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OPTIMIZATION AND PERFORMANCE EVALUATION OF THE CONTINUOUS ALGAL TOXICITY TEST

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Abstract—Responses of *Selenastrum capricornutum* to cadmium in a chemostat are evaluated under various test conditions defined by the nitrogen to phosphorus ratio, phosphate strength, dilution rate, and ethylenediaminetetraacetic acid content. Based on the test results, these parameters were optimized to achieve superior test sensitivity. This continuous system was also modified to allow testing on a weekly basis. The performance of the modified testing technique was evaluated, employing six different metal toxicants. Compared with batch test results reported by other researchers and our own experiment, the continuous test achieved superior sensitivities and reproducibility. The test method presented in this study is believed to be an ideal technique both for research and regulatory purposes.

Keywords—Algae Chemostat EC50 Sensitivity Reproducibility

INTRODUCTION

In recent years, toxicity testing with microalgae has been used extensively in ecotoxicogical studies. Several standard algal test protocols were developed for the comparison of the relative toxicity of chemicals and/or waste discharges [1–4]. These methods, although employing different medium compositions, are quite similar in some respects: they are all basically batch tests and the growth mediums are all enriched with macronutrients including phosphate and nitrogen. In addition, considerable amounts of chelating agents such as ethylenediaminetetraacetic acid (EDTA) were added to the growth mediums.

Previous research indicated that the sensitivity of algal toxicity tests is governed by several factors. The composition of the medium was found to significantly affect the sensitivity of the test algae [5,6]. Furthermore, the type of limiting nutrients was also a possible factor affecting toxicity. Hall et al. [7] reported that algae were more sensitive under phosphorus limitation than they were under nitrogen limitation. Stauber and Florence [6] suggested that chelators, which may alter metal toxicity through complexation, should not be included in the medium. Vasseur and Pandard [8] found that toxicity decreased when the quantity of algal inoculum increased. Nyholm and Kallqvist [9] indicated that excessively high biomass could lead to high pH levels due to poor gas-exchange conditions, which might eventually affect toxicity.

The choice of response variables (i.e., biomass or growth rate) also makes a difference in test sensitivity. Analysis of experimental results from the International Standards Organization's ring-tests [10,11] show that median effective concentrations (EC50s) based on biomass were generally lower and could differ by a factor of two from EC50s based on growth rate. Nevertheless, some scientists prefer to use growth rate because it allows greater reproducibility of results between laboratories [12,13]. Considering the variation in test results, various authors [14,15] reported that the intralaboratory precision of batch tests ranged from 20 to 32%. Interlaboratory comparisons, however, showed that EC50 values could vary by several orders of magnitude [16]. Nyholm and Kallqvist [9], in their critical review of batch-culture toxicity tests, indicated that results from these test methods varied extensively.

Parallel to the development of batch testing techniques, continuous culture in a chemostat was also applied to algal toxicity testing [17,18]. The experimental results from chemostats demonstrate that continuous tests generally exhibit much better sensitivities than do batch tests. Nevertheless, Kayser [19] reported that the difference in sensitivity obtained by the two testing methods was not highly significant. The fact that contradictions occur is not suprising as they may arise from differences in the growth medium composition or operating conditions (dilution rate, temperature, light intensity, etc.) of the different test systems. On the other hand, our previous studies indicated that the sensitivity of the continuous test was correlated with nutrient concentration, which was determined by the dilution rate of the system [20].

The above discussions reveal that medium composition, chelator concentration, cell density, and dilution rate of continuous cultures are also likely to be crucial factors in determining the sensitivity of toxicity testing with a chemostat. Our main objective in this study was to evaluate the effects of these parameters in the chemostat and to optimize them for better sensitivities. Another objective was to standardize the continuous technique for algal toxicity testing so as to minimize any discrepancies in test results between different researchers. The evaluation consists of two parts, the optimization of the aforementioned parameters and the performance evaluation of the optimized test system.

MATERIALS AND METHODS

The alga *Selenastrum capricornutum* (UTEX 1648) was obtained from the University of Texas, Austin, Texas, USA. The alga was grown in a transparent glass reactor with the volume of the culture medium kept at 400 ml. The growth

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Table 1. Composition of U.S. Environmental Protection Agency's (EPA) medium and the revised algal growth medium

Compound	Concentration (mg/L)	Compound	Concentration (μg/L)
$NaNO_3$	25.5[a] (12.75[b])	H_3BO_3	186
$NaHCO_3$	15.0	$MnCl_2 \cdot 4H_2O$	415.6
K_2HPO_4	1.04[a] (0.52[b])	$ZnCl_2$	3.27
$MgSO_4 \cdot 7H_2O$	14.7	$CoCl_2 \cdot 6H_2O$	1.428
$MgCl_2 \cdot 6H_2O$	12.16	$CuCl_2 \cdot 2H_2O$	0.012
$CaCl_2 \cdot 2H_2O$	4.41	$Na_2MoO_4 \cdot 2H_2O$	7.26
		$FeCl_3 \cdot 6H_2O$	160.0
		$Na_2EDTA \cdot 2H_2O$	300[a] (30[b])

[a] The original concentration in the EPA medium.
[b] Concentration revised by this study.

medium was continuously supplied by variable-speed pumps. 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 4,304 lux (±10%). A detailed description of the experimental setup can be found in the authors' earlier works [20,21]. Samples for analysis were taken directly from the effluent pore. The population density of the algae was determined on a 24 h basis using an electronic particle counter (Culter Electronics, Inc., Luton, England). Growth rate was chosen as the response parameter. The system was first allowed to reach a steady state with no toxicant involved. Then toxicant was added both to the reactor and the medium influent until the required concentration level was reached. This addition created a nonsteady state in the reactor. In a chemostat at steady state, the specific growth rate (μ) is equal to the dilution rate (D) and the population density remains constant. If a toxicant reduces the growth rate, cells wash out faster than they are replaced by growth (i.e., $\mu < D$ or $\mu/D < 1$) and the population density declines. The new growth rate μ (disturbed growth rate) can be calculated from the rate of population decline according to the following equation:

$$\mu = \left[\ln\left(\frac{x_2}{x_1}\right) \Big/ (t_2 - t_1)\right] + D$$

where x_1 and x_2 are densities, in terms of cells/ml, at time t_1 and t_2, respectively. The steady state growth rate, which is equal to D, can also be considered as the chemostat's unperturbed growth rate. Hence, the EC50 is calculated as the toxicant concentration that reduces μ to one-half of its unperturbed value. A more detailed discussion regarding the equation and the definition of the EC50 can be found in the first author's previous work [20].

In order to develop a relatively simple and short-term technique, the exposure time for the nonsteady state test was set at only 24 h. Furthermore, after completing the first part of evaluation (the optimization of medium composition and test conditions), an 8-L chemostat reactor was run under the same conditions as the 400-ml test vessels. This larger reactor provided a steady supply of algae for toxicity tests conducted in the 400-ml vessels.

For comparison purposes, batch tests were also conducted following the U.S. Environmental Protection Agency's (EPA) method [2]. Again, algal growth rate was selected as the response parameter. For each toxicant concentration and the control, three flasks were used for the batch tests. The initial inoculum cell density was approximately 15,000 cells/ml. In our past experiments, inconsistent test results were obtained when inoculum cell density was below 10,000 cells/ml.

All glassware was thoroughly cleaned with phosphorus-free detergent and rinsed with tap water. This was followed by an acid rinse (10% HCl), after which the glassware was neutralized with a saturated solution of sodium bicarbonate, and finally rinsed with double-distilled deionized water. Growth medium was filter-sterilized through a 0.45-μm membrane filter. Orthophosphate content was analyzed according to the stannous chloride method [22]. The NO_3-N concentration was also analyzed by ion chromatography. All chemicals used were of reagent grade.

RESULTS

The EPA medium was selected as the initial basis for this study, simply because it was used in our previous work. Table 1 lists the original composition of the medium. In chemostats, cell density is controlled by both dilution rate and medium strength. Thus, the parameters to be optimized were the nitrogen to phosphorus (N:P) ratio, dilution rate (D), medium strength, and EDTA concentration. Optimization was governed by two major criteria: the growth condition during the steady state and the sensitivity to toxicants during the second stage of nonsteady state tests.

Optimization of the test system

Table 2 illustrates the three different N:P ratios ranging from 11.3 to 45.0 that were tested at $D = 0.3$/d. The nutrient concentrations in the influent are NO_3-N and PO_4-P. Tests for different conditions were carried out in duplicate. Cell density was measured at steady state. In these tests only nitrogen and phosphate contents were changed. 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 appeared to have no significant effect on algal growth. After establishing steady states, a 1-d nonsteady state toxicity test was conducted with cadmium (cd) (0.075

Table 2. Effects of nitrogen to phosphorus (N:P) ratios on a toxicity test (at $D = 0.3$/d and Cd = 0.075 mg/L)[a]

N:P	NO_3-N (mg/L)	PO_4-P (mg/L)	Cell density (10^6 cells/ml)	μ/D		IR (%)	
				1	2	1	2
11.3	4.2	0.372	8.18	0.59	0.65	41	35
22.5	4.2	0.186	4.39	0.24	0.19	76	81
45.0	8.4	0.186	4.17	0.25	0.21	75	79

[a] D = dilution rate: Cd = cadmium; NO_3-N and PO_4-P = nutrient concentrations in the influent; μ = disturbed growth rate; 1 and 2 = replicates; IR = inhibition rate; IR (%) = $(1 - \mu/D) \times 100$.

Table 3. Effects of phosphate strength on toxicity test (at D = 0.5/d and Cd = 0.075 mg/L)[a]

Phosphate strength (%)	Cell density (10^6 cells/ml)	μ/D		IR (%)	
		1	2	1	2
100	3.56	0.30	0.33	70	67
50	1.70	0.21	0.22	79	78
20	0.72	0.03	0.04	97	96

[a] D = dilution rate; Cd is cadmium; μ = disturbed growth rate; 1 and 2 = replicates; IR = inhibition rate; IR (%) = $(1 - \mu/D) \times 100$.

mg/L), and results in relative growth rates (μ/D) and the corresponding inhibition rates were obtained. The choice of 0.075 mg Cd/L was made because significant inhibition was observed in our past experiment. Comparisons were made to determine whether one test condition consistently achieved better sensitivities than others. It was found that N:P ratios of 22.5 and 45.0 did not significantly differ from each other. Algae in medium with an N:P ratio of 11.3 were the least sensitive, with a mean inhibition rate of only 38% (Table 2). Because increasing nitrogen content did not improve the test sensitivity, for the following experiments the N:P ratio was kept the same as that in the original EPA medium (i.e., 22.5).

Table 2 indicates that phosphorus content in the medium directly controls cell density. Hence, evaluating the effects of medium strength (or cell density) on toxicity requires altering only the phosphate concentration in the medium. The primary concern here is to prevent other nutrients or trace elements from becoming limited while the strength of the entire medium is being reduced. Therefore, the term ''medium strength'' should be more appropriately replaced by ''phosphate strength.'' However, the nitrogen concentration must be adjusted with the phosphate content to maintain the desired N:P ratio. Table 3 presents the effects of phosphate strength on toxicity tests at D = 0.5/d and Cd = 0.075mg/L. One hundred percent phosphate strength represents the original EPA medium and 50% strength implies that the nitrogen and phosphorus contents were reduced to one-half of that in the EPA medium, while the amounts of other nutrients remained the same. Cell density was generally proportional to phosphate strength. In terms of sensitivity, we observed that inhibition rate increased with a decrease in cell density. Such a phenomenon can be explained as follows: higher cell density means lower toxicant load per individual cell and, thus, a lower degree of inhibition. Vasseur and Pandard [8] made a similar observation that greater EC50 values are associated with a higher density of inoculum.

Table 4 presents a summary of a series of comparisons with respect to different dilution rates. Orthophosphate and nitrate contents were the equilibrium concentrations in the reactor and were determined when the system reached steady state. At D = 0.5/d, no significant difference was observed for the equilibrium phosphorus concentration between the three phosphate strengths listed. This phenomenon suggests that the medium's phosphate strength does not affect the phosphorus concentration in the chemostat under the same dilution rate. The pH values were all maintained within the range of 7.5 to 8.5. The rating of the growth conditions was based primarily on the frequency of obvious microbial contamination, which was manifested in a change of the color of the culture from a blue-green hue to a slightly yellowish one. Test systems that easily or occasionally induced the above condition were rated as poor or fair, respectively. Good growth conditions were those where no obvious contamination occurred and where a steady state could be easily maintained for long periods (e.g., 3–6 months). Significant microbial contamination generally occurred under low-strength and low-dilution-rate conditions. Previous studies have indicated that bacterial contamination is unavoidable for chemostates under long-term operation. However, the bacterial biomass is relatively small compared to that of the total culture, and its effect on the algal culture is almost undetectable [23].

Table 4. Effects of dilution rate and phosphate strength on algal growth and toxicity test (Cd = 0.075 mg/L)[a]

Phosphate strength (%)	Cell density (10^6 cells/ml)	pH	PO_4-P (mg/L)	NO_3-N (mg/L)	Growth condition	μ_{ave}/D	Mean inhibition rate (%)	Sensitivity
				D = 0.3/d				
100	4.06	8.30	0.002	1.992	Fair	0.22	78	Good
50	1.73	7.78	0.002	1.000	Fair	0.16	84	Good
20	—	—	—	—	Poor	—	—	—
				D = 0.5/d				
100	3.56	8.24	0.007	2.020	Good	0.31	69	Good
50	1.70	7.66	0.005	1.008	Good	0.21	79	Good
20	0.72	7.52	0.005	0.453	Poor	0.03	97	Good
				D = 0.7/d				
100	3.34	8.15	0.011	2.222	Good	0.60	40	Poor
50	1.56	7.60	0.011	1.173	Good	0.48	52	Fair
				D = 0.9/d				
100	3.20	8.23	0.016	2.279	Good	0.73	27	Poor
50	1.51	7.58	0.015	1.218	Good	0.53	47	Poor

[a] Cd = cadmium; PO_4-P and NO_3-P = equilibrium concentrations in the reactor; μ_{ave} = average disturbed growth rate (day^{-1}); D = dilution rate (day^{-1}).

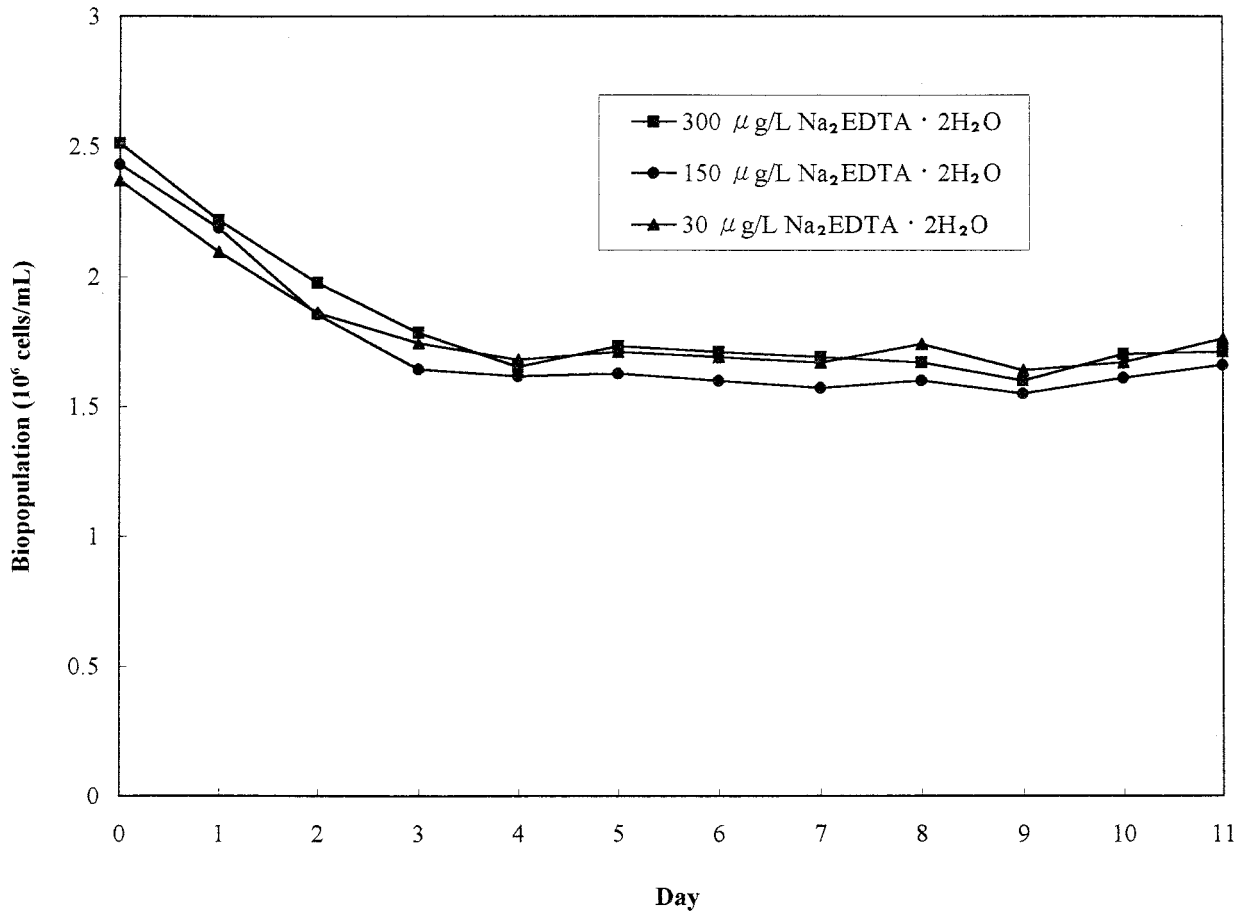

Fig. 1. Growth conditions of *Selenastrum capricornutum* under three different concentrations of ethylenediaminetetraacetic acid in a chemostat with dilution rate (D) = 0.5/d.

For the case of D = 0.3/d and 20% phosphate strength, after several trials, steady state was never attained. Toxicity testing with 0.075 mg/L of Cd indicated that the inhibition rate increased with decreasing phosphate strength for the four different dilution rates (see Table 4). In addition, the degree of inhibition increased as the dilution rate diminished. This is in accordance with our previous report [20], which concluded that tolerance in algal cells is reduced as nutrients become more limiting. The differences in tolerance of various dilution rates can also be compared (see Table 4). At 100% phosphate strength, cell densities under D = 0.5/d and D = 0.7/d were not significantly different. However, the inhibition rate for the former was 69%, whereas that for the latter was only 40%. This indicates that the average tolerance of algal cells decreases at lower dilution rates. It can be concluded that the sensitivity of algal toxicity tests is mainly determined by two factors: dilution rate and cell density. The dilution rate determines the general tolerance in a microbial culture. Under a specific dilution rate, the cell density of an algal culture, which is regulated by the phosphate strength, determines the individual toxicant load of the cell. The case of D = 0.5/d and 50% phosphate strength, which provided both good growth condition and sensitivity, was thus selected for further optimization evaluation. The test condition at 100% strength (D = 0.5/d) was not selected because of the apparent lower inhibition rate.

Table 5. Effects of concentration of ethylenediaminetetraacetic acid (EDTA) on the algal growth and toxicity test (at D = 0.5/d, 50% phosphate strength, and Cd = 0.075 mg/L)[a]

$Na_2EDTA\cdot 2H_2O$ (μg/L)	Cell density ($\times 10^6$ cells/ml)	Inhibition rate (%)[b]	
		1	2
300	1.67 ± 0.10	75.3	80.3
150	1.61 ± 0.07	90.6	87.4
30	1.70 ± 0.06	97.0	93.6

[a] D = dilution rate; Cd = cadmium.
[b] 1 and 2 are replicates.

Based on the above test conditions and medium composition, the next task was to determine the appropriate concentration for chelators. Figure 1 depicts the growth conditions, from initial inoculation to reaching a steady state, employing three different $Na_2EDTA\cdot 2H_2O$ concentrations. Medium with 150 μg/L yielded the lowest biopopulation (1.61×10^6 cells/ml) at a steady state, but it did not differ significantly from the other two cases. Comparisons based on a student's t test at the α = 0.05 level also showed no differences between $Na_2EDTA\cdot 2H_2O$ = 150 and 30 μg/L. Reducing $Na_2EDTA\cdot 2H_2O$ content to 30 μg/L apparently did not adversely affect algal growth. Table 5 lists algal responses to 0.075 mg/L of Cd. As expected, data from duplicate tests consistently showed a better sensitivity at lower EDTA concentrations. Hence, 30 μg/L of $Na_2EDTA\cdot 2H_2O$ was considered adequate for the chemostat

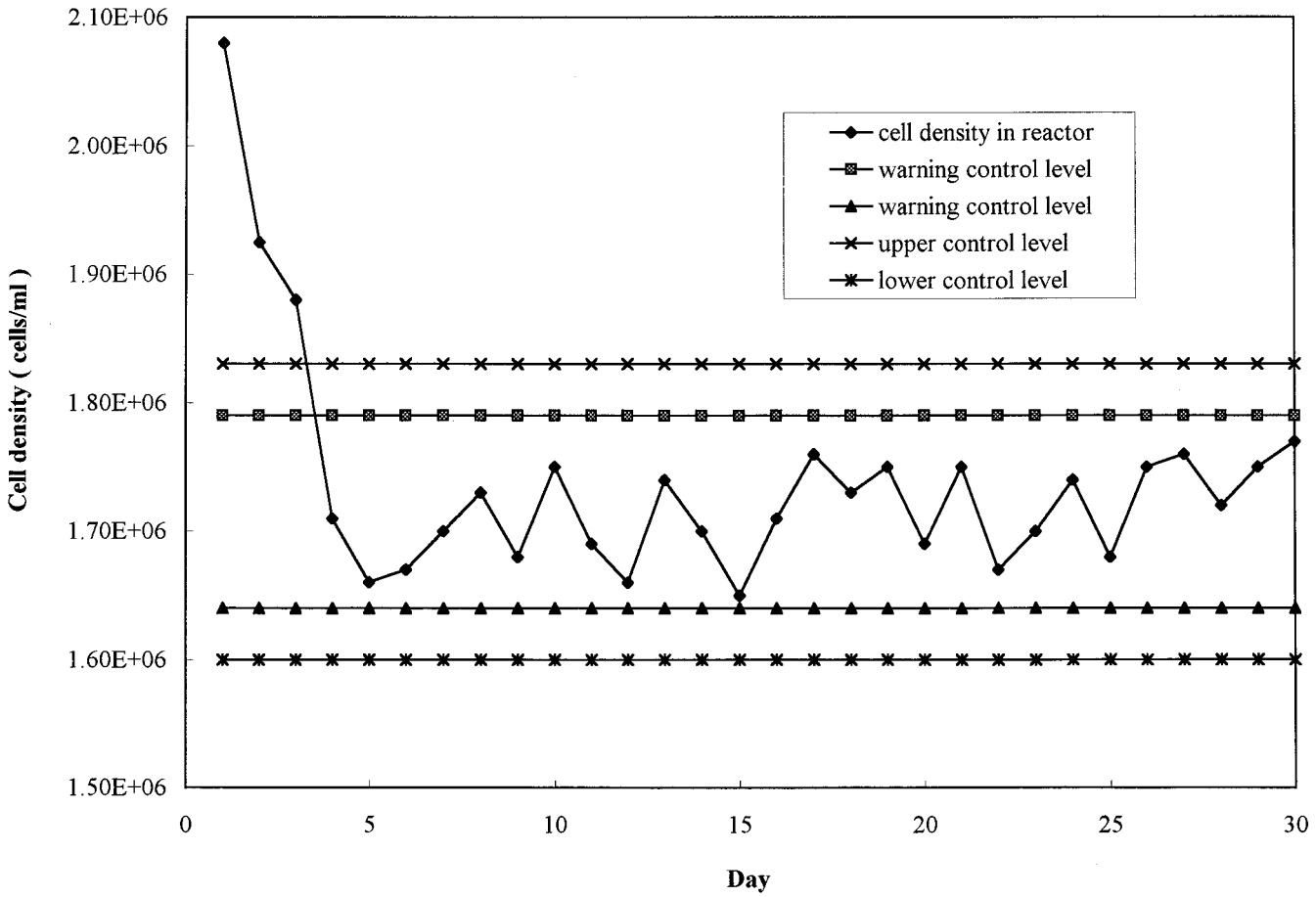

Fig. 2. Control chart based on cell density in the algal incubator.

system. Table 1 also lists the revised algal growth medium, which can be compared to the original EPA medium.

Obviously, our selection of the optimum test condition was not entirely based on the test's sensitivity. The final choice, in fact, was to sacrifice part of the sensitivity in exchange for a more stable system. Such a sacrifice is aimed at ensuring that a steady state can be easily maintained for long-term operations. The significance of such a choice is revealed in the operation of an algal incubator, which will be discussed below.

Performance evaluation

Using the revised algal medium given in Table 1, an 8-L algal incubator was run at $D = 0.5$/d. Records of the first month of operation were collected in order to establish quality assurance criteria for toxicity testing. Figure 2 is the control chart for cell densities in the chemostat, which shows that the variation of the biopopulation was within $1.7 \times 10^6 \pm 6\%$ cells/ml. The pH values were 7.65 ± 0.15 at steady state. Nutrient concentrations were periodically checked and were quite similar to the corresponding values given in Table 4. If cell density and pH were within the normal range for three consecutive days, toxicity testing was conducted by dispensing algal liquor into six 400-ml chemostat reactors (one control and five treatments) for the nonsteady state test. A preliminary 2-d comparison showed that no significant differences were observed for cell densities, pH, and nutrient concentrations, within the 400-ml vessels after being transferred from the larger reactor. The purpose of performing a control run was to determine whether any unidentified variations occurred that might significantly affect algal growth. The 24-h toxicity test was performed in exactly the same manner as mentioned earlier: at the beginning of the test, a toxicant was added to the reactor and the medium influent until the required concentration level was reached. For each test run, 2.4 L of algal liquor was removed from the 8-L incubator (30% volume). A previous study indicated that a sample withdrawal up to 30% of the chemostat volume does not have a significant impact on the algal population [23].

Six metal toxicants (Cd, lead [Pb], zinc [Zn], copper [Cu], nickel [Ni], and mercury [Hg]) were tested to evaluate the general sensitivity of the optimized continuous system. Table 6 lists the test results in terms of EC50, standard deviation (SD) of the EC50, and the coefficient of variation (CV). The CV values for various metals varied from 8.5 to 22.6%. In addition, for comparative purposes, Table 6 lists data derived from the batch method as reported in the literature [24–28]. These data were mainly calculated on biomass unless otherwise specified. Obviously, for Cd, Zn, Pb, and Hg, the literature EC50 values were two to four times greater than our values, which were calculated on growth rate. Similar sensitivities were found for Cu. In addition, based on a growth rate calculation, the literature value for Cu was 20 times greater than that from the continuous test. On the other hand, the data for Ni indicated that the batch test was more sensitive than the continuous technique. Chiaudani and Vighi [27] used an inoculum density of 4,000 cells/ml to obtain such a low EC50 value. According to the findings of Vasseur and Pandard [8], such superior sensitivity could be related to the low cell density

Table 6. Sensitivity and reproducibility of the continuous algal toxicity test[a]

Metal toxicant	EC50 (mg/L)	*n*	SD	CV (%)	Reported[b] EC50 (mg/L)
Cd	0.013	5	0.0011	8.5	0.04 [23]
Cu	0.021	3	0.0031	14.5	0.04 [23]
					0.4 [24][c]
Zn	0.015	3	0.0017	11.3	0.06 [23]
Pb	0.256	3	0.0580	22.6	0.5 [25]
Ni	0.125	3	0.0125	10.2	0.012 [26]
Hg	0.027	3	0.0053	19.2	0.06 [27]

[a] EC50 = median effective concentration; *n* = numbers of replicate tests; SD = standard deviation; CV = coefficient of variation; Cd = cadmium; Cu = copper; Zn = zinc; Pb = lead; Ni = nickel; Hg = mercury.
[b] Reference numbers are given in brackets.
[c] EC50 based on growth rate.

Table 7. Reproducibility of median effective concentrations (EC50s) (for copper) obtained with *Selenastrum capricornutum* using various methods[a]

Method	*n*	EC50 (μg/L)	CV (%)	Source
Conventional	6	13.8	32.1	Van Coillie et al. [15]
Conventional	3	29.9	22.6	Blaise et al. [14]
MP	3	54	20.4	Blaise et al. [14]
MP	3	60	25.0	Blaise et al. [14]
Chemostat	3	21.3	14.5	Our data [this study]

[a] Conventional method = the algal bottle test; MP method = the microplate algal test; *n* = number of replicates; CV = coefficient of variation.

in the inoculum. However, the latest ASTM method [4] recommends the use of an inoculum density of 2×10^4 cells/ml.

Table 7 compares the test reproducibilities of different test methods using Cu. The CV values from chemostat tests were much lower than those obtained by batch methods (i.e., conventional bottle test and microplate techniques). One possible explanation is that, for chemostat testing, algae were always maintained at the steady state in similar physiological conditions, whereas for batch tests, the variously prepared initial inoculums could result in different physiologic states.

To create a uniform basis for comparison, batch tests were conducted following the EPA method [2]. The EC50s calculated based on growth rate were compared with the result from the continuous tests, as given in Table 8. The continuous test was found to be more sensitive than the batch test for Cd, Cu, Zn, Pb, and Ni. Ratios of EC50s between the two types of tests ranged from 1.83 to 26.3. The 95% confidence intervals for the two sets of EC50s did not overlap. Hence, we may assert that the differences are obvious at a 5% level of significance. The continuous method was found to be less sensitive to Hg, as its EC50 was three times greater than that of the batch test. The difference, however, was not significant at the 5% level. The lower sensitivity of the continuous test to Hg may be due to the formation of more volatile forms of Hg from reactions between Hg and organic compounds released by algae. Aeration may cause a rapid decrease in the amount of Hg in solution [29]. Comparisons were also made according to the free ionic forms of the metals, which were considered to be the most toxic. Free metal concentrations in standard EPA medium and in our revised medium were calculated using the MINEQL program [30]. The EC50s based on free ion concentrations are calculated and listed in Table 8. Similarly, the continuous test resulted in lower EC50 values for most cases but not for Hg and Cu. The literature has indicated that certain metal–EDTA complexes (CuEDTA and ZnEDTA) are also quite toxic [31]. Hence, free Cu ions may be the most toxic form, but certainly are not the major toxic form of Cu. Therefore, irrespective of whether relating to total concentration analysis or to ionic form concentration analysis, EC50s obtained from the continuous test are generally lower than those obtained from the batch test. This means that the continuous test is more sensitive than is the batch test. Figure 3 depicts the concentration–response curves based on the total Cd concentrations from both batch and continuous tests. The slope of the concentration–response curve is very steep for the continuous test, which implies that the tolerances for algae are distributed within a narrow range. In comparison, the moderate slope of the batch test indicates that the tolerance distribution is wider. Our interpretation of such a difference in slopes is as follows: the algae cultured in the chemostat probably have more uniform tolerances because they are kept by the steady state at similar physiological conditions. We believe such uniformity may be one of the reasons for the good reproducibilities shown in Tables 6 and 7.

To identify any inconspicuous changes that might have occurred in the algal culture, Cd was selected as the reference toxicant. As shown in Table 6, the five tests for Cd were conducted within a period of 6 months. The CV value was only 8.5%, which indicates that the general tolerance for the algal culture was very consistent during long-term operations.

Table 8. Median effective concentration (EC50) values and 95% confidence intervals (CIs) for continuous and batch toxicity tests

	Continuous test		Batch test		
Metal toxicant	EC50 (mg/L)	95% CI (mg/L)	EC50 (mg/L)	95% CI (mg/L)	Ratio of batch EC50 to continuous EC50
Cd	0.013 (0.0040[b])	0.012–0.017	0.341[c] (0.238)	0.110–1.672	26.3
Cu	0.021 (0.0029)	0.018–0.031	0.038 (—)[d]	0.031–0.054	1.83
Zn	0.015 (0.013)	0.011–0.026	0.178 (0.132)	0.110–0.271	11.9
Pb	0.256 (0.0044)	0.285–0.356	2.655 (0.0163)	1.983–4.539	10.4
Ni	0.125 (0.113)	0.106–0.173	0.233 (0.173)	0.174–0.311	1.91
Hg	0.027 (4.67×10^{-12})	0.011–0.085	0.009 (1.50×10^{-12})	0.002–0.034	0.33

[a] Cd = cadmium; Cu = copper; Zn = zinc; Pb = lead; Ni = nickel; Hg = mercury.
[b] The EC50 was estimated based on free metal concentration.
[c] Value also presented in Lin and Chen [21].
[d] Ninety-nine percent of the Cu was complexed with ethylenediaminetetraacetic acid.

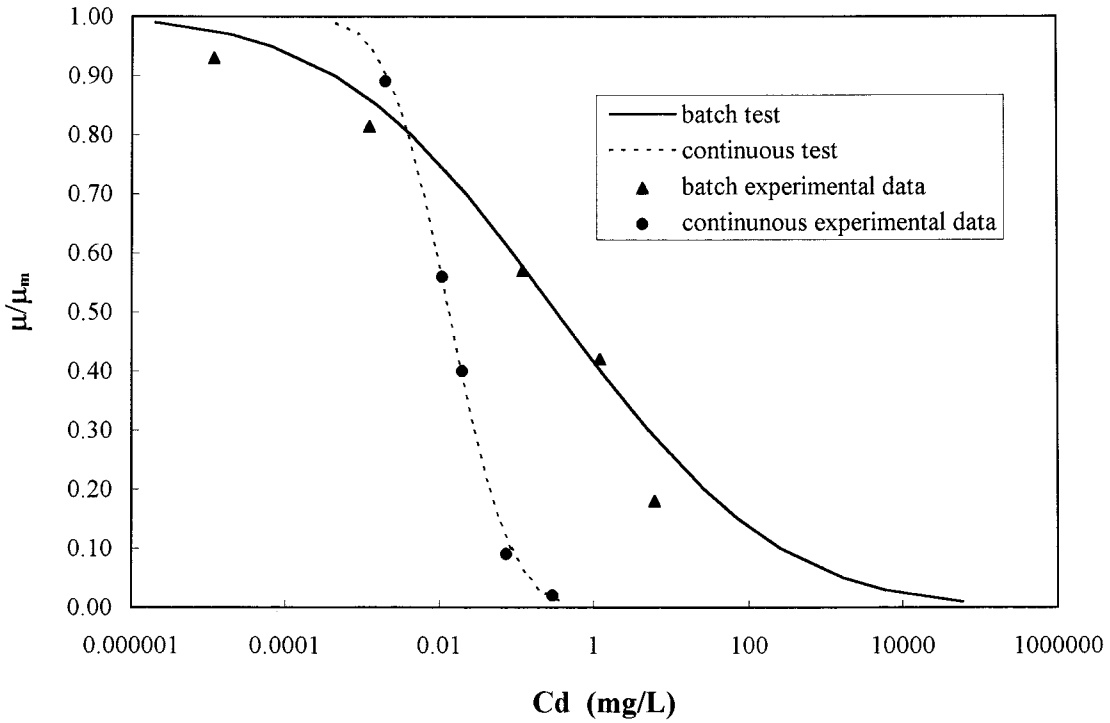

Fig. 3. The concentration–response relationships for *Selenastrum capricornutum* in batch and continuous toxicity tests.

Additional quality assurance criteria, including cell density and pH for the 8-L incubator, were discussed above. The continuous tests in our performance evaluation were conducted without replicates, primarily because of the maximum 30% withdrawal of the chemostat volume. However, as revealed in Tables 2, 3, and 5, the continuous tests produced minute variations among replicates. This is not always found in batch test results. For such a stable system, tests can be run with no replication because any abnormal results can still be determined from the relative inhibition rates under different toxicant concentrations. On the other hand, the volume of the test vessel could also be reduced to 200 ml to allow for duplicate tests.

The continuous toxicity test system developed in this study poses the following advantages over batch tests: the test duration is only 24 h instead of the 96 h required by traditional tests and this method is also considerably more sensitive and has superior reproducibility. Previously, the major disadvantage of the chemostat system, thereby limiting its application in algal toxicity assessment, was 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. Our modification of adding the 8-L incubator, however, minimized the disturbance of the steady state brought about by toxicity testing and enables us to conduct tests on a weekly basis. This is almost equivalent to the total run time for the batch test (including the time for preparing the inoculum). In addition, according to our own experience, the batch test is not necessarily less labor-intensive than the continuous one. We believe the modified test system is ideal for laboratories or regulatory agencies that are routinely involved in algal toxicity testing.

CONCLUSIONS

This study describes the effects of the N:P ratio, dilution rate, phosphate strength, and EDTA concentrations on algal growth and algal sensitivity to metal toxicants in a chemostat. The N:P ratio for the original EPA medium, (22.5) was found to be adequate. A low phosphate strength resulted in low cell density and, consequently, better algal sensitivity. Likewise, a low dilution rate for the chemostat improved test sensitivity. The EDTA content was reduced to 30 μg/L without any adverse effect on algal growth. Based on these results, we have optimized the continuous algal testing system with respect to the above parameters. This optimized system can be considered as the basis for standardizing future toxicity testing employing the continuous system, to minimize discrepancies arising between laboratories. We modified the continuous technique by introducing an algal incubator so that testing can be conducted more frequently. In addition, several quality assurance criteria were developed to insure better laboratory precision of the test. The performance of the continuous testing technique developed by this study was evaluated with six different metal toxicants. Compared to batch test results reported in the literature as well as our own experiment, the continuous test achieved superior sensitivities and reproducibility compared to the batch test. We believe the test method discussed herein is an ideal technique for both research and regulatory purposes.

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