Harmful	Harmful	Toxic	Harmful
9	3,4-DCA	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
10	3,5-DCA	LPV	—	Toxic	Harmful	Toxic	Harmful
11	2,3,4-TCA	#	—	Toxic	Harmful	Toxic	Toxic
12	2,4,5-TCA	LPV	—	Toxic	Toxic	Toxic	Very toxic
13	2,4,6-TCA	#	—	Toxic		Toxic	Very toxic
14	3,4,5-TCA	#	—	Toxic	Harmful	Toxic	Toxic
15	2-MA	HPV	+ (N)	Not harmful	Not harmful	Toxic	Harmful
16	3-MA	HPV	+ (N)	Harmful	Harmful	Toxic	Harmful
17	4-MA	HPV	+ (N)	Harmful	Harmful	Toxic	Harmful
18	2,3-DMA	LPV	—	Harmful	Not harmful	Toxic	Harmful
19	2,4-DMA	HPV	—	Harmful	Harmful	Toxic	Toxic
20	2,5-DMA	LPV	—	Harmful	Harmful	Toxic	Toxic
21	2,6-DMA	HPV	+ (N)	Not harmful	Harmful	Toxic	Toxic
22	3,4-DMA	LPV	—	Toxic	Toxic	Toxic	Toxic
23	3,5-DMA	LPV	—	Harmful	Harmful	Toxic	Harmful
24	2,4,6-TMA	#	—	Harmful	Harmful	Toxic	Toxic
25	2-EA	LPV	—	Harmful	Harmful	Toxic	Harmful
26	3-EA	#	—	Harmful	Harmful	Toxic	Harmful
27	4-EA	#	—	Toxic	Toxic	Toxic	Harmful
28	2,6-DEA	LPV	+	Harmful	Toxic	Toxic	Toxic
29	P	HPV	+	Not harmful	Not harmful	Harmful	Harmful
30	2-CP	HPV	+ (N)	Harmful	Harmful	Harmful	Harmful
31	3-CP	#	+ (N)	Harmful	Harmful	Harmful	Very toxic
32	4-CP	HPV	+ (N)	Harmful	Toxic	Harmful	Harmful
33	2,3-DCP	LPV	—	Harmful	Harmful	Harmful	Harmful
34	2,4-DCP	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
35	2,5-DCP	LPV	—	Toxic	Harmful	Toxic	Toxic
36	2,6-DCP	LPV	—	Harmful	Harmful	Harmful	Toxic
37	3,4-DCP	#	—	Toxic	Toxic	Toxic	Toxic
38	3,5-DCP	#	—	Toxic	Toxic	Toxic	Harmful
39	2,3,4-TCP	#	—	Toxic	Very toxic	Toxic	Toxic
40	2,3,5-TCP	#	—	Toxic	Very toxic	Toxic	Toxic
41	2,3,6-TCP	#	—	Toxic	Toxic	Toxic	Very toxic
42	2,4,5-TCP	#	+ (N)	Toxic	Very toxic	Toxic	Toxic
43	2,4,6-TCP	HPV	+ (N)	Toxic	Toxic	Toxic	Toxic
44	2-MP	HPV	+	Not harmful	Harmful	Harmful	Harmful
45	3-MP	HPV	+	Not harmful	Harmful	Harmful	Toxic
46	4-MP	HPV	+	Harmful	Toxic	Harmful	Harmful
47	2,3-DMP	#	+ (N)	Harmful	Harmful	Harmful	Harmful
48	2,4-DMP	LPV	+ (N)	Harmful	Toxic	Harmful	Toxic
49	2,5-DMP	LPV	+ (N)	Harmful	Harmful	Harmful	Toxic
50	2,6-DMP	HPV	+ (N)	Harmful	Harmful	Harmful	Toxic
51	3,4-DMP	#	+ (N)	Harmful	Toxic	Harmful	Toxic
52	3,5-DMP	HPV	+	Harmful	Harmful	Harmful	Harmful
53	2,3,5-TMP	#	—	Harmful	Harmful	Toxic	Harmful
54	2,3,6-TMP	HPV	—	Harmful	Harmful	Harmful	Very toxic
55	2,4,6-TMP	#	—	Toxic	Harmful	Harmful	Toxic
56	2-EP	#	—	Harmful	Harmful	Harmful	Harmful
57	3-EP	#	—	Harmful	Toxic	Harmful	Very toxic
58	4-EP	LPV	—	Harmful	Very toxic	Harmful	Harmful

Notes:
– This substance has not been reported as an HPVC or LPVC.
+ (N) – substance is included in Annex I of directive 67/548/EEC and is classified as dangerous for the environment.
+ – substance is included in Annex I of directive 67/548/EEC, but is not classified as dangerous for the environment.
— substance is not included in Annex I of directive 67/548/EEC and does not have harmonised classification in EU.

[a] Abbreviations are explained in Table 1.

[b] ESIS – European Chemical Substances Information System (http://ecb.jrc.ec.europa.eu/esis/); HPVC – High Production Volume Chemical, production or import volume in EU exceeds 1000 tonnes per year per producer or importer; LPVC – Low Production Volume Chemical, production or import volumes in EU is between 10 tonnes and 1000 tonnes per year per producer or importer.

[c] EC, 1967.

[d] Chemicals are categorized as: very toxic – L(E)C50 $\leqslant$ 1 mg L^{-1}, toxic – 1 mg L^{-1} < L(E)C50 $\leqslant$ 10 mg L^{-1}, harmful - 10 mg L^{-1} < L(E)C50 $\leqslant$ 100 mg L^{-1}. In addition, L(E)C50 > 100 mg L^{-1} were designated as "not classified".

[e] Experimentally determined toxicity to *Pseudokirchneriella subcapitata*, 72-h EC50.

[f] Experimentally determined toxicity to *Vibrio fischeri*, 15-min EC50.

[g] Predicted toxicity, calculated using ECOSAR QSAR models of anilines and phenols for green algae, 96-h EC50.

[h] http://130.226.165.14/User_Manual_Danish_Database.pdf (ECB, 2005).

appeared that 34 of them had not been evaluated on the EU-level under previous legislation and 18 were classified as dangerous to the environment (symbol of danger "N"; Table 4). The list contains 19 high and 19 low production volume chemicals (HPVC and LPVC) for which there was no harmonized classification. This means that the information on environmental and health properties has to be obtained. However, available aquatic toxicity data on algae, daphnids and fish were far from complete, especially data obtained with standard test protocols. Notably, algae were the least represented group, with EC50 values available for only 10 substances, for daphnia and fish respectively 36 and 40 chemicals were covered. In the case of *V. fischeri* almost a complete set of toxicity data for the selected chemicals was available (112 EC50 values for 55 chemicals) (Table S3).

As QSARs are proposed for the hazard classification of chemicals, the 58 studied chemicals were classified using the ECOSAR and the Danish (Q)SAR database. The result of this analysis is presented in Table 4. The ECOSAR classified all anilines as toxic in disagreement with classification based on our experimental data on algae. In case of phenols, ECOSAR classified all 30 phenols as harmful or toxic, in 25 cases matching the classification based on our algal data. The results according to the Danish (Q)SAR database were equally inaccurate for both anilines and phenols, predicting the hazard class in roughly half of the cases (Table 4).

As mentioned, the toxicity of chemicals to bacteria is not taken into account in ecotoxicity assessment for regulatory purposes. However, comparison of the toxicity data for bacteria and algae (Fig. S1) shows that the EC50 values for the majority of the tested compounds for both organisms were between 1 and 100 mg L^{-1}. According to EU classification criteria described in Annex VI of Directive 67/548/EEC (EC, 1991), these chemicals could be considered "harmful" (10–100 mg L^{-1}) or "toxic" (1–10 mg L^{-1}; Table 4). Four compounds would be classified as "very toxic" (<1 mg L^{-1}) only based on bacterial data. The classification would overlap for 59% of substances (for 34 out of the 58 tested substances). This suggests that *V. fischeri* toxicity data may be useful for environmental toxicity screening. The outlook of replacing some of the time-consuming and expensive toxicity testing with the rapid bacterial bioluminescence assay is worth further consideration. In addition, the need to include bacterial data in the ecotoxicological risk assessment has been highlighted by Vighi et al. (2009), who have showed similarities between the QSAR models for *V. fischeri* with those for fish, algae and *Daphnia*.

4. Conclusions and outlook

Often the limiting factor in the development of QSARs is the availability of high quality toxicity data for congeneric chemicals, preferably measured in a single laboratory and using standardized test protocols. Probably the best homogenous toxicity dataset, extensively used for QSARs modeling, contains *Tetrahymena pyriformis* growth inhibition data for 2400 industrial organic compounds (Dimitrov et al., 2003). Indeed, bibliometric analysis (Table S2) shows that currently most of the QSARs have been constructed using toxicity data on fish and protozoa *Tetrahymena,* followed by *Daphnia* and the bacterium *V. fischeri*. Remarkably less QSARs have been developed on algal data. Thus the number of available QSARs is in correlation with the amount of experimental data available and not with the regulatory need.

In this paper a set of homogenous experimental toxicity data was generated for 58 substituted anilines and phenols using algae *P. subcapitata* and bacteria *V. fischeri*. For the 15 HPVC and 17 LPVC in our experimental set, the toxicity data obtained using the OECD 201 algal growth inhibition test were published for the first time. Only five of the tested 58 chemicals showed inhibitory effect to algae at concentrations >100 mg L^{-1}, i.e. could be classified as "not harmful", 32 chemicals as "harmful" (10–100 mg L^{-1}) and 21 as "toxic" (1–10 mg L^{-1}). Comparison of the experimental toxicity data with the predictions made using the existing QSAR models suggests that the toxicity of phenols to algae may be modeled with a simple hydrophobicity-based equation. Aniline toxicity to algae as well as toxicity of both anilines and phenols to *V. fischeri* depended on other characteristics in addition to logK_{ow}.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.05.023.

References

Chen, C.Y., Lin, J.H., 2006. Toxicity of chlorophenols to Pseudokirchneriella subcapitata under air-tight test environment. Chemosphere 62, 503–509.
Chen, C.Y., Ko, C.W., Lee, P.I., 2007. Toxicity of substituted anilines to *Pseudokirchneriella subcapitata* and quantitative structure-activity relationship analysis for polar narcotics. Environ. Toxicol. Chem. 26, 1158–1164.
Costanza, R., Hartung, T., 2009. Re-evaluation of animal numbers and costs for in vivo tests to accomplish REACH legislation requirements for chemicals–a report by the Transatlantic Think Tank for Toxicology (t4). ALTEX 26, 187–208.
Cronin, M.T.D., Schultz, T.W., 1997. Validation of *Vibrio fischeri* acute toxicity data: mechanisms of action–based QSARs for non-polar narcotics and polar narcotic phenols. Sci. Total. Environ. 204, 75–88.
Dearden, J.C., Cronin, M.T.D., Kaiser, K.L.E., 2009. How not to develop a quantitative structure–activity or structure–property relationship (QSAR/QSPR). SAR QSAR Environ. Res. 20, 241–266.
Dimitrov, S.D., Mekenyan, O.G., Sinks, G.D., Schultz, T.W., 2003. Global modeling of narcotic chemicals: ciliate and fish toxicity. J. Mol. Struct.: THEOCHEM 622, 63–70.
EC, 1967. Council Directive 67/548/EEC of 27 June 1967 on the Approximation of Laws, Regulations and Administrative Provisions Relating to the Classification, Packaging and Labelling of Dangerous Substances. Official J. of the European Communities P 196, European Commision, Brussels, Belgium.
EC, 1991. Annex VI to Directive 67/548/EEC of 9 July 1991. General Classification and Labelling Requirements for Dangerous Substances and Preparations. Official J. of the European Communities No. L180/45, European Commission, Brussels, Belgium.
EC, 2006. Regulation No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official J. of the European Union, L 396/1-849, European Commission, Brussels, Belgium.
EC, 2008. Regulation No. 1272/2008 of the European Parliament and of the Council of 16 December 2008 on Classification, Labelling and Packaging of Substances and Mixtures, Amending and Repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No. 1907/2006. Official J. of the European Union, L 353, European Commission, Brussels, Belgium.
ECB, 2003. Technical Guidance Document (TGD) on Risk Assessment of Chemical Substances, second ed. EUR 20418 EN/2, European Chemicals Bureau, Ispra, Italy.
ECB, 2005. User Manual for the Internet Version of the Danish (Q)SAR Database. European Chemicals Bureau, Ispra, Italy. <http://130.226.165.14/User_Manual_Danish_Database.pdf> (accessed 08.04.11).
ECHA, 2008. Guidance on Information Requirements and Chemical Safety Assessment, Chapter R.10: Characterisation of Dose [concentration]-Response for Environment. European Chemicals Agency, Helsinki, Finland. <http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r10_en.pdf?vers=20_08_08> (accessed 08.04.11).
Golbraikh, A., Tropsha, A., 2002. Beware of q2! J. Mol. Graph. Model. 20, 269–276.

Golbraikh, A., Shen, M., Xiao, Z., Xiao, Y.D., Lee, K.H., Tropsha, A., 2003. Rational selection of training and test sets for the development of validated QSAR models. J. Comput. Aided Mol. Des. 17, 241–253.

Hartung, T., 2009. Toxicology for the twenty-first century. Nature 460, 208–212.

Hutchinson, T.H., Barrett, S., Buzby, M., Constable, D., Hartmann, A., Hayes, E., Huggett, D., Länge, R., Lillicrap, A.D., Straub, J.O., Thompson, R.S., 2003. A strategy to reduce the numbers of fish used in acute ecotoxicity testing of pharmaceuticals. Environ. Toxicol. Chem. 22, 3031–3036.

ISO, 2007. ISO 11348-3:2007, Water Quality – Determination of the Inhibitory Effect of Water Samples on the Light Emission of *Vibrio fischeri* (Luminescent bacteria test) – Part 3: Method using Freeze-dried Bacteria. International Organization for Standardization, Geneva, Switzerland.

ISO, 2010. ISO 21338:2010, Water Quality – Kinetic Determination of the Inhibitory Effects of Sediment, other Solids and Coloured Samples on the Light Emission of *Vibrio fischeri* (Kinetic Luminescent Bacteria test). International Organization for Standardization, Geneva, Switzerland.

Jeram, S., Riego Sintes, J.M., Halder, M., Baraibar Fentanes, J., Sokull-Klüttgen, B., Hutchinson, T.H., 2005. A strategy to reduce the use of fish in acute ecotoxicity testing of new chemical substances notified in the European Union. Regul. Toxicol. Pharmacol. 42, 218–224.

Kahru, A., Dubourguier, H.-C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269, 105–119.

Kaiser, K.L., 1998. Correlations of *Vibrio fischeri* bacteria test data with bioassay data for other organisms. Environ. Health Perspect. 106, 583–591.

Kaiser, K.L.E., Devillers, J., 1994. Ecotoxicity of chemicals to *Photobacterium Phosphoreum*. Handbooks of ecotoxicological data. Gordon and Breach Science Publishers SA, Amsterdam.

Kaiser, K.L.E., Palabrica, V.S., 1991. *Photobacterium phosphoreum* toxicity data index. Water Poll. Res. J. Canada 26, 361–431.

Keith, L.H., Telliard, W.A., 1979. Priority pollutants 1: a perspective view. Environ. Sci. Technol. 13, 416.

Klopman, G., Stuart, S.E., 2003. Multiple computer-automated structure evaluation study of aquatic toxicity III. *Vibrio fischeri*. Environ. Toxixol. Chem. 22, 466–472.

Lessigiarska, I., Worth, A.P., Netzeva, T.I., 2005. Comparative Review of (Q)SARs for Acute Toxicity. EUR 21559 EN – 2005.

Lin, S.-H., Juang, R.-S., 2009. Adsorption of phenol and its derivatives from water using synthetic resins and low-cost natural adsorbents: a review. J. Environ. Manage. 90, 1336–1349.

Mortimer, M., Kasemets, K., Heinlaan, M., Kurvet, I., Kahru, A., 2008. High-throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles. Toxicol In Vitro 22, 1412–1417.

Netzeva, T., Pavan, M., Worth, A., 2007. Review of Data Sources, QSARs and Integrated Testing Strategies for Aquatic Toxicity. European Commission (EC), Joint Research Centre, Institute for Health and Consumer Protection, Ispra, Italy. <http://ecb.jrc.ec.europa.eu/documents/QSAR/EUR_22943_EN.pdf> (accessed 10.01.11).

OECD, 1992. OECD Guideline 203 for the Testing of Chemicals, Fish, Acute Toxicity Test. Organisation for Economic Cooperation and Development, Paris, France.

OECD, 2004. OECD Guideline 202 for the Testing of Chemicals, *Daphnia sp.* Acute Immobilisation Test. Organisation for Economic Cooperation and Development, Paris, France.

OECD, 2006. OECD Guideline 201 for the Testing of Chemicals, Alga, Growth Inhibition Test. Organisation for Economic Cooperation and Development, Paris, France.

Pedersen, F., de Bruijn, Jack, Munn, S., van Leeuwen, Kees, 2003. Assessment of Additional Testing Needs under REACH. Effects of (Q)SARS, Risk based Testing and Voluntary Industry Initiatives. European Commission (EC), Joint Research Centre, Institute for Health and Consumer Protection, Ispra, Italy.

Rappoport, Z., 2007. Patai series: The Chemistry of Functional Groups. The Chemistry of Anilines. Part I. Wiley.

Reuschenbach, P., Silvani, M., Dammann, M., Warnecke, D., Knacker, T., 2008. ECOSAR model performance with a large test set of industrial chemicals. Chemosphere 71, 1986–1995.

Vaal, M., Van der Wal, J.T., Hoekstra, J., Hermens, J., 1997. Variation in the sensitivity of aquatic species in relation to the classification of environmental pollutants. Chemosphere 35, 1311–1327.

Van Leeuwen, C.J., Van der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., Hermens, J.L.M., 1992. Application of QSARs, extrapolation and equilibrium partitioning in aquatic assessment: I Narcotic industrial pollutants. Environ. Toxicol. Chem. 11, 267–282.

Verhaar, H.J.M., Urrestarazu Ramos, E., Hermens, J.L.M., 1996. Classifying environmental pollutants. 2: Separation of class 1 (baseline toxicity) and class 2 (''polar narcosis'') type compounds based on chemical descriptors. J. Chemom. 10, 149–162.

Verhaar, H.J.M., Hermens, J.L.M., Mulder, W., 1995. QSAR for Ecotoxicity. EV5V-CT92-0211, RITOX, Utrecht, The Netherlands.

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

Vighi, M., Migliorati, S., Monti, G.S., 2009. Toxicity on the luminescent bacterium *Vibrio fischeri* (Beijerinck) I: QSAR equation for narcotics and polar narcotics. Ecotoxicol. Environ. Saf. 72, 154–161.

Vindimian, E., MSExcel macro REGTOX_EV7.0.5.xls; 2009. <http://www.normalesup.org/~vindimian/> (accessed 21.01.10).

Weyer, A., Sokull-Klüttgen, B., Baraibair-Fentanes, J., Vollmer, A., 2000. Acute toxicity data: A comprehensive comparison of results from fish, *Daphnia* and algae tests with new substances notified in the European Union. Environ. Toxicol. Chem. 19, 1931–1933.

Woo, Y.-T., Lai, D.Y., 2004. Aromatic Amino and Nitro–Amino Compounds and their Halogenated Derivatives. Patty's Toxicology. John Wiley & Sons Inc..

Zhao, Y.H., Cronin, M.T.D., Dearden, J.C., 1998. Quantitative structure-activity relationships of chemicals acting by non-polar narcosis – theoretical considerations. Quant. Struct.-Act. Relat. 17, 131–139.