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AN ALGAL TOXICITY DATABASE OF ORGANIC TOXICANTS DERIVED BY A CLOSED-SYSTEM TECHNIQUE

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Abstract—The current study presents the toxicity data of 90 organic compounds with various modes of actions to *Pseudokirchneriella subcapitata*. The assessment was conducted using a closed-system technique, and a biomass-type end point based on the cell density was employed. The above toxicity data were compared with test results from ciliate (*Tetrahymena pyriformis*), water flea (*Daphnia magna*), fish (*Pimephales promelas*), and luminescent bacteria (*Photobacterium phosphoreum*). Satisfactory correlation relationships between toxicity data from algae and other aquatic organisms were found (r^2 = 0.66–0.82). *Pseudokirchneriella subcapitata* revealed considerably higher sensitivity to organic toxicants compared with other organisms. Benzenes, aldehydes, and alkanes also were highly selective to the test alga. In addition, the results show that conventional algal batch tests tend to underestimate the toxicity of organic compounds, except in the case of 4-chlorophenol. Toxicity observed from the closed-system test is approximately 2- to 380-fold higher than that estimated by conventional batch tests. Such a phenomenon can be found in nearly all organic compounds, regardless of the chemical's Henry's law constant. In the risk assessment of chemicals, following the European Union's practice, approximately 30% (7 of 23) of the cases may result in a more strict classification when the batch test is replaced by the closed-system test. More effort therefore is needed to revise the algal toxicity database using the closed-system test method.

Keywords—Toxicity *Pseudokirchneriella subcapitata* Biomass Correlation Risk

INTRODUCTION

Algal toxicity tests have been widely used in bioassay test batteries to assess the relative toxicity of various toxic chemicals. Algal biomass and growth rate are two traditional response end points applied most commonly in previous studies of phytoplankton [1–6]. Analysis of experimental results from International Standards Organization ring tests [1,2] shows that median effective concentrations (EC50s) based on biomass ($EC50_b$) were generally lower and could differ by a factor of two compared with EC50s based on growth rate ($EC50_r$) [3,4]. Nevertheless, growth rate end point is still preferred by some toxicologists, because it is more stable, comparable, and ecologically relevant [3,5,6].

Algal toxicity tests also have been adopted by the European Commission as a standard test for risk assessment of existing and new chemical substances [7]. In the current practice for classification of new substances in the European Union (EU), the chemical risk is assessed by three aquatic standard test organisms, such as fish, *Daphnia* sp., and algae. Faucon et al. [8] analyzed 74 EU notifications of chemicals to explore the influences of different end points (fish median lethal concentration [LC50], *Daphnia* EC50, algal $EC50_b$, and algal $EC50_r$) for chemical classification and labeling of new substances. They concluded that the order of influence on classification and labeling is *Daphnia* > algae ($EC50_b$) > fish > algae ($EC50_r$). Therefore, the end point of $EC50_r$ was found to be the least influential parameter because of its relatively low sensitivity [8]. Weyers and Vollmer [5] conducted a similar analysis using the entire 694 data sets, which contained information regarding all three acute aquatic toxicity tests (fish, *Daphnia* sp., and algae), available in 1999 in the EU. They found that algal growth rate would still determine the classification more often than fish and daphnids would and, thus, support the use of the growth rate end point. Their analysis, however, also showed that $EC50_b$ would result in the strictest classification in 159 cases. When using the less-sensitive $EC50_r$, the algal test resulted in strictest classification in only 107 cases. Clearly, $EC50_b$ is a more influential end point than $EC50_r$ when species sensitivity is the major concern for chemical's classification.

The traditional batch-type test, which is adopted by most standard algal test protocols, has been criticized as being unsuitable for assessing the effects of volatile compounds [9,10]. Several closed-system tests had been proposed by previous researchers [11–14]. Generally speaking, most of these closed-system tests are considerably more complicated in experimental design compared to conventional batch techniques. Therefore, algal toxicity data derived from closed-system tests are still quite scarce. The authors' recent works have proposed a closed-system algal toxicity test technique with no headspace and with low bicarbonate buffer content [15,16]. The experimental design is quite simple, and the test revealed satisfactory sensitivities to both metallic and organic toxicants. The test technique has been successfully applied to assess the toxicity of aldehydes, chlorophenols, anilines, benzenes, alkanes, alcohols, ketones, and nitriles using algal growth rate and dissolved oxygen production as the test end points [15–21].

In the existing toxicity database, a significant portion of the data is based on various biomass-type end points, such as algal biomass, population, fluorescent absorbance, and so on. These data have significant influence on risk assessment of organic chemicals; therefore, the adequacy of these data requires careful review. The objective of the present study is to present a toxicity database for organic toxicants on *Pseudokirchneriella subcapitata* based on a biomass end point (the net production of algal cell density). Data for other aquatic organisms were selected to compare species sensitivity and to derive species correlation with *P. subcapitata*. Furthermore, chemicals with various modes of toxic action are employed to derive an overall

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comparison between the conventional batch test and the closed-system test. The adequacy of batch test results was examined based on the following toxicological characteristics: test sensitivity, species correlation, and the baseline toxicity relationship (quantitative structure–activity relationship based on the chemical's hydrophobicity). The possible impact on risk assessment of organic chemicals when the closed-system test technique is adopted by current practice also is analyzed.

MATERIALS AND METHODS

Toxicity testing

Algal inoculum (*P. subcapitata*, UTEX 1648 [University of Texas at Austin, Austin, TX, USA], a freshwater green alga) was withdrawn from a chemostat operated under steady state and transferred into 300-ml, biochemical-oxygen-demand (BOD) test bottles together with dilution water (with growth medium) and toxicants. The BOD bottles were completely filled, with no headspace left. A water seal was provided to ensure a closed-test environment. The bottles were then placed on an orbital shaker operated at 100 rpm. Temperature and light intensity were kept at 24 ± 1°C and 65 $\mu Em^{-2}s^{-1}$ (±10%), respectively. Algal growth medium [22] with no ethylenediaminetetra-acetic acid content was used for toxicity testing. The initial pH for the growth medium was 7.5, and the initial inoculated cell density was 15,000 cells/ml. All tests were conducted in triplicate, with a test duration of 48 h. The population density of the algae was determined using an electronic particle counter (Coulter Electronics, Luton, UK). The inhibition rate on the net increase of algal cell density was calculated as follows:

$$\% \text{ Inhibition} = [(X_t - X_0)/(X_c - X_0)]\cdot 100 \quad (1)$$

where X_t and X_c denote the final cell densities for treatment and control, respectively, and X_0 is the cell density at the beginning of the test for both treatment and control. The EC50 was determined using probit analysis. A detailed description regarding the test technique can be found in the authors' previous works [16].

Test chemicals

Seventy sets of data, including aldehydes, nitriles, anilines, chlorophenols, benzenes, alkanes, and alcohols, were derived from the raw data of the authors' previous works [15–21]. Median effective concentrations based on cell density were reanalyzed using Equation 1. In addition, 14 nonpolar narcotics (ethylbenzene, 2-chloro-*p*-xylene, nitrobenzene, 2-chlorotoluene, methylene chloride, trichloromethane, tetrachloromethane, 1,2-dichloropropane, 1,3-dichloropropane, 1-chlorobutane, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, trichloroethylene, and tetrachloroethylene), four polar narcotics (2-nitrophenol, 3-nitrophenol, 4-nitrophenol, and 2,4-dimethylphenol), and two reactive toxicants (2,4-dinitrophenol and 2,6-dinitrotoluene) were tested in the present study to improve the significances for species correlation and baseline toxicity analyses. All chemicals used were of reagent grade. The toxicant concentrations used in the present study are in the form of nominal concentrations. Before commencing the experiment, stock solution was freshly prepared, and its concentration was analyzed using high-performance liquid chromatography (HPLC; 2996 Photodiode Array Detector; Waters, Milford, MA, USA). All compounds were tested at least twice (i.e., range-finding test and definitive test). A rough estimate of the EC50 was made according to results from the range-finding test. At the beginning of the definitive test, one treatment concentration, which was closest to the estimated EC50, was selected for concentration check. This concentration check was conducted following the exact same procedures used for toxicity testing described above, except that algal inoculum was not added to the BOD bottle. The concentration of toxicant was then analyzed by HPLC. Normally, the difference between the nominal and measured concentration was within the range of 1 to 6%. Tests with a difference of greater than 6% were repeated.

Selection of literature data

Literature data for ciliate (*Tetrahymena pyriformis*), water flea (*Daphnia magna*), fish (*Pimephales promelas*, fathead minnow), luminescent bacteria (*Photobacterium phosphoreum*, Microtox; Azur Corp., Carlsbad, CA, USA), and alga (*Pseudokirchneriella subcapitata*) were selected from previous studies for correlation analyses and comparison. For a specific test organism, toxicity data of the same exposure time were selected, if possible, to eliminate any unnecessary discrepancy during correlation analyses. A brief description regarding data selection is as follows: *T. pyriformis*, EC50s with a test duration of 24 to 60 h (end points: Growth, mortality, and behavioral changes); *D. magna*, 48-h EC50 (end points: Immobile, mortality, distance moved, reproduction, and enzyme activity); fathead minnow, 96-h LC50 (end points: Mortality and equilibrium); *P. subcapitata*, 96-h EC50 (end points: Biomass, population, and chlorophyll content); Microtox: EC50s with 5, 15, or 30 min of exposure time (end point: Light emission). The sources for the literature data are listed at the bottom of the Appendix.

RESULTS AND DISCUSSION

The Appendix lists the EC50, mode of toxic action, molecular weight, 1-octanol/water partition coefficient, Henry's law constant, and solubility for 90 organic compounds as evaluated by the closed-system algal toxicity test. Literature data for *P. subcapitata*, based on the conventional batch tests, together with toxicity data for ciliate, water flea, fathead minnow, and the luminescent bacteria (Microtox) also are presented in the Appendix for comparison. The modes of action for the 90 chemicals were classified into five categories—polar narcosis, nonpolar narcosis, oxidative phosphorylation uncoupling, electrophilic/proelectrophilic, and respiratory inhibition—following the definitions of previous researchers [23,24]. Compounds from the third, fourth, and fifth categories also may be classified as reactive toxicants. For a specific compound, the most-sensitive organism was marked with an asterisk.

Figure 1 compares species sensitivity of various aquatic organisms (i.e., ciliate, water flea, fathead minnow, and luminescent bacteria) with *P. subcapitata* as assessed by the closed-system technique. The unit for EC50 or LC50 is mmol/L. The ciliate (*T. pyriformis*) is obviously quite insensitive to organic toxicants, because all data points in Figure 1a are located below the diagonal line, indicating that *P. subcapitata* is more sensitive to the test compounds. Furthermore, approximately 66% of the data in Figure 1b show that algae (*P. subcapitata*) are more sensitive than water fleas (*D. magna*). On the other hand, most polar narcotic compounds (or, more precisely, anilines) displayed an apparently higher toxicity to water fleas than to algae. For fathead minnow and luminescent bacteria (*Photobacterium phosphoreum*), 82 and 80% of the cases (from a total of 50 sets of data), respectively,

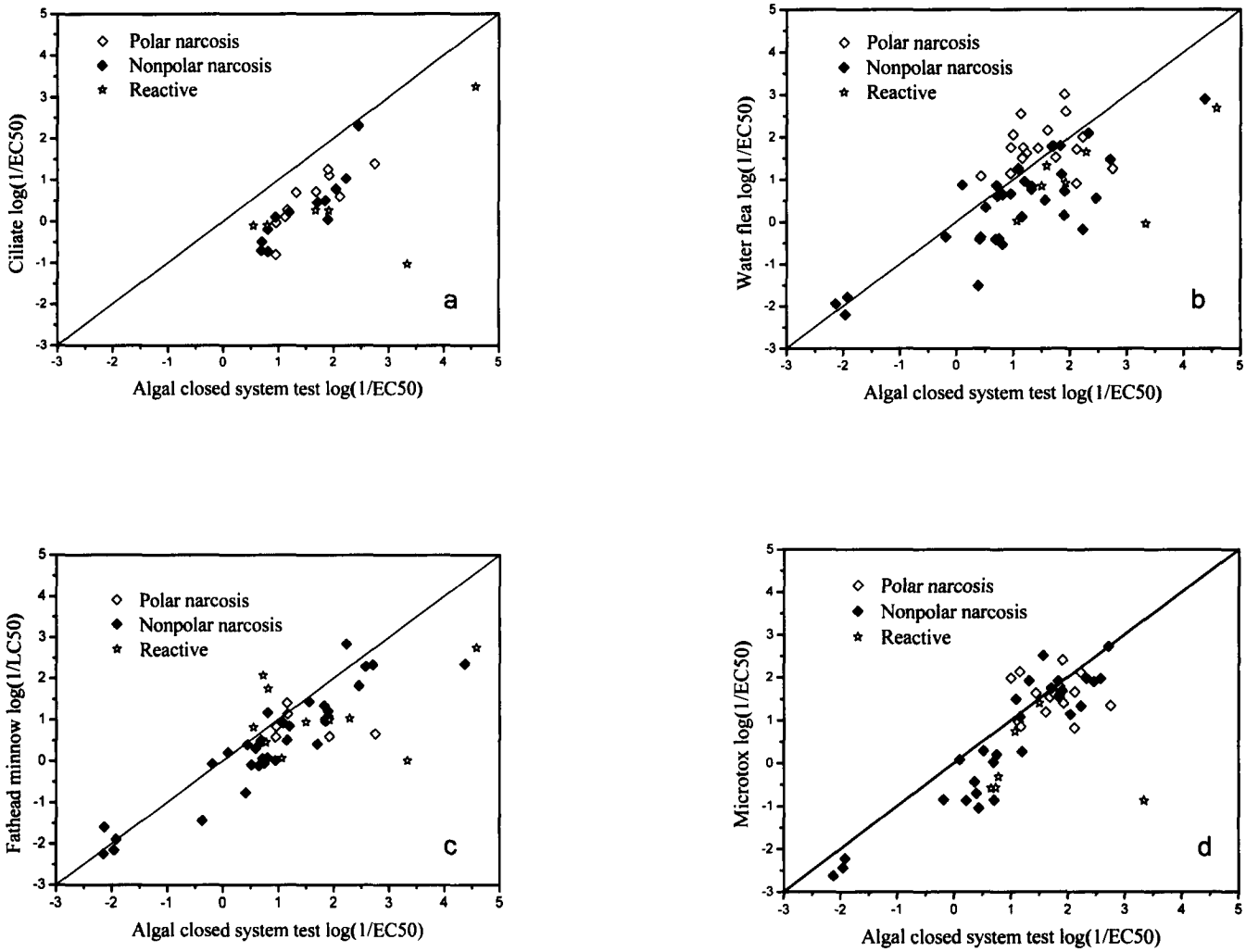

Fig. 1. Comparisons of species sensitivity. (**a**) Algae versus ciliate. (**b**) Algae versus water flea. (**c**) Algae versus fathead minnow. (**d**) Algae versus Microtox. EC50 = median effective concentration; LC50 = median lethal concentration.

showed that these two organisms were less sensitive than algae (Fig. 1c and d). Based on the available data in Appendix 1, the relative sensitivity for aquatic organisms to organic compounds is estimated as follows: *Pseudokirchneriella subcapitata* water flea (*D. magna*) > *Photobacterium phosphoreum* (Microtox) ≥ fathead minnow (*Pimephales promelas*) > ciliate (*T. pyriformis*). Because literature data were derived using different test methods and/or end points, the above relationship is just a rough estimation based on the overall performances of various organisms and is subject to change when more data are available.

Table 1. A summary of the most-sensitive counts for aquatic organisms to different types of organic compounds[a]

Chemical	Alga (closed-system test)	Ciliate	Water flea	Fathead minnow	Microtox
Anilines (13)[b]	3	—	10 (77%)[c]	—	—
Phenols (12)	7	—	3	—	2
Benzenes (14)	12 (85.7%)	—	1	1	—
Alkanes (18)	10 (55.6%)	—	2	2	4
Alcohols (3)	2	—	1	—	—
Ketones (2)	2	—		—	—
Nitriles (5)	2	—	—	3 (60%)	—
Aldehydes (9)	8 (88.9%)	—	—	1	—
Total (76)	46	0	17	7	6

[a] Scientific names for species: Algae, *Pseudokirchneriella subcapitata*; ciliate, *Tetrahymena pyriformis*; water flea, *Daphnia magna*; fathead minnow, *Pimephales promelas*; and Microtox, *Photobacterium phosphoreum*.
[b] Number of data for a specific category of chemicals.
[c] Percentage of the most-sensitive count for a species in relation to the total available data.

Table 1 summarizes the most-sensitive counts for various aquatic organisms (the numbers of chemicals marked by an asterisk in the Appendix) with respect to different types of organic compounds. Among the 76 chemicals listed in Table 1, algae were the most-sensitive species in 46 cases (60%). These counts generally reflect the order of the relative sensitivity relationship and are consistent with discussions in the previous paragraph. Furthermore, the most-sensitive counts in Table 1 provide a clear picture regarding chemical selectivity to different aquatic organisms. For example, the alga *Pseudokirchneriella subcapitata* is most sensitive to more than 85% of the benzenes and aldehydes. Second, 77% of the anilines are highly toxic to water fleas. One also may find that more than 55% of the alkanes are very toxic to algae. Finally, nitriles seemed to display strong selectivity to fathead minnow, because 60% (three of five) of the nitriles showed such a tendency. The selectivity of nitriles, however, still requires further verification based on more data.

Table 2. Species correlation analyses with respect to *Pseudokirchneriella subcapitata*[a]

Species	Closed-system test							Batch test				
	α	β	*n*	r^2	*F*	*p*	Outlier (ID)[b]	*n*	r^2	*F*	*p*	Outlier (ID)
Ciliate												
Overall	0.95	−1.05	27	0.82	110.46	1.14×10^{-10}	73	13	0.73	27.39	3.82×10^{-4}	
Polar	0.95	−0.97	10	0.69	18.09	2.71×10^{-3}		5	0.91	21.19	4.41×10^{-2}	
Nonpolar	1.14	−1.37	12	0.77	33.80	1.66×10^{-4}		6	0.53	4.54	0.1	
Water flea												
Overall	0.73	−0.03	57	0.54	64.56	7.91×10^{-11}	73	25	0.39	13.83	1.19×10^{-3}	
	0.71	−0.25	45	0.66	84.43	1.09×10^{-11}	73, 1~13	23	0.35	11.42	2.80×10^{-3}	3, 6
Polar	0.15	1.50	20	0.02	0.38	5.49×10^{-1}		7	0.27	1.46	2.93×10^{-1}	
Nonpolar	0.72	−0.39	31	0.69	63.64	8.64×10^{-9}		15	0.23	3.88	7.06×10^{-2}	
Fathead minnow												
Overall	0.74	−0.18	50	0.74	138.03	9.71×10^{-16}	73	23	0.29	8.16	9.77×10^{-3}	
	0.75	−0.26	48	0.81	193.62	3.97×10^{-18}	73, 84, 90					
Polar	0.13	1.12	7	0.07	0.36	5.81×10^{-1}		5	0.12	0.28	6.48×10^{-1}	
Nonpolar	0.82	−0.30	34	0.86	193.51	4.02×10^{-15}		15	0.21	3.40	8.82×10^{-2}	
Reactive	0.38	0.61	9	0.33	3.45	1.06	73					
	0.57	−0.02	7	0.85	27.58	3.33×10^{-3}	73, 84, 90					
Microtox												
Overall	1.07	−0.47	49	0.78	169.47	3.42×10^{-17}	73	18	0.12	2.08	1.70×10^{-1}	
Polar	0.03	1.51	15	0.001	0.01	9.12×10^{-1}		6	0.62	4.96	1.12×10^{-1}	
Non-polar	1.08	−0.50	29	0.86	169.49	3.91×10^{-13}		12	0.06	0.64	4.42×10^{-1}	

[a] Scientific names for species: Green algae, *Pseudokirchneriella subcapitata*; ciliate, *Tetrahymena pyriformis*; water flea, *Daphnia magna*; fathead minnow, *Pimephales promelas*; and Microtox, *Photobacterium phosphoreum*. Equation: $y = \alpha\chi + \beta$; p = level of significance.
[b] Outliers for the correlation relationship: 1–13, anilines; 73, acetaldehyde; 84, chloroacetonitrile, 90, malononitrile.

Species correlations

Species correlations between toxicity data of *P. subcapitata* and other aquatic organisms were analyzed (Table 2). Acetaldehyde was found to exert extremely high toxicity to *P. subcapitata* and, thus, was removed from all correlation analyses. From Table 2, one may find that algal toxicity data provide satisfactory correlations with other organisms, with r^2 varying from 0.74 to 0.82, except in the case of water fleas. Although not highly impressive, the above r^2 values were good enough considering that toxicants with various modes of action were included in the regression analyses. The correlation relationship between *P. subcapitata* and water fleas was considerably lower as compared to cases of other organisms, such as ciliate, fathead minnow, and luminescent bacteria (Microtox), with r^2 values equal to only 0.54. The high selectivity of anilines to water fleas, however, provides a good justification to exclude anilines from the regression, and doing so improves the r^2 value to 0.66. Furthermore, as shown in the Appendix, chloroacetonitrile and malononitrile were very toxic to fathead minnow (EC50, 1.35 and 0.56 mg/L, respectively). The above two toxicants exerted their toxic effects through the mechanism of respiratory inhibition, which should be valid only for fish or other animals. The r^2 value between algae and fathead minnow could thus be improved from 0.74 to 0.81 when the above two compounds were considered as outliers for the regression. To further justify the adequacy of removing chloroacetonitrile and malononitrile from the regression, analyses were conducted based on toxicity data related to reactive toxicants only. As shown in Table 2, the correlation relationship is poor, with r^2 equaling only 0.33. When chloroacetonitrile and malononitrile were removed, however, the r^2 value was raised to 0.85. This remarkable change in the r^2 value indicates that chloroacetonitrile and malononitrile have a unique pathway in fish, as they do in algal cells. Therefore, the identification of these two compounds as outliers is appropriate.

Table 2 shows more detailed analyses of species correlation, conducted with respect to nonpolar and polar narcotic compounds. Good correlations (r^2 = 0.69–0.86) existed between algae and other organisms for nonpolar narcotic chemicals. For polar narcotic compounds, correlations generally are poor (r^2 = 0.001–0.07), except in the case between algae and ciliate (r^2 = 0.69). Vaal et al. [25,26] pointed out that the reason for the high sensitivity of water fleas to anilines could be that anilines may act by a different mode of action in daphnids. Because two-thirds of polar narcotic compounds in the Appendix are anilines, results from the above studies [25,26] provide an explanation for the poor species correlations of polar narcotic compounds.

Species correlation analyses based on batch test results (Table 2), on the other hand, show no valid relationships with data for other aquatic organisms, except in the case of ciliate. The correlations between ciliate and algae (batch test) are less statistically significant, however, because the significance level (p) for these relationships is from 0.1 to 0.00038. The lack of species correlation for the batch test results (r^2 = 0.12–0.39, except in the case of ciliate), in contrast to the good species correlations (r^2 = 0.66–0.81) revealed by the closed-system test, suggests that organic toxicants were not properly assessed by the batch tests.

Baseline toxicity relationship

A large portion of the chemicals in common use by industries has been classified as nonpolar narcotic compounds [27]. Quantitative structure–activity relationships based on the chemical's hydrophobicity were established based on various organisms, such as fish, protozoa, and bacteria [28,29]. Such relationships are most important and fundamental in ecotoxicological studies and also are referred to as the baseline toxicity [28–30]. Figure 2 depicts the relationship between the observed toxicity with the logarithm of the 1-octanol/water

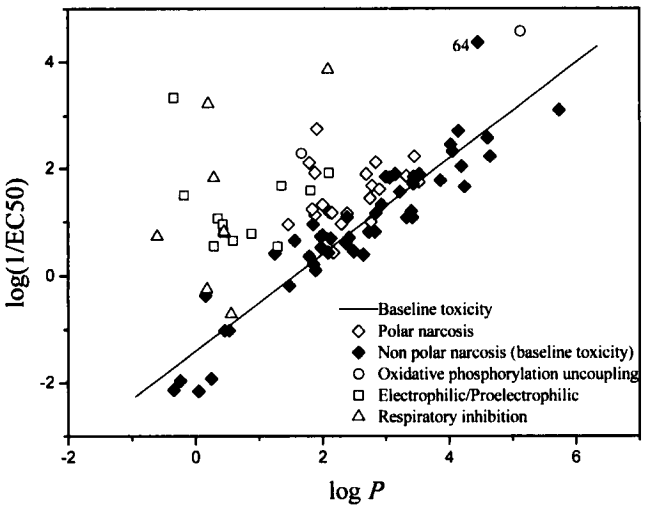

Fig. 2. Relationships between log(1/median effective concentration [EC50]) and log P based on the closed-system technique. The EC50s are reported in terms of mmol/L. Oxidative phosphorylation uncoupling, electrophilic/proelectrophilic, and respiratory inhibition are different mechanisms for reactive toxicants.

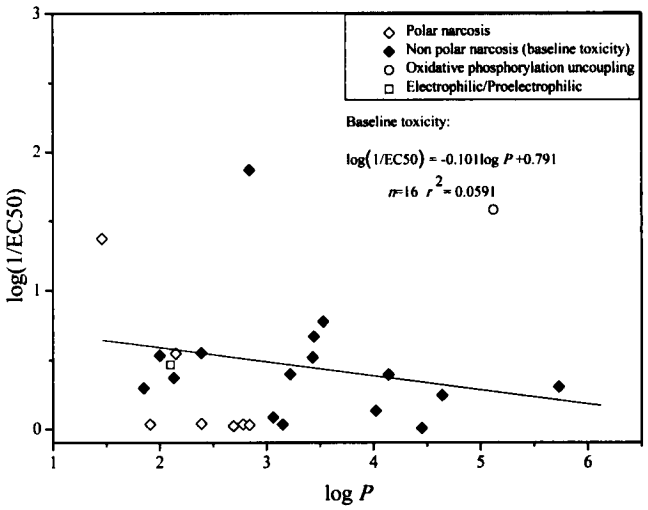

Fig. 3. A plot of log(1/median effective concentration [EC50]) and log P based on traditional batch tests. The EC50s are reported in terms of mmol/L. Oxidative phosphorylation uncoupling and electrophilic/proelectrophilic are different mechanisms for reactive toxicants.

partition coefficient (log P) for 48 nonpolar narcotic compounds. The baseline toxicity based on the end point of algal cell density is as follows:

$$\text{48-h log(1/EC50)} = 0.90 \log P - 1.40$$
$$n = 48, \qquad r^2 = 0.87 \qquad (2)$$

As shown in Figure 2, all experimental points for nonpolar chemicals fit reasonably well with Equation 2, except for 2,3,4,6-tetrachlorophenol (ID 64), which had a toxicity approximately 58-fold higher than that estimated by the baseline toxicity. Results from previous studies on ciliate and fish [31,32] also showed that 2,3,4,6-tetrachlorophenol displayed high excess toxicity compared to the baseline toxicity relationship. Schultz et al. [31] concluded that phenols with four or five halogen atoms, with pK_a values of between 4.3 and 6.3, should be classified as reactive toxicants instead of nonpolar narcotic chemicals. On an average basis, polar and reactive toxicants were 5.5- and 125-fold more toxic than estimated by the baseline toxicity relationship. The excess toxicity observed, revealed by polar and reactive toxicants, is consistent with observations based on fish or water flea [33–35].

A similar analysis based on data from algal batch tests is presented in Figure 3. The correlation between toxicity and log P is poor (r^2 = 0.06), in contrast to the good correlation observed from the closed-system test. A more serious fault is that more than 50% (five of nine cases) of the polar and reactive toxicants displayed an apparently lower toxicity than that estimated by the baseline toxicity equation shown in Figure 3. The above inconsistency in terms of species correlation and baseline toxicity relationship indicates that the reliability for the present algal toxicity database, which was derived primarily by conventional batch tests, is questionable. The closed-system technique, on the other hand, provides a more adequate assessment regarding the effects of organic toxicants.

Henry's law constant

Differences in test sensitivities between the conventional batch test and the closed-system technique were previously discussed in the authors' works using dissolved oxygen production or algal growth rate as the test end point [15–21]. Generally speaking, the closed-system technique is 2- to 200-fold more sensitive than the batch tests [15–21]. The Organization for Economic Co-Operation and Development, in its recent Guidance Document, states that "the losses due to volatilization may become significant for substances with Henry's law constants of 1–10 Pa m^3/mol under vigorous mixing conditions where the opportunity for water/air exchange is high" [10]. Thus, in the following paragraph, comparisons between batch and closed-system tests were conducted with special emphasis on chemical's volatile characteristics.

The EC50 ratio ($EC50_{batch}/EC50_{closed\ system}$) is applied to compare the performances of batch and closed-system tests. Figure 4 displays the plot of EC50 ratio versus the Henry's law constant. In Figure 4, EC50 ratios were mainly within the range of 2 to 380, except in the case of 4-chlorophenol (EC50 ratio, 0.57). It is not yet clear for 4-chlorophenol, however, why the batch test yielded a smaller EC50 than that from the closed-system test. Results from regression analyses showed that neither the Henry's law constant nor the water solubility revealed any correlation relationship with the EC50 ratio (r^2 = 0.01 and 0.02, respectively). Regression analysis using both parameters (the Henry's law constant and the water solubility) also

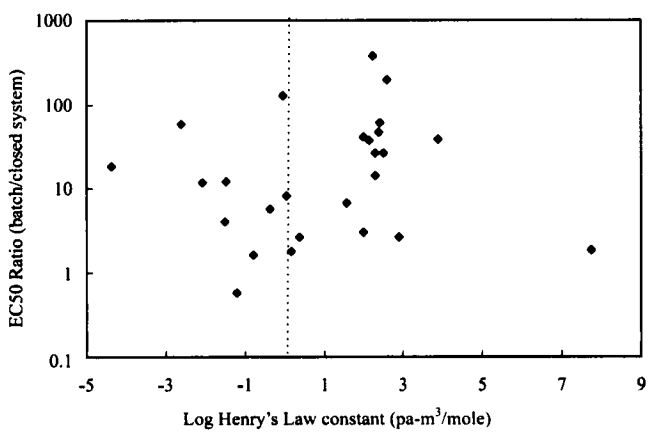

Fig. 4. Median effective concentration (EC50) ratios in relation to the Henry's law constant.

Table 3. Risk classifications of chemicals using batch or closed-system tests[a]

No.	Chemical	Algae (closed system) EC50 (mg/L)	Algae (batch test, open system) EC50 (mg/L)	Water flea EC50 (mg/L)	Fathead minnow LC50 (mg/L)	Analysis 1 (batch test)	Analysis 2 (closed system)	Remark
6	3,4-Dichloroaniline	2.02	3.67	0.16	9.96	R50	R50	—
14	Phenol*	10.53	129.00	6.60	24.60	R51	R51	—
15	2-Chlorophenol	8.63	70.00	3.91	9.41	R51	R51	—
16	4-Chlorophenol*	8.80	5.01	4.10	5.00	R51	R51	—
20	4-Nitrophenol*	0.25	4.89	7.68	30.40	R51	R50	New classification (small HL constant)
22	Benzene	15.77	29.0	200.00	24.60	R52	R52	—
23	Chlorobenzene	7.82	210.00	86.00	35.40	R52	R51	New classification
24	1,2-Dichlorobenzene	2.84	76.10	2.40	57.00	R51	R51	—
25	1,3-Dichlorobenzene	1.86	114.00	28.00	9.12	R51	R51	—
26	1,4-Dichlorobenzene	2.07	98.10	11.00	14.20	R52	R51	New classification
28	1,2,4-Trichlorobenzene	0.63	24.10	50.00	2.76	R51	R50	New classification
31	1,2,4,5-Tetrachlorobenzene	1.26	52.90	320.00	0.32	R50	R50	—
37	Ethylbenzene	1.34	3.60	75.00	9.09	R51	R51	—
39	Nitrobenzene	13.89	36.60	27.00	119.00	R52	R52	—
48	1,1,2,2-Tetrachloroethane	13.72	92.30	9.30	20.40	R51	R51	—
49	Pentachloroethane	5.61	80.30	63.00	7.34	R51	R51	—
50	Hexachloroethane	0.46	93.20	8.10	1.10	R51	R50	New classification
52	1,3-Dichloropropane	19.93	60.10	280.00	131.00	R52	R52	—
63	2,4-Dichlorophenol*	2.42	14.00	2.60	7.43	R51	R51	—
64	2,3,4,6-Tetrachlorophenol*	0.01	1.30	0.29	1.03	R50	R50	—
70	Pentachlorophenol*	0.007	0.42	0.55	0.48	R50	R50	—
71	2,4-Dinitrophenol*	0.94	10.90	4.10	17.00	R51	R50	New classification (small HL constant)
83	2,6-Dinitrotoluene*	2.19	84.91	21.70	18.50	R52	R51	New classification (small HL constant)

[a] Scientific names for species: Algae, *Pseudokirchneriella subcapitata*; water flea, *Daphnia magna*; and fathead minnow, *Pimephales promelas*. An asterisk indicates chemicals with a Henry's law (HL) constant of less than 1 Pa·m³/mol. EC50 = median effective concentration; LC50 = median lethal concentration; R50 = very toxic to aquatic organisms (EC50/LC50, <1 mg/L); R51 = toxic to aquatic organisms (EC50/LC50, 1–10 mg/L); R52 = harmful to aquatic organisms (EC50/LC50, 10–100 mg/L).

failed to establish any relationship. Therefore, for the following discussion, all data in Figure 4 were divided into two groups by the dotted line representing Henry's law constant equal to 1 Pa·m³/mol. Chemicals with Henry's law constant greater than 1 Pa·m³/mol have an average EC50 ratio of 52.8 (1.80–378.0; 95% confidence range = 7.2–98.4). For chemicals with Henry's law constants smaller than 1 Pa·m³/mol, the mean EC50 ratio is equal to 27.2 (0.57–130; 95% confidence range = −0.66–55.0). It is obvious that chemicals with high volatility (>1 Pa·m³/mol) did result in greater EC50 ratios compared to the less volatile compounds. The difference between the two groups of chemicals, however, is not statistically significant. Furthermore, the average EC50 ratio of 27.2 for compounds with a small Henry's law constant is already sufficient evidence showing that the toxicity of these chemicals was seriously underestimated by the conventional batch technique. Hence, results from the present study suggest that closed-system test should be adopted for evaluation of the hazardous effects of organic chemicals despite the magnitude of the Henry's law constant.

Risk assessment of chemicals

To explore the impact on risk assessment of chemicals when the batch test is replaced by the closed-system technique, three aquatic standard test organisms (fish, *Daphnia* sp., and algae) were chosen for analyses, following the current practice for classification of new substances in the EU. The Appendix lists 23 existing chemicals that can be applied for the comparison, as shown in Table 3. Among these 23 chemicals, only eight compounds have a Henry's law constant smaller than 1 Pa·m³/mol. Analysis 1 used the conventional batch test, together with results from fish and water flea tests, to establish the classifications of chemicals. In Analysis 2, the batch test results were replaced by the toxicity data based on the closed-system test. From the two series of classification, one finds that approximately 30% of the cases (7 of 23) were different. All seven cases became one step more critically classified when the closed-system test was applied. The proportion of the less volatile compounds is only about one-third in Table 3, but 43% of the new classifications are related to these compounds. There seems to be no clear difference between chemicals with high or low volatile characteristics.

CONCLUSION

The results of the present study show that the alga *P. subcapitata* is considerably more sensitive than other aquatic organisms, such as ciliate (*T. pyriformis*), water flea (*D. magna*), fish (*Pimephales promelas*), and luminescent bacteria (*Photobacterium phosphoreum*, Microtox), to organic toxicants. In addition, data from the closed-system test display good correlation relationships with test results from other aquatic organisms when a small fraction of outliers known to have different modes of action in organisms is removed. The conventional batch tests tend to underestimate the phytotoxicity of

organic compounds. Such a phenomenon can be found with nearly all organic compounds, regardless of the Henry's law constant of the chemical. The lack of species correlation and the inconsistency in the baseline toxicity relationship also are indications for the inadequacy of the current algal toxicity database, which was derived primarily by conventional batch tests. Furthermore, in risk assessment of chemicals, the present study shows that nearly 30% of the cases may result in stricter classifications when the batch test is replaced by the closed-system test. The above impact on risk assessment still needs to be verified by more extensive research in the future, but more effort clearly is needed to revise the algal toxicity database using the closed-system test technique.

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APPENDIX 1. Toxicity data and physical properties of 90 chemicals[a]

ID	Chemical	MOA	MW	log P[b]	Henry's law constant (Pa·m³/mol)[c]	Water solubility (mg/L)	Green algae: Closed-system EC50 (mg/L)	Green algae: Batch[d] EC50 (mg/L)	Ciliate[e] EC50 (mg/L)	Water flea[f] EC50 (mg/L)	Fathead minnow[g] LC50 (mg/L)	Microtox[h] EC50 (mg/L)
1	3-Chloroaniline	P	127.57	1.88	1.01E-01	5,400	9.48		100.00	0.35*		13.99
2	4-Chloroaniline	P	127.57	1.88	1.18E-01	3,900	1.54		10.00	0.31*	32.50	5.08
3	2,4-Dichloroaniline	P	162.01	2.78	1.60E-01	620	3.38	5.60	31.00	2.70*		4.67
4	2,5-Dichloroaniline	P	162.01	2.75	1.62E-01	230	5.94			2.92*		3.80
5	2,6-Dichloroaniline	P	162.01	2.76	1.06E-01	295	16.30			1.40*		1.70
6	3,4-Dichloroaniline	P	162.01	2.69	1.48E+00	92	2.02	3.67	9.00	0.16*	9.96	0.65
7	3,5-Dichloroaniline	P	162.01	2.9	1.60E-01	784	3.98			1.12*		10.46
8	2,4,5-Trichloroaniline	P	196.46	3.45	7.85E-02	51.6	1.17*			1.91		1.49
9	2,4,6-Trichloroaniline	P	196.46	3.52	1.36E-01	40	3.47*			6.00		4.61
10	3,4,5-Trichloroaniline	P	196.46	3.32	7.85E-02	66.6	2.73*					3.34
11	2-Bromoaniline	P	172.02	2.11	7.69E-02	949	11.26			3.00*		
12	2,3-Dimethylaniline	P	121.18	2.17*	2.53E-01	5,050	45.40			10.0**		
13	3,4-Dimethylaniline	P	121.18	1.84	1.88E-01	3,800	6.93			2.90*		
14	Phenol	P	94.11	1.46	3.37E-02	8.28E+04	10.53	129.00	600.00	6.60*	24.60	
15	2-Chlorophenol	P	128.55	2.15	1.13E+00	1.13E+04	8.63	70.00	67.97	3.91*	9.41	18.00
16	4-Chlorophenol	P	128.55	2.39	6.35E-02	2.40E+04	8.80	5.01		4.10	5.00	0.95*
17	2,3-Dichlorophenol	P	163.00	2.84	3.12E-02	3,600	1.23*	5.00		3.10		3.60
18	2-Nitrophenol	P	139.11	1.79	1.30E+00	2,500	1.08*		35.00	17.00		21.00
19	3-Nitrophenol	P	139.11	2.00	2.03E-04	1.35E+04	6.71*		28.00	24.00		
20	4-Nitrophenol	P	139.11	1.91	4.20E-05	1.16E+04	0.25*	4.89	5.50	7.68	30.40	6.40
21	2,4-Dimethylphenol	P	122.16	2.30	9.63E-02	7,870	13.50		130.51	2.10*	18.10	
22	Benzene	NP	78.11	2.13	5.62E+02	1,790	15.77*	29.0	391.35	200.00	24.60	75.00
23	Chlorobenzene	NP	112.55	2.84	3.15E+02	498	7.82*	210.00		86.00	35.40	9.40
24	1,2-Dichlorobenzene	NP	147.0036	3.43	1.94E+02	156	2.84	76.10	51.00	2.40*	57.00	2.70
25	1,3-Dichlorobenzene	NP	147.0036	3.53	2.66E+02	125	1.86*	114.00	130.00	28.00	9.12	3.10
26	1,4-Dichlorobenzene	NP	147.0036	3.44	2.44E+02	81.3	2.07*	98.10		11.00	14.20	4.30
27	1,2,3-Trichlorobenzene	NP	181.44	4.05	1.27E+02	18	0.84*			1.45		1.90
28	1,2,4-Trichlorobenzene	NP	181.44	4.02	1.44E+02	49	0.63*	24.10	0.91	50.00	2.76	2.30
29	1,3,5-Trichlorobenzene	NP	181.44	4.19	1.91E+02	6.01	1.67*		30.00			13.00
30	1,2,3,4-Tetrachlorobenzene	NP	215.89	4.60	7.70E+01	5.92	0.57*				1.10	2.30
31	1,2,4,5-Tetrachlorobenzene	NP	215.89	4.64	1.01E+02	0.595	1.26	52.90	20.00	320.00	0.32*	10.00
32	Hexachlorobenzene	NP	284.78	5.73	1.72E+02	0.0062	0.22*	87.00				
33	Toluene	NP	92.14	2.73	6.73E+02	526	14.19*		142.82	310.00	77.40	
34	2-Chlorotoluene	NP	126.58	3.42	3.62E+02	374	10.65					
35	4-Chlorotoluene	NP	126.58	3.33	4.44E+02	106	10.53					
36	2,4-Dichlorotoluene	NP	161.030	4.24	4.20E+02	16.2	3.53					
37	Ethylbenzene	NP	106.167	3.15	7.98E+02	169	1.34*	3.60		75.00	9.09	
38	2-Chloro-*p*-xylene	NP	140.61	3.86	4.92E+02	42.1	2.34					
39	Nitrobenzene	NP	123.11	1.85	2.43E+00	2,090	13.89*	36.60	98.00	27.00	119.00	
40	Methylene chloride	NP	84.9328	1.25	3.29E+02	13,000	33.09*			220.00	502.00	
41	Chloroform	NP	119.3779	1.97	3.72E+02	7,950	22.86*			29.00	103.00	
42	Carbon tetrachloride	NP	153.82	2.83	2.80E+03	793	23.59		830.00	35.00	10.40*	
43	1,1-Dichloroethane	NP	98.95	1.79	5.69E+02	5,040	42.92*					270.00
44	1,2-Dichloroethane	NP	98.95	1.48	1.20E+02	8,600	154.93			220.00	116.00*	700.00
45	1,1,1-Trichloroethane	NP	133.40	2.49	1.74E+03	1,290	47.43*				52.80	
46	1,1,2-Trichloroethane	NP	133.40	1.89	8.35E+01	4,590	105.42			18.00*	81.60	110.00
47	1,1,1,2-Tetrachloroethane	NP	167.84	2.93*	2.53E+02	1,070	8.05			24.00		2.00*
48	1,1,2,2-Tetrachloroethane	NP	167.84	2.39	3.72E+01	2,830	13.72	92.30		9.30	20.40	5.40*
49	Pentachloroethane	NP	202.29	3.22	1.97E+02	480	5.61	80.30		63.00	7.34	0.63*
50	Hexachloroethane	NP	236.74	4.14	3.94E+02	50	0.46	93.20		8.10	1.10	0.45*
51	1,2-Dichloropropane	NP	112.98	1.98	2.86E+02	2,800	34.42*			52.00	140.00	59.00

APPENDIX 1. Continued

ID	Chemical	MOA	MW	log P[b]	Henry's law constant (Pa·m³/mol)[c]	Water solubility (mg/L)	Green algae: Closed-system EC50 (mg/L)	Green algae: Batch[d] EC50 (mg/L)	Ciliate[e] EC50 (mg/L)	Water flea[f] EC50 (mg/L)	Fathead minnow[g] LC50 (mg/L)	Microtox[h] EC50 (mg/L)
52	1,3-Dichloropropane	NP	112.98	2	9.89E+01	2,750	19.93*	60.10		280.00	131.00	71.00
53	1-Chlorobutane	NP	92.56	2.64	1.69E+03	1,100	37.55*			3,020.00		480.00
54	Trichloroethene	NP	131.38	2.42	9.98E+02	1,280	26.24		410.00	18.00*	45.00	960.00
55	Tetrachloroethene	NP	165.83	3.4	1.79E+03	206	10.54*		100.00	18.00	23.80	90.00
56	*cis*-1,2-Dichloroethene	NP	96.94	1.86	4.13E+02	6,410	59.69*					720.00
57	*trans*-1,2-Dichloroethene	NP	96.94	2.09	9.50E+02	4,520	36.36*			220.00		1,100.00
58	1-Propanol	NP	60.095	0.25	7.51E-01	1.00E+06	4,947.90			3,644.00*	4,630.00	9,900.00
59	2-Propanol	NP	60.095	0.05	8.21E-01	1.00E+06	8,468.50*				10,400.0	
60	1-Octanol	NP	130.22	3	2.48E+00	540	1.85*		41.16		14.40	3.40
61	Acetone	NP	58.079	−0.24	4.02E+00	1.00E+06	5,276.10*			9,218.00	8,120.00	15,900
62	2-Octanone	NP	128.21	2.37	1.90E+01	900	32.20*				63.00	
63	2,4-Dichlorophenol	NP	163.003	3.06	4.35E-01	4,500	2.42	14.00		2.60	7.43	2.00*
64	2,3,4,6-Tetrachlorophenol	NP	231.89	4.45	8.95E-01	23	0.01*	1.30		0.29	1.03	
65	Acetonitrile	NP	41.052	−0.34	3.49E+00	1.00E+06	5,509.10			3,600.00	1,640.00*	17,500
66	Propionitrile	NP	55.079	0.16	3.75E+00	1.03E+05	127.72*				1,520.00	
67	Butyronitrile	NP	69.10	0.53	5.30E+00	3.30E+04	724.20					
68	Isobutyronitrile	NP	69.10	0.46	5.46E+00	5.50E+04	728.50					
69	Benzonitrile	NP	103.12	1.56	5.28E+00	2,000	23.33*				135.00	
70	Pentachlorophenol	OPU	266.33	5.12	2.48E-03	14	0.007*	0.42	0.15	0.55	0.48	
71	2,4-Dinitrophenol	OPU	184.10	1.67	8.71E-03	2,790	0.94*	10.90		4.10	17.00	
72	Formaldehyde	EP	30.026	0.35	3.41E-02	4.00E+05	2.55*			29.00	26.30	5.60
73	Acetaldehyde	EP	44.053	−0.34	6.76E+00	1.00E+06	0.02*		475.0	48.25	43.10	328
74	Propionaldehyde	EP	58.079	0.59	7.44E+00	3.06E+05	12.92*					228
75	Butyraldehyde	EP	72.10	0.88	1.16E+01	7.10E+04	11.95*				25.80	150
76	Glutaraldehyde	EP	100.11	−0.18*	1.11E-02	1.67E+05	3.15*			14.60	11.60	3.95
77	2-Pyridinecarboxaldehyde	EP	107.11	0.44	1.78E-03	4.68E+04	16.85*		131.23			
78	3-Pyridinecarboxaldehyde	EP	107.11	0.29	1.52E-02	6.29E+04	30.00		134.43		16.40*	
79	4-Pyridinecarboxaldehyde	EP	107.11	0.43	1.79E-02	4.77E+04	11.64					
80	2-Hydroxybenaldehyde	EP	122.12	1.81	5.68E-01	1.70E+04	3.12*			5.80		
81	3-Hydroxybenaldehyde	EP	122.12	1.29	2.53E-04	7,190	34.90					
82	4-Hydroxybenaldehyde	EP	122.12	1.35	5.18E-05	8,450	2.53*		66.18			
83	2,6-Dinitrotoluene	EP	182.13	2.1	7.57E-02	182	2.19*	84.91	100.00	21.70	18.50	
84	Chloroacetonitrile	RI	75.49	0.45	1.09E+00	1.00E+05	11.45				1.35*	
85	Dichloroacetonitrile	RI	109.94	0.29*	3.84E-01	3.35E+04	1.64					
86	Trichloroacetonitrile	RI	144.38	2.09	1.36E-01	715	0.02					
87	Bromoacetonitrile	RI	119.94	0.2*	No data	No data	0.07					
88	3-Chloropropionitrile	RI	89.52	0.18	1.45E+00	4.76E+04	159.54					
89	4-Chlorobutyronitrile	RI	103.55	0.56	1.92E+00	2.06E+04	526.19					
90	Malononitrile	RI	66.062	−0.6	1.33E-02	1.33E+05	12.40				0.56*	244

[a] Scientific names for species: Green algae, *Pseudokirchneriella subcapitata*; ciliate, *Tetrahymena pyriformis*; water flea, *Daphnia magna*; fathead minnow, *Pimephales promelas*; and Microtox, *Photobacterium phosphoreum*. An asterisk indicates the most sensitive species. EC50 = median effective concentration; EP = electrophilic/proelectrophilic; ID = identification number; LC50 = median lethal concentration; MOA = mode of action; MW = molecular weight; NP = nonpolar narcosis; OPU = oxidative phosphorylation uncoupling; P = polar narcosis; RI = respiratory inhibition.

[b] Log P data from KOWWIN V1.67 software (SAS®, Cary, NC, USA).

[c] 1 atm = 101,300 Pa.

[d] U.S. Environmental Protection Agency (EPA) ECOTOX (http://www.epa.gov/ecotox/) original references 5336, 19285, 13171, 16259, 4189, and 9607.

[e] U.S. EPA ECOTOX original references 11258, 11553, and 9812.

[f] U.S. EPA ECOTOX original references 846, 5375, 15149, 19263, 10936, 847, 10915, 5184, 607, 13669, 212, 13070, 6516, 2193, 344, 2017, and 5087.

[f] U.S. EPA ECOTOX original references 15031, 12859, 10432, 875, 14128, 10183, 17138, 12447, 12567, 10432, 14339, 11227, 973, 12004, 12448, 4154, 3217, 923, 2189, 2965, 344, 12858, and 10141.

[h] Blum and Speece [36], Ribo and Kaiser [37], and Chen and Yeh [38].