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# Toxicity of chlorophenols to *Pseudokirchneriella subcapitata* under air-tight test environment

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**Abstract**

A closed-system algal toxicity test with no headspace was applied to evaluate the toxicity of chlorophenols to *Pseudokirchneriella subcapitata*. The dissolved oxygen production and the growth rate based on cell density were the response endpoints. Phenol and seven chlorophenols were tested using the above test technique. Median effective concentrations (EC50) range from 0.004 to 25.93 mg/l (based on DO production) and 0.0134 to 20.90 mg/l (based on growth rate). No-observed-effect concentration (NOEC) is within the range of 0.001–8.19 mg/l. In general, growth rate is a more sensitive response endpoint than the oxygen production, except for the case of pentachlorophenol. However, the differences in sensitivity between the two parameters were marginal. Furthermore, quantitative structure–activity relationships (QSAR's) based on the *n*-octanol/water partition coefficient ($\log P$) and the acid dissociation constant ($pK_a$) values were established with $R^2$ ranged from 0.90 to 0.96. From literature data also based on *P. subcapitata*, the new test method is 1.65–108 times more sensitive than the conventional algal batch tests. A completely different relative-sensitivity relationship among various aquatic organisms was thus observed. The results of this study indicate that the toxicity data of volatile organic chemicals derived by conventional algal toxicity tests may severely underestimate the impact of these toxicants. Our results show that alga is very sensitive to chlorophenols compared to other aquatic organisms such as the luminescent bacteria (the Microtox test), *Daphnia magna*, and rainbow trout.


*Keywords:* Algae; Chlorophenols; EC50; Photosynthesis; *Pseudokirchneriella subcapitata*

## 1. Introduction

Batch technique is traditionally adopted by most standard algal test protocols for assessing the relative toxicity of chemicals and/or wastewater discharges (OECD, 1984; ISO, 1987; ASTM, 1994; US EPA, 1996). Several studies indicated that algal toxicity tests revealed excellent sensitivity to heavy metals but responded poorly to organic toxicants. On the contrast, fish, daphnia and luminescent bacteria (the Microtox test) were capable of detecting the presence of organic toxicants but were relatively insensitive to metals as compared to algae (Munkittrick et al., 1991; Toussaint et al., 1995; Peterson and Peterson, 1996). For algal toxicity tests, the main reason causing the low sensitivity to organic toxicants can be related to the open test environment and vigorous mixing usually employed by these protocols. The above experimental design causes the loss of volatile organic toxicants and, consequently,

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underestimations of the toxicity of volatile organic chemicals. The European Centre for Ecotoxicology and Toxicology of Chemicals (1996) has concluded that current algal toxicity test protocols are unsuitable for assessing the effects of volatile compounds. Several studies solved the above problem by adopting the closed system and providing large headspace for additional carbon supply (Galassi and Vighi, 1981; Herman et al., 1990; Brack and Rottler, 1994; Halling-Sørensen et al., 1996). Large headspace may cause a significant portion of the volatile compound to partition from the aqueous phase into the headspace until equilibrium is reached. Mayer et al. (2000) pointed out that the exposure concentration might thus be altered significantly. Therefore, they proposed the use of a closed test system with no headspace and an enriched bicarbonate buffer in the growth medium (300 mg/l of $NaHCO_3$). The experimental design for the above closed-system tests is still quite complicated and troublesome. Furthermore, enriched carbonate buffer may also result in increased ionic strength and lower test sensitivity (Brack and Rottler, 1994; Lin et al., 2005). The Organization for Economic Co-Operation and Development has suggested that sealed exposure system should be used for testing volatile compounds (OECD, 2000). However, the closed-system test technique has not yet been standardized.

The no-observed-effect concentration (NOEC) is traditionally adopted as the highest tested concentration that yields no adverse effect to the test organism. The relevance and utility of the NOEC has been seriously criticized by recent scientific publications. Chapman and his coworkers pointed out that NOEC is highly variable between tests and is not a good estimate of the no-effect concentration (NEC). On the contrary, EC50s and other point estimates are more consistent, more reliable, and less variable estimates than NOECs (Chapman et al., 1996; Chapman and Chapman, 1997). Moore and Caux (1997), based on the analyses of 198 toxicity data sets, showed that most NOECs represent 10–30% reductions from the control responses. They believe that the point estimates (e.g., EC10) is a better approach than hypothesis testing for estimating low toxic effects. On the other hand, Shief et al. (2001) showed that the response endpoint and the nature of the toxicity test could be an important consideration for the selection of NOEC or EC10. A more precise test (or response endpoint) associated with smaller within-group-variances would produce a NOEC that may offer better protection than EC10.

Due to the aforementioned limitations of conventional algal toxicity test protocols, the effects of organic toxicants on phytoplankton have not been well studied. The few available data may also be derived from inappropriate test design. Furthermore, studies of the low toxic effects of organic chemicals on algae are rarely found. The objective of this study was to evaluate the toxicity of chlorophenols using an air-tight algal toxicity test without headspace. The test method applied in this study has a simple experimental design and short exposure period (48 h). Two response endpoints, i.e., algal growth rate and dissolved oxygen (DO) production, were used to evaluate the toxic effects of various toxicants.

## 2. Materials and methods

### 2.1. Algal incubation

The alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*, UTEX 1648) was grown in a 4-l transparent chemostat incubator. The growth medium was continuously supplied by a variable-speed pump. Air agitation was used to achieve adequate mixing. The chemostat reactors were placed in a constant-temperature room at $24 \pm 1$ °C. Light intensity was set at 65 μE $m^{-2}$ $s^{-1}$ (±10%). Growth medium composition is basically the same as that described by the EPA bottle technique (US EPA, 1996). However, according to our previous work (Chen and Lin, 1997), $NaNO_3$, $K_2HPO_4$, and EDTA contents were reduced to 12.75 mg/l, 0.52 mg/l, and 30 μg/l, respectively. The dilution rate ($D$) for the chemostat was set at 0.25/day to ensure a nutrient-limited condition. Quality assurance (QA) procedures were routinely conducted by plotting control charts of cell density and pH to verify that steady state was achieved and well maintained.

### 2.2. Toxicity testing

After the algal incubator has reached the steady state, toxicity testing was conducted by transferring adequate amounts of algal suspension, dilution water (with growth medium), and toxicants into 300-ml BOD bottles. The BOD bottles were completely filled up with no headspace left. 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 the same as the algal incubator. US EPA (1996) bottle medium with no EDTA content was used for toxicity testing. The dilution water was stripped by nitrogen gas to reduce the dissolved oxygen level. In addition, the $N_2$ gas contained 0.5% carbon dioxide as an extra carbon source. The DO level at the beginning of the test was approximately 1–3 mg/l. Two response endpoints were used to evaluate the toxicity of toxicants; dissolved oxygen production (ΔDO) and algal growth rate based on cell density. The median effective concentration (EC50) was defined as the toxicant concentration that reduced the final DO or algal growth rate to half of that obtained by the control. The initial inoculated cell density was 15000 cells/ml

and the duration of the test was 48 h. With proper control of the inoculum cell density and the exposure time, exponential growth was maintained during the entire test period. Therefore, we may conclude that carbon source was not limiting during the test period.

Eight organic toxicants were tested in this study: phenol, 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2,3-dichlorophenol (2,3-DCP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TTP), and pentachlorophenol (PCP). All chemicals used were of reagent grade and all tests were performed in triplicate. Stock solutions of toxicants were prepared in foil-wrapped glass containers. Before commencing the experiment, stock solution was freshly prepared and its concentration was analyzed using a HPLC analyzer.

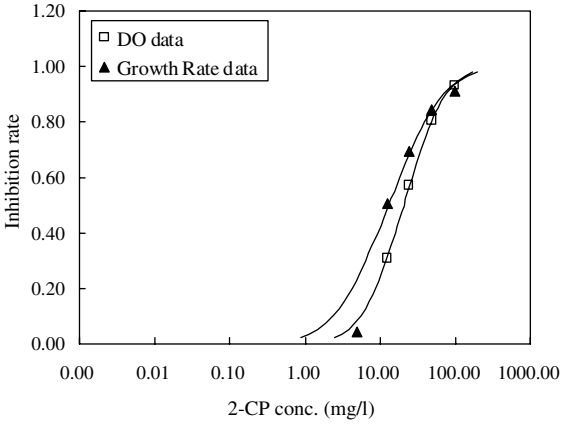


Fig. 1. Dose–response curves of *Pseudokirchneriella subcapitata* to 2-CP.

## 3. Results and discussion

Table 1 displays a typical set of algal responses with respect to the toxicity of 2-Chlorophenol (2-CP). For the test control, the dissolved oxygen concentration increased from 1.59 mg/l at the beginning to a final DO concentration of 6.80 mg/l. The cell density increased from an initial value of 15 000 cells/ml to a final yield of 209 400 cells/ml. Generally speaking, at a specific 2-CP concentration, the inhibition rate based on delta cell density is greater than that based on delta DO. Concentration response curves for the aforementioned response endpoints are shown in Fig. 1. These curves were obtained through linear regression assuming a log-normal distribution (probit model) of the tolerances. Based on the probit analyses, EC50 values were found to be equal to 13.01 mg/l (growth rate based on cell density) and 20.55 mg/l (ΔDO), respectively. According to the dose–response curves in Fig. 1, we may also conclude that endpoint based on growth rate is slightly more sensitive than DO production.

The no-observed–effect concentration (NOEC) was determined using the one-tail Dunnett's test at $p = 0.05$. Individual treatments that were statistically different from the control were marked with an asterisk. Therefore, based on DO production, the lowest-observed-effect concentration (LOEC) for 2-CP is 12.3 mg/l and the NOEC is thus equal to 4.93 mg/l. An identical NOEC value (4.93 mg/l) was found with respect to the endpoint of algal growth rate.

Table 2 lists the EC50 values for phenol and chlorophenols. EC50 values range from 0.004 to 25.93 mg/l (based on ΔDO) and 0.0134 to 20.90 mg/l (based on growth rate). For both endpoints, we may find an apparent trend that the toxicity order is PCP > TTP > TCP > DCP > MCP > Phenol. The toxicity of chlorophenols is directly related to the number of chlorine molecules contained by the compound. This phenomenon is in consistency with previous conclusions drawn by other researchers (Boyd et al., 2001). In general, growth rate is a more sensitive response endpoint than the oxygen production, except for the case of pentachlorophenol. However, the differences in sensitivity between the two parameters were marginal. Toxicity data from pentachlorophenol indicates that PCP is three times more

Table 1
Algal responses to various 2-CP concentrations

| Conc. (mg/l) | Initial DO (mg/l) | Final DO (mg/l) | ΔDO (mg/l) | Final cell density (cells/ml) | Growth rate $\mu$ ($day^{-1}$) | Inhibition rate | |
|---|---|---|---|---|---|---|---|
| | | | | | | DO | Growth rate |
| Control | 1.59 | 6.80 | 5.22 | 209400.00 | 1.318 | 0 | 0 |
| 98.6 | 1.97 | 2.29 | 0.31* | 18966.67 | 0.117* | 0.940 | 0.911 |
| 49.3 | 1.88 | 2.91 | 1.02* | 22500.00 | 0.203* | 0.804 | 0.846 |
| 24.6 | 1.77 | 4.01 | 2.23* | 33700.00 | 0.405* | 0.572 | 0.693 |
| 12.3 | 1.71 | 5.33 | 3.62* | 54766.67 | 0.648* | 0.306 | 0.508 |
| 4.93 | 1.64 | 7.16 | 5.52 | 186400.00 | 1.260 | −0.059 | 0.044 |
| | | | | | EC50 | 20.55 | 13.01 |

* Significantly different from the controls at $p = 0.05$ using the one-tail Dunnett's test.

Table 2
EC50, log $P$ and p$K_a$ values for various chlorophenols

| Toxicant | EC50(DO) | | EC50(GR) | | Physicochemical descriptors | |
|---|---|---|---|---|---|---|
| | EC50 (mg/l) | 95% Confidence limit | EC50 (mg/l) | 95% Confidence limit | log $P$ | p$K_a$ |
| Phenol | 25.93 [CV = 12.4%, $n = 3$] | | 20.90 [CV = 15.3%, $n = 3$] | | 1.57 | 9.9 |
| 2-CP | 20.55 | 20.4–20.7 | 13.01 | 5.20–19.4 | 2.29 | 8.3 |
| 4-CP | 20.88 | 15.8–26.0 | 14.75 | 3.96–26.5 | 2.53 | 9.2 |
| 2,3-DCP | 3.03 | 2.92–3.13 | 2.53 | 1.85–3.14 | 3.26 | 7.6 |
| 2,4-DCP | 4.20 | 3.51–4.77 | 3.82 | 1.05–6.72 | 3.20 | 7.8 |
| 2,4,6-TCP | 0.801 | 0.45–1.14 | – | – | 3.67 | 6.0 |
| 2,3,4,6-TTP | 0.072 | 0.04–0.08 | 0.061 | 0.018–0.10 | 4.24 | 5.2 |
| PCP | 0.004 | 0.0030–0.0041 | 0.0134 | 0.0025–0.027 | 5.02 | 4.7 |

CV: coefficient of variation.

toxic to DO production than algal growth. This suggests that certain chemical compounds may have special preference (or selectivity) to photosynthesis reactions. Furthermore, for the growth-rate endpoint, 2,4,6-TCP depicted no linear inhibitory effect with respect to its concentrations. Hence, its corresponding EC50 and 95% confidence intervals could not obtain through linear regression. Previous report indicated that 2,4,6-TCP might decompose through photolysis (Svenson and Hynning, 1997). It is possible that the photolysis products may exhibit different toxic effects to algal growth as compared to their parent compound (2,4,6-TCP). However, toxicity data based on DO production did not show the above phenomenon and satisfactory results were obtained through linear regression. The use of two different endpoints for the analyses of toxic effects of toxicants can thus provide more thorough assessment to the effects of organic toxicants. Finally, the reproducibility of the proposed technique was evaluated according to repeated tests on phenol. Coefficients of variation (CV value) for DO and growth rate endpoints were 12.4% and 15.3%, respectively.

Table 3 displays the no-observed-effect concentrations for the test compounds determined by the one-tail Dunnett's test. NOECs are within the range of 0.001–<8.19 mg/l. The two test endpoints applied herein have almost identical NOEC values except for pentachlorophenol. The inhibition rates at NOEC levels are also given in Table 3. The degree of inhibition at NOEC level ranges from 0.2% to 6.3%. The applications of NOEC have long been criticized as not being able to provide sufficient protection to aquatic organisms than the point estimator EC10 (Moore and Caux, 1997). The low inhibition rates showed in Table 3 indicates that the proposed test method has pretty small variations among replicates. In other words, the with-in-group variance for the Dunnett's test will also be small (Shief et al., 2001). Unlike other tests using organisms such as fish or invertebrate, NOECs from our algal test technique may provide better protection than EC10.

Correlation analyses were conducted to establish the relationship between EC50 values and physicochemical parameters such as the $n$-octanol/water partition coefficient (log $P$) and the acid dissociation constant (p$K_a$). Traditionally, for the analyses of quantitative structure–activity relationships (QSAR's), EC50 is expressed in terms of mol/l. As shown in Figs. 2 and 3, both log $P$ and p$K_a$ have very good linear relationships with EC50 values based on DO production and biomass. The $R^2$ value ranges from 0.90 to 0.96, which means that good

Table 3
No-observed-effect concentrations and the corresponding inhibition rate

| Toxicant | DO production | | Growth rate | |
|---|---|---|---|---|
| | NOEC (mg/l) | Inhibition rate at NOEC (%) | NOEC (mg/l) | Inhibition rate at NOEC (%) |
| Phenol | <8.19 | – | <8.19 | – |
| 2-CP | 4.93 | −5.9 | 4.93 | 4.4 |
| 4-CP | 5.00 | 0.2 | 5.00 | 4.8 |
| 2,3-DCP | 0.5 | −1.2 | 0.5 | 4.4 |
| 2,4-DCP | 0.97 | −1.5 | 0.97 | 4.2 |
| 2,4,6-TCP | <0.5 | – | <0.5 | – |
| 2,3,4,6-TTP | <0.1 | – | <0.1 | – |
| PCP | 0.001 | 6.3 | 0.002 | 2.1 |

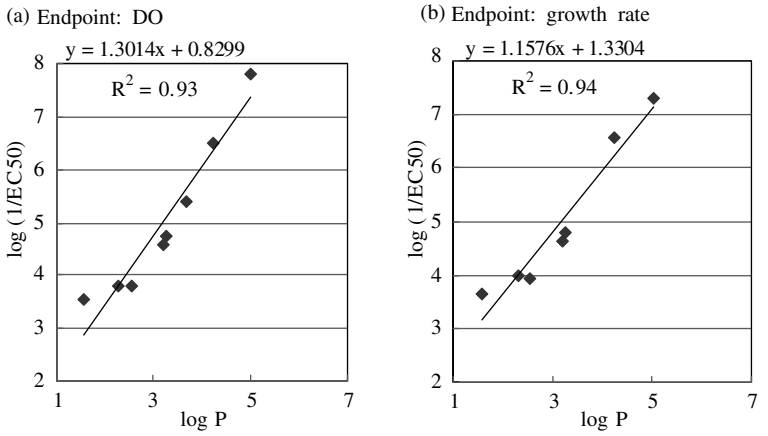


Fig. 2. Correlations between EC50 values and log $P$.

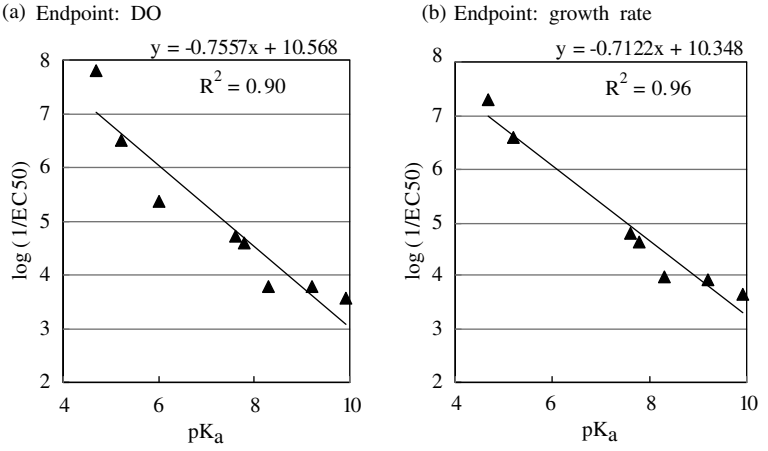


Fig. 3. Correlations between EC50 values and p$K_a$.

correlations exist. Toxicity of chlorophenols is positively correlated with log $P$ values and negatively correlated with p$K_a$ values. Results in Figs. 2 and 3 indicate that the toxicity of chlorophenols on *P. subcapitata* can be estimated successfully with log $P$ or p$K_a$ values. The quantitative structure–activity relationships (QSAR's) can be formulated as follows:

Endpoint: DO

$$\log(1/\mathrm{EC50}) = 1.3014\log P + 0.8299 \quad (R^2 = 0.93)$$

$$\log(1/\mathrm{EC50}) = -0.7557\mathrm{p}K_a + 10.568 \quad (R^2 = 0.90)$$

Endpoint: Growth rate

$$\log(1/\mathrm{EC50}) = 1.1576\log P + 1.3304 \quad (R^2 = 0.94)$$

$$\log(1/\mathrm{EC50}) = -0.7122\mathrm{p}K_a + 10.348 \quad (R^2 = 0.96)$$

EC50 values are compared with literature data to evaluate the test sensitivity of the applied technique (Table 4). For both endpoints (DO and growth rate), the closed-system test reveals apparently smaller EC50 values as compared to that derived by the conventional algal batch tests (Shigeoka et al., 1988). Using the DO endpoint as a basis, our test is 1.65–108 times more sensitive than the conventional test methods (based on the same test alga—*P. subcapitata*). The above differences may be due to the reason that, for the bath tests, significant lose of volatile compounds may take place. The type of algal culture (chemostat incubation vs. batch culture) for the inoculums might also contribute to the differences in sensitivities for the two kinds of tests. In particular, the EC50 value for pentachlorophenol from the batch test is 108 times larger than that based on the DO endpoint. Such a difference indicates that the test endpoint (growth rate) used in Shigeoka's study (Shigeoka et al., 1988) could not adequately reflect PCP's toxicity on photosynthesis reactions. In Table 4, a ranking system was used to compare the relative sensitivities for various aquatic organisms: for each specific

Table 4
Comparisons of algal toxicity data with literature data

| Toxicant | EC50 | | LC50 or EC50 from literature data | | | | |
|---|---|---|---|---|---|---|---|
| | DO | Growth rate | Algae[a] *P. subcapitat* | Daphnia[b] | Microtox[c] | Rainbow trout[d] | *P. reticulate*[a] |
| Phenol | 25.93 [4] | 20.90 [2] | 150 [7] | 21.0 [3] | 35.7[e] [5] | 9.90 [1] | 43.0 [6] |
| 2-CP | 20.55 [4] | 13.01 [1] | 70.0 [6] | 17.7[c] [3] | 33.8 [5] | – | 14.0 [2] |
| 4-CP | 20.88 [4] | 14.75 [3] | 38.0 [5] | – | 8.29 [1] | – | 8.50 [2] |
| 2,3-DCP | 3.03 [2] | 2.53 [1] | 5.00 [5] | 4.10 [3] | 4.92 [4] | – | – |
| 2,4-DCP | 4.20 [4] | 3.82 [3] | 14.0 [7] | 2.50 [2] | 5.50 [5] | 1.70 [1] | 5.50 [5] |
| 2,4,6-TCP | 0.801 [1] | – | 3.50 [4] | 3.70 [5] | 7.70 [6] | 1.10 [2] | 2.30 [3] |
| 2,3,4,6-TTP | 0.072 [2] | 0.061 [1] | 1.30 [5] | – | 1.30[e] [4] | – | 1.10 [3] |
| PCP | 0.004 [1] | 0.0134 [2] | 0.42 [4] | 0.67 [6] | 0.05 [3] | – | 0.44 [5] |
| Ave. Ranking | 2.75 | 1.86 | 5.38 | 3.67 | 4.13 | 1.33 | 3.71 |

All EC50 and LC50 values are in mg/l. [ ]: sensitivity ranking.
[a] Shigeoka et al. (1988).
[b] Kuhn et al. (1989).
[c] Shannon et al. (1991).
[d] Liu et al. (1982).
[e] Todeschini et al. (1996).

test compound, the most sensitive organism was assigned with the smallest numerical value for ranking, e.g., [1]. Larger ranking values were then given to the other species of test organism that are less sensitive, accordingly. The order of the relative sensitivity for different species of test organisms can be judged according to the average ranking values. Considering literature data alone, the relative sensitivity based on the average ranking values is in the following order: rainbow trout > *Daphnia magna* > *Poecilia reiculata* > Microtox test > algae (batch). One may thus conclude that *P. subcapitata* is quite insensitive to chlorophenols. On the other hand, a new order of relative sensitivity based on data from the present study will be as follows: rainbow trout > algae (growth rate) > algae (DO production) > *D. magna* > *Poecilia reiculata* > Microtox test. Therefore, the alga *P. subcapitata* is actually very sensitive to chlorophenols and the previous impression that algae can be very resistant to organic toxicants may not be appropriate. Furthermore, it is quite obvious that, for TCP, TTP, and PCP, the ranking values for the DO endpoint are very small. This might indicates that chlorophenols containing more chlorine molecules have stronger inhibitory effects on algal photosynthesis reactions. The above discussions also indicate that the air-tight test technique applied in this study is quite superior in terms of test sensitivity.

## 4. Conclusions

This study presents an algal toxicity testing technique conducted under closed test environment. The new technique is very simple in experimental design and reveals good test sensitivity and reproducibility. In addition, this paper presents the toxicity data of chlorophenols on *P. subcapitata*. Median effective concentrations (EC50) range from 0.004 to 25.93 mg/l (based on DO production) and 0.0134 to 20.90 mg/l (based on growth rate). No-observed-effect concentration (NOEC) is within the range of 0.001 to 8.19 mg/l. Quantitative structure–activity relationships were established to correlate EC50 values with log$p$ and p$K_a$ values ($R^2 = 0.90$–0.96). From literature data also based on *P. subcapitata*, the new test method is 1.65–108 times more sensitive than the conventional algal batch tests. Such differences suggest that previous data regarding the toxicity of volatile organic toxicants derived based on batch-type protocols may severely underestimate the impact on phytoplankton. Therefore, there is a need to re-evaluate the effects of volatile organic chemicals using the closed-system testing technique. Finally, results from the present study show that algae is very sensitive to chlorophenols compared to other aquatic organisms such as the luminescent bacteria (the Microtox test), *D. magna*, and rainbow trout.

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