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Metal toxicity to *Chlorella pyrenoidosa* assessed by a short-term continuous test

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Abstract

This study presents the metal toxicity as revealed by *Chlorella pyrenoidosa* using a short-term continuous test and the conventional batch test. Toxicity tests were conducted in a chemostat with a test duration of 1 day. Toxicity data of five different metals (Cd, Pb, Co, Ni, and Zn) derived from the continuous test were compared against results from the conventional batch tests. The batch test is characterized by its saturated nutrient status for algal growth and, on the other hand, the continuous test is conducted at a nutrient-limiting condition simulating more closely to the aquatic field environment. The continuous test consistently yielded smaller EC_{50} values, which indicated that alga in the chemostat is approximately four to six times more sensitive than those from the batch culture. Similar phenomena between the two types of tests were observed based on the NOEC values. Compared to literature data (US EPA ECOTOX Database) from the same species of alga, differences in test sensitivity between the batch tests and chemostat technique varied from 9.25 to 160 times. Such distinct differences indicate that current toxicity database may not adequately reflect the impact of various metal toxicants on *C. pyrenoidosa.* The continuous test method presented herein is a sensitive and rapid assessment technique and, therefore, has a potential for more general applications such as routine checkup for regulatory purposes.

Keywords: Toxicity; Algae; *Chlorella pyrenoidosa*; EC_{50}; NOEC

1. Introduction

Chlorella pyrenoidosa is a green unicellular alga and an important primary producer commonly found in many small ponds. *C. pyrenoidosa* has been widely used for detecting metal concentration in natural water as well as in waste effluent. The inhibitory effects of various metal toxicants on *C. pyrenoidosa* have been studied by previous researchers [1–4]. In addition, *C. pyrenoidosa* was also used quite extensively to evaluate the impacts from pesticides and organic compounds [2,5,6]. Basically *C. pyrenoidosa* is not a highly sensitive alga compared to other aquatic organisms used extensively for toxicity assessment. Hence, toxic effects revealed by *C. pyrenoidosa* could indicate hazards to other organisms at higher trophic levels [7].

Batch technique is conventionally adopted by most standard algal test protocols for assessing the relative toxicity of chemicals and/or wastewater discharges [8–11]. The growth mediums for these test methods are all enriched with macronutrients including phosphate and nitrogen. During the test period, the excessive amounts of nutrients allow test algae to grow under a nutrient-saturated condition. Continuous algal toxicity test is another alternative for ecotoxicologists to evaluate the impact of toxicants to phytoplankton. Hall et al. concluded that continuous tests generally exhibit much better sensitivities than batch tests [12]. Nevertheless, Kayser reported that the difference in sensitivity obtained by the two testing methods was not highly significant [13]. Our previous study indicated that the general tolerance of an algal culture decreases as the phosphorus or nitrogen status becomes limiting. The sensitivity of algae to a toxicant thus increased significantly [14,15]. Other research also pointed out that changes in algal nitrogen status might alter algal lipid content and, eventually, affect the bioconcentration of hydrophobic organic compounds [16]. The author's previous works have proposed a short-term continuous algal toxicity test technique, whereby algae were grown and tested under nutrient-limited conditions. Toxicity tests employing different

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metal toxicants revealed that, with respect to both the median effective concentration (EC_{50}) and the no-observed-effect concentration (NOEC), algae cultured in a continuous reactor are more severely inhibited than those cultured by the batch technique [17,18]. Furthermore, batch and continuous techniques were found to depict completely different relationships in relative toxicity [19]. Since algae in the field are grown under low concentrations of nutrients and chelators, toxicity orders among various toxicants as determined by protocols may not be adequate when extrapolating toxicity data for field applications [19].

The major disadvantage of the traditional continuous test methods (chemostat or tubidostat) is that it is quite time-consuming for the system to reach the steady state. Subsequent toxicity testing often severely inhibits culture growth and causes the recovery to take a longer time. Therefore, their application in algal toxicity assessment is quite limited. On the other hand, our short-term technique evaluates the toxic response under non-steady state and the test duration is only 1 day [17]. The technique thus has a potential for more general applications such as routine checkup for regulatory purposes. The objective of the present study was to apply the aforementioned short-term test technique for assessing the toxicity of various metal toxicants to *C. pyrenoidosa* by identifying the appropriate incubation and testing conditions (i.e., medium composition and dilution rate) for *C. pyrenoidosa* to grow in a chemostat. Furthermore, the batch and continuous culture tests were used to compare the response of *C. pyrenoidosa* to various heavy metals.

2. Materials and methods

2.1. Batch tests

The test alga used for this study was *C. pyrenoidosa* Chick (UTEX 1806), which was obtained from the University of Texas, Austin. Alga was grown in modified bristol's medium (Table 1) under continuous cool-white fluorescent light with a light intensity of 5000 lx (±10%). The temperature was set at 24 ± 1 °C. Algal culture at exponential growth phase was then used as inoculums for subsequent toxicity testing. The initial pH and cell density for toxicity tests were approximately equal to 6.8 and 20,000 cells/ml, respectively. Tests were conducted using 250-ml Erlenmeyer flasks containing 50 ml modified bristol's medium. Eethylenediaminetetraacetic acid (EDTA) content was removed from the growth medium following the US EPA Guideline [10]. Median effective concentrations (EC_{50}) based on algal growth rate were calculated using the probit model. Cell density and mean cell volume (MCV) were measured by an electronic particle counter (Coulter Electronics, Luton, UK), which was connected with a channelizer. The specific growth rate can be defined as $\mu = \ln(x_2/x_1)/(t_2 - t_1)$, where x_1 and x_2 denote cell densities at time t_1 and t_2, respectively. For each toxicant concentration (treatment) and the control, three replicates were used for the tests. No-observed-effect concentrations (NOEC) based on the final cell density were determined using Dunnett's test at 5% level of significance.

Table 1
Composition of media (mg/l)

Macronutrient	Micronutrient
$CaCl_2{\cdot}2H_2O$: 25	$Na_2EDTA{\cdot}2H_2O$: 4.36
$MgSO_4{\cdot}7H_2O$: 25	$FeCl_3{\cdot}6H_2O$: 3.15
$NaNO_3$: 250	$CuSO_4$: 0.01
K_2HPO_4: 75	$ZnSO_4{\cdot}7H_2O$: 0.022
KH_2PO_4: 175	$C0Cl_2{\cdot}6H_2O$: 0.01
	$MnCl_2{\cdot}4H_2O$: 0.18
	$Na_2MoO_4{\cdot}2H_2O$: 0.006
	H_3BO_4: 1.0

2.2. Continuous tests

Alga was grown in an 8-l transparent chemostat reactor (the 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 ± 1 °C. Light intensity was set at 5000 lx (±10%). The composition of modified bristol's medium was adjusted at different stages of study which will be discussed below. Samples were taken directly from the effluent pore for analyses. For the 8-l incubator, quality assurance procedures were routinely conducted by plotting control charts of cell density and pH to verify that steady state was well maintained. At steady state, toxicity testing was conducted (with a test duration of 24 h) by dispensing the algal suspension into six 400-ml chemostat reactors (the test vessels). These test vessels were operated at the same conditions (temperature, light intensity, and dilution rate) as the 8-l incubator. Toxicants were then added both to the reactors and the medium influent until the desired nominal concentration was reached. The specific growth rate (μ) during the test period can be calculated from the rate of population decline according to the following equation $\mu = \ln(x_2/x_1)/(t_2 - t_1) + D$, where x_1 and x_2 denote cell densities at time t_1 and t_2, respectively. D is the dilution rate of the test vessel. EC_{50} was calculated according to the inhibitory effects on algal growth rate using the probit analyses. Two response endpoints, 24-h cell density and mean cell volume (MCV) were used to estimate NOEC values using the control chart method (one-sample t-test). A detail description for NOEC determination can be found from our previous study [18].

In the preliminary batch studies, the maximum specific growth rate was found to be approximately equal to 1.1 day^{-1}. Therefore, *C. pyrenoidosa* was grown in the continuous reactor at dilution rate $D = 0.3$–$0.8\ day^{-1}$. The highest dilution rate for this study was chosen to be $0.8\ day^{-1}$ to prevent a possible washout phenomenon.

Growth medium was prepared using double-distilled deionized water and then filter-sterilized through a 0.45-μm Millipore membrane. The metal toxicants studied were $CdCl_2$, $NiCl_2$, $PbCl_2$, $CoCl_2$ and $ZnCl_2$. Toxicant concentrations (nominal) presented were in the form of total metal concentration. Orthophosphate content was analyzed according to the stannous chloride method (APHA [20]), and the concentration of NO_3-N was analyzed by ion chromatography.

Table 2
The nutrient content and cell density from steady-state operations at dilution rate = 0.5 day^{-1} (EDTA content: 30 μg/l)

$[NO_3\text{-}N]_i$[a] (mg/l)	$[PO_4\text{-}P]_i$ (mg/l)	N/P	$[NO_3\text{-}N]_e$[b]	$[PO_4\text{-}P]_e$	Cell density (cells/ml)
8.2	0.28	29	1.02	0.01	4569800
8.2	0.14	58	0.53	0.008	2265300
4.1	0.14	29	0.51	0.009	2196500

[a] Influent nitrate concentration.
[b] Concentration in the reactor and effluent.

Table 3
The nutrient content, cell density and EC_{50} values from steady-state operations for various dilution rates

D (day^{-1})[a]	Cell density (cells/ml)	NO_3-N (mg/1)	PO_4-P (mg/1)	EC_{50} (mg/l)
0.8	1.91×10^6	0.59	0.022	0.110
0.5	2.09×10^6	0.51	0.009	0.066
0.3	2.27×10^6	0.31	0.005	0.042

Toxicant: cadmium.
[a] D: dilution rate (day^{-1}).

3. Results and discussions

3.1. Algal incubation and testing conditions

The growth conditions for *C. pyrenoidosa,* in the chemostat, at three different steady states are show in Table 2. In these tests only nitrogen and phosphate contents were changed and the N/P ratio varied from 29 to 58. The dilution rates for these tests were all maintained at $D = 0.5\ day^{-1}$. The concentration of $Na_2EDTA{\cdot}2H_2O$ was reduced to 30 μg/l following our previous approach [17,18]. One may find that the cell density dropped from 4.6 to 2.2×10^6 cells/ml as the PO_4-P content decreased from 0.28 to 0.14 mg/l. On the other hand, based on the last two tests in Table 2, variations of nitrogen concentration did not result in any change in cell density. Therefore, it is clear that the phosphate content was directly proportional to cell density, indicating that phosphorus was the limiting factor for algal growth. Nitrogen concentration, however, appeared to have no significant effect on algal growth. For the following studies, NO_3-N and PO_4-P concentrations were thus chosen as 4.1 and 0.14 mg/l, respectively, to assure a phosphorus-limiting test condition.

Table 3 shows the toxic effects of cadmium on *C. pyrenoidosa* observed from three different dilution rates (0.3, 0.5 and 0.8 day^{-1}). With decreasing dilution rate, EC_{50} decreased from 0.11 to 0.042 mg/l, while the phosphorus concentration varied from 0.022 to 0.005 mg/l. Generally speaking, one expects that higher cell density will result in greater EC_{50} value because higher cell density means lower toxicant load per individual cell. Vasseur and Pandard have similar observation that greater EC_{50} values are associated with a higher density of inoculum [21]. However, in Table 3, a contradicting relationship is observed as the test condition associated with the highest cell density ($D = 0.3\ day^{-1}$) depicts the smallest EC_{50} value. The above phenomenon shows that the average tolerance of algal cells decreased as the nutrient (phosphate) become more limiting. A good correlation with $R^2 = 0.98$ between EC_{50} and phosphorus concentration was observed (Fig. 1) indicating that EC_{50} values or tolerance were correlated with phosphate content. Generally speaking, stable growth conditions were maintained for all three dilution rates. Hence $D = 0.3\ day^{-1}$, which is the most sensitive condition for toxicity testing, was selected for the following studies. The pH for the chemostat, under steady state, was within the range of 6.3–6.5.

It has well been recognized that metal toxicity was significantly influenced by chelating agents. Since our batch tests were conducted without EDTA, an attempt was made to further decrease the EDTA concentration for the continuous testing, so that, comparisons could be made on a less-biased basis. Fig. 2 displays the growth conditions of the chemostat ($D = 0.3\ day^{-1}$) under three different EDTA concentrations. Medium with 4360 μg/l yielded the highest biopopulation (2.34×10^{-6} cells/ml), but it did not differ significantly from the case of 30 μg/l. Comparisons based on a Student's t-test at the $\alpha = 0.05$ level also showed no differences between $Na_2EDTA{\cdot}2H_2O = 4360$ and 30 μg/l. It shows that the EDTA content in the original modified bristol's medium is unnecessarily high. On the other hand, medium with 15 μg/l of $Na_2EDTA{\cdot}2H_2O$ yielded the lowest biopopulation (1.86×10^{-6} cells/ml). Although reducing $Na_2EDTA{\cdot}2H_2O$ content to 15 μg/l has resulted approximately 20% decrease in biopopulation, steady state could still be maintained for a long period time. Hence medium containing 15 μg/l of

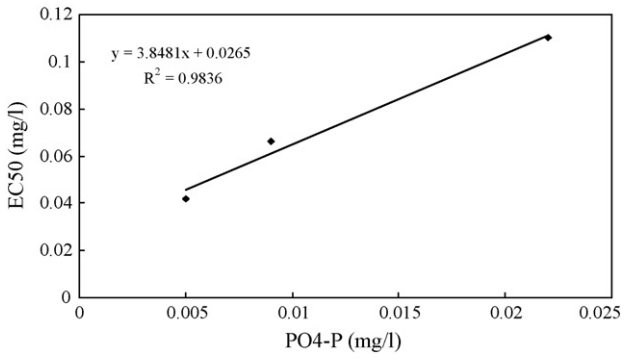

Fig. 1. The correlation coefficient between EC_{50} and PO4-P.

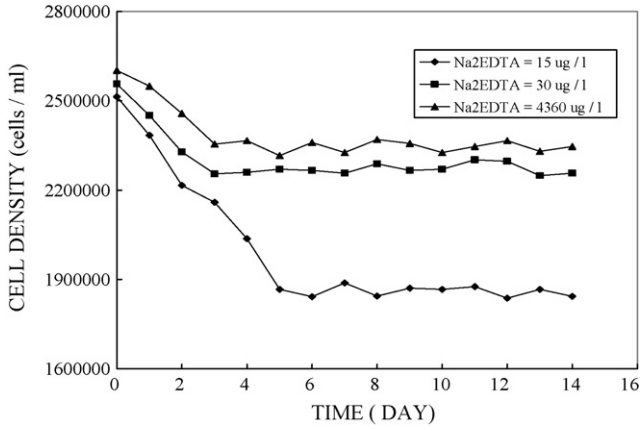

Fig. 2. Growth conditions of *C. pyrenoidosa* under three different concentrations of EDTA in a chemostat with dilution rate (D) = 0.3 day^{-1}.

$Na_2EDTA{\cdot}2H_2O$ was considered as adequate for the following studies.

3.2. Comparisons between batch and continuous tests

Table 4 shows the effect of cadmium on *C. pyrenoidosa* from continuous tests. The specific growth rate was calculated based on the final cell density after 24 h of exposure. The inhibition rate was determined using the following equation: inhibition rate (%) = $(1 - \mu/D) \times 100$. The median effective concentration (EC_{50}) is equal to 0.028 mg/l based on probit analysis.

Fig. 3 depicts the control charts for mean cell volume and cell density in the chemostat under steady state. The numerical values for upper and lower control limits are specified in the diagrams. The upper and lower control limits are at +3 and −3 standard deviations from the mean value of observations. The test statistics is to determine whether or not a specific observation drawn from an individual treatment falls within the range defined by the upper and lower control limits as shown in Fig. 3. If it does, then, no toxic effect is observed from the algal culture in the 400-ml test vessel. The highest toxicant concentration that does not produce a significant effect will be considered as the NOEC. From Table 4, significant differences were found at Cd concentration of 0.006 mg/l and the NOEC was equal to 0.003 mg/l for both endpoints; i.e., cell density and mean cell volume. Theoretically, the level of significance (P) for the above statistical approach is less than or equal to 0.027% [18].

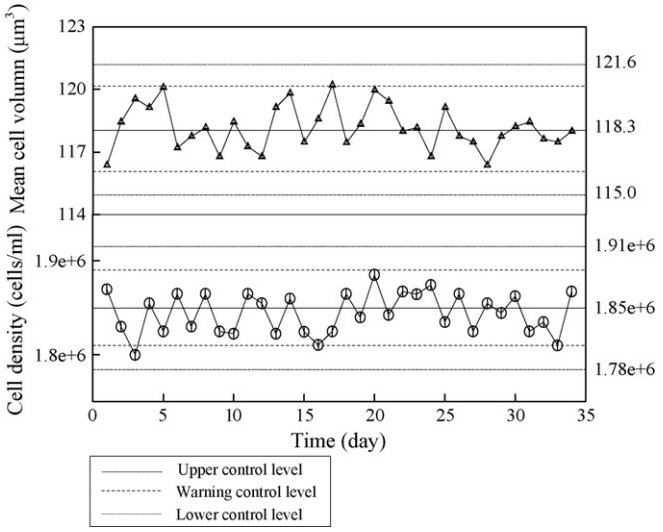

Fig. 3. Control charts for the determination of NOEC values.

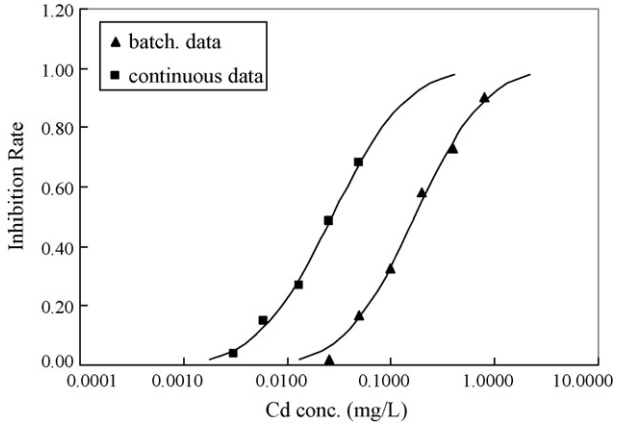

Fig. 4. Effects of cadmium on *C. pyrenoidosa* (growth rate) in batch and continuous tests.

EC_{50} values calculated based on growth rate were compared between batch and continuous tests, as given in Table 5. The continuous test was found to be more sensitive than batch test for all test metal toxicants. Fig. 4 depicts the concentration–response curves of cadmium obtained from batch and continuous tests. Based on the ratios of EC_{50}s between the two types of tests, we may assert that algae in the chemostat are 4.2–6.1 times more sensitive than those in the batch culture. Since batch tests were conducted without EDTA, it is likely that the difference in

Table 4
Effects of cadmium on *C. pyrenoidosa* from continuous tests at D = 0.3 day^{-1}

Cd (mg/l)	Cell density (×10^6 cells/ml)	MCV (μm^3)	μ (1 day^{-1})	Inhibition rate (%)
Control	1.84	118.5	0.300	0
0.003	1.82	119.4	0.289	3.67
0.006	1.76*	122.5*	0.255	15.0
0.013	1.70*	125.3*	0.220	26.7
0.025	1.59*	130.8*	0.154	48.7
0.05	1.50*	134.1*	0.096	68.0
NOEC	0.003	0.003		EC_{50} = 0.028

* Statistically different (P < 0.027) from the controls.

Table 5
Comparison of EC_{50} values between the batch and continuous tests

	EC_{50}[a] (mg/1)		Ratio (batch/continuous)	EE_{50} (μg/10^6 μm^3)		Ratio (batch/continuous)
	Batch	Continuous		Batch	Continuous	
Cd	0.17	0.028	6.1	118	0.128	922
Pb	0.68	0.14	4.9	429	0.648	662
Co	0.52	0.098	5.3	331	0.447	740
Ni	0.41	0.09	4.6	244	0.413	591
Zn	0.24	0.057	4.2	150	0.261	574

[a] EC_{50} values based upon growth rate of cell density.

Table 6
Comparison of NOEC values between the batch and continuous tests

Toxicant	NOEC (mg/l)		Ratio (batch/continuous)
	Batch	Continuous[a]	
Cd	0.025	0.003	8.3
Pb	0.1	0.038	2.6
Co	0.038	0.025	1.5
Ni	0.045	0.025	1.8
Zn	0.025	0.020	1.3

[a] NOEC values based on cell density and MCV are identical.

sensitivity between the two kinds of culture is even greater than what we observed from Table 5.

Stratton and Gilles [22] pointed out that initial biovolume in the inoculum plays an important role on the observed toxicity. Greater biovolume could result in less sensitive tests and larger EC_{50} values. They proposed the use of a new parameter—the required exposure effect at 50% response (EE_{50}), for more reasonable comparison. The EE_{50}, which unify the effects of different amounts of algal inoculum, can be calculated as EC_{50} divided by the total cell volume of the inoculum. At the beginning of toxicity tests, the total cell volumes for batch and continuous tests were approximately equal to 1.58×10^6 and 218×10^6 μm^3, respectively. The difference in sensitivity between batch and continuous tests becomes even more apparent based on the ratio of EE_{50} (574–922 times).

Table 6 presents the NOEC values for five metal toxicants (Cd, Pb, Co, Ni, and Zn) in both batch and continuous test. NOEC values from the batch tests were determined using Dunnett's test at $P = 0.05$. The continuous tests, on the other hand, were conducted with 15 μg/l of $Na_2EDTA{\cdot}2H_2O$ in the growth medium and the level of significance $P = 0.027$. Theoretically, it will be more difficult to produce a statistically significant difference in the continuous test than the batch test, especially when the metal concentration is low (considering the effects of EDTA). The ratio of NOEC values between batch and continuous tests varies from 1.3 to 8.3, which also indicates that the continuous test is more sensitive to the stresses of metal toxicants.

Literature data for *C. pyrenoidosa* from US EPA ECOTOX Database [23] were listed in Table 7 and compared with results from the chemostat tests. For EC_{50} values, the differences in sensitivity between two test methods varied from 9.25 to 52.5 times. The toxicity datum for zinc (0.5272 mg/l [2]) is at large comparable with our batch test result (0.24 mg/l). On the other hand, a difference of 160 times can be found for the NOEC values based on nickel.

The difference in sensitivity between the continuous and conventional batch tests can be attributed to the following reasons: The over-enriched growth medium employed by the batch test may cause significant detoxification effects to metal ions [24]. Phosphorus is also known to sequester excessive metals in polyphosphate bodies of freshwater algae [25–27]. Furthermore, as microalgae have been well recognized to have a tremendous ability to uptake heavy metals through biosorption [28–30], more metal ions are expected to be absorbed by algae in the continuous reactor than that in the batch test. Generally speaking, algae in the field are grown under a more diluted and nutrient-limited conditions [31]. Hence, results from the continuous technique provide more realistic description for the impact of toxicants to phytoplankton.

Table 7
Comparisons of continuous test results with literature data (US EPA ECOTOX Database)

Toxicant	Continuous data (this study)	Literature data		Ratio (literature data/continuous data)
	Concentration (mg/l)	Concentration (mg/l)	Endpoint and effects	
Cd	0.028 (24 h-EC_{50})	1.47 [1]	11 days-EC_{50}, GPOP[a]	52.5
		1.37 [1]	21 days-EC_{50}, GPOP	48.9
Zn	0.057 (24 h-EC_{50})	0.5272 [2]	96 h-EC_{50}, PGRT	9.25
		1.1 [3]	96 h-EC_{50}, GGRO	19.3
		1.8 [3]	96 h-EC_{50}, GGRO	31.6
Ni	0.025 (24 h-NOEC)	4 [4]	14 days-NOEC, GPOP	160

[a] GPOP: population change, PGRT: population growth rate, GGRO: growth, general.

The test method presented in this study is rapid and highly sensitive.

4. Conclusion

Toxicity data of five different metals (Cd, Pb, Co, Ni, and Zn) derived from a one-day continuous test were compared against results from the conventional batch tests. The batch test is characterized by its saturated nutrient status for algal growth and, however, the continuous test is conducted at a nutrient-limiting condition simulating more closely to the aquatic field environment. The continuous test consistently yielded smaller EC_{50} values, which indicated that alga in the chemostat is approximately four to six times more sensitive than those from the batch culture. Similar phenomena between the two types of tests were observed based on the NOEC values. Compared to literature data based on the same species of alga, differences in test sensitivity between the batch tests and chemostat technique varied from 9.25 to 160 times. Such distinct differences indicate that current toxicity database may not adequately reflect the impact of various metal toxicants on *C. pyrenoidosa.* Previously, continuous algal toxicity tests (chemostat or tubidostat) were rather time-consuming and were seldom applied for regulatory purposes. The short-term continuous test method applied in this study, however, was found to be more sensitive and rapid as compared to the conventional batch test.

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