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Discrepancies between different response parameters in batch and continuous algal toxicity tests

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Abstract

The test sensitivities and reproducibilities based on different response endpoints (cell density and total cell volume) were compared using both bath and continuous algal toxicity tests. Parameters related to algal cell density are found to be more sensitive and reproducible than total cell volume which bears a direct correlation to algal biomass or dry weight. The magnitude of differences in the median effective concentration values could be upto six folds. The main cause for the above discrepancies is because the cell density and total cell volume are two response endpoints reflecting the degree of inhibition on the binary fission mechanism and overall photosynthesis, respectively. At low toxicant concentrations, the inhibitory effect is primarily on the cell division rather than on the cellular photosynthesis. Thus, more severe toxic effects are expected to be observed based on the cell density than the total cell volume. The results of this study clearly demonstrate that sensitivity and reproducibility can be significantly improved if response parameters are calculated based on the cell density. Traditionally, cell density data are often converted into biomass before assessing the effects of toxicants. This procedure may not be necessary and may reduce the test sensitivity.

Keywords: Toxicity; Algae; *Raphidocelis subcapitata*; Cell density; Biomass

1. Introduction

Microalgae have been used extensively as biological indicators to assay pollutant toxicity. Algal growth estimated from biomass is a traditional response parameter in most studies of phytoplankton. Algal biomass can be estimated by methods such as dry weight, absorbance, chlorophyll, and total volume of algal cells. For unicellular organisms, biomass determination is often replaced by direct counting of the cell number. The total cell volume (TCV),

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which is a product of the mean cell volume (MCV) and the total cell number, is a surrogate measurement for algal biomass. The final yield of algae and the growth rate are two types of response endpoints which have been commonly employed to describe the inhibitory effects of toxicants [1]. Analysis of the experimental results from International Standards Organization's (ISO) ring-tests reveal that EC50s based on biomass (the final yield of TCV) were generally lower and could differ from those based on growth rate by a factor of 2 [2,3]. However, some researchers prefer growth rate owing to the greater reproducibility of results [1,4].

Researchers examining the effects of toxic chemicals on microalgae have found that cells respond to levels of toxicants through cell volume regulation. Abdel-Hamid et al. [5] reported significant increase in MCV of *Raphidocelis subcapitata* with the increase in incubation time at higher concentrations of industrial effluents. Hence, they suggested that changes in MCV during the period of toxicity testing could be used as a criterion for monitoring the waste impact on the overall cellular metabolic activities of the test alga. Similar effects were reported for various kinds of algae exposed to metal and/or organic toxicants [6–10]. On the contrary, Fernandez-Leborans and Novillo [11] reported that the presence of cadmium resulted in smaller *Olisthdiscus luteus* cells with morphological abnormality. Such reduction in biovolume has also been observed by other workers, and it was rationalized as the metabolic cost of the detoxification process [12,13].

The morphological abnormality of algal cells could unavoidably create some discrepancies in the toxicity test results, when toxic responses are estimated based either on cell density or total cell volume (TCV). The purpose of this study is therefore to evaluate different endpoints of the algal toxicity test, in terms of the test sensitivity and variability, using batch and continuous culture techniques. For batch tests, two response endpoints, namely, cell density and TCV are studied. The median effective concentration (EC50) values are estimated according to the final yields or the growth rates of the two types of response end points. Continuous tests were conducted in chemostat reactors as described previously by Chen and Lin [14]. Due to the nature of the experimental design, only two parameters (i.e. growth rates based on cell density and TCV) were evaluated.

2. Materials and methods

2.1. Batch tests

The alga *R. subcapitata* (formerly known as *Selenastrum capricornutum*, UTEX 1648) was obtained from the University of Texas, Austin. Batch tests were conducted following the EPA method [15]. The initial inoculum cell density was approximately 10,000 cells/ml. The cultures used in this study were already in their exponential phase when toxicants were introduced. Four response parameters were studied, namely, the 96 h cell density (cells/ml), the 96 h total cell volume (TCV, m^3/ml), the growth rate based on cell density (per day) and the growth rate based on TCV (per day). Cell density and mean cell volume (MCV) were measured by a Coulter Counter (model ZM) connected to a channelyzer. The Coulter Counter was calibrated using an Isoton II solution containing one drop of well-mixed Organic Calibration Material (Lot# 13020). TCV was calculated by multiplying cell density

Table 1
Composition of 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] Concentration applied in the batch tests.
[b] Concentration applied in the continuous study.

with MCV. The specific growth rate can be defined as

$$\mu = \frac{\ln(x_2/x_1)}{t_2 - t_1} \tag{1}$$

where x_1 and x_2 denote cell densities (or TCV) at times t_1 and t_2, respectively. The number of replicates for the control and treatments were equal to three. For toxicants cadmium (Cd) and nickel (Ni), tests were repeated three times in order to obtain the values of the coefficient of variation (CV). Experimental data were fitted into the probit model and EC50 values were determined accordingly.

2.2. Continuous tests

Algae (*R. subcapitata*) were grown in an 8-liter transparent chemostat reactor (the incubator). The growth 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 $24 \pm 1^{\circ}$C. Light intensity was set at 65⌈$Em^{-2}\,s^{-1}$ (±10%). Table 1 lists the growth medium composition which is basically the same as that described by the EPA bottle technique [15]. However, modifications of phosphorus, nitrogen, and EDTA contents were made according to our previous study [14]. The dilution rate (D) for the chemostat was set at 0.5 per day to ensure a nutrient-limited condition. At the steady-state, the phosphorus and EDTA concentrations within the chemostat were 0.007 mg/l and 30 μg/l, respectively.

Samples were taken directly from the effluent pore for analysis. For the 8-liter incubator, quality assurance (QA) 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 test was conducted (with a test-duration of 24 h) by dispensing the algal suspension into seven 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-liter incubator. Toxicants were then added both to the reactors and to the medium influent until the desired

concentration level was reached. The specific growth rate during the test period can be calculated from the rate of population decline according to the following equation:

$$\mu = \frac{\ln(x_2/x_1)}{t_2 - t_1} + D \tag{2}$$

where x_1, x_2, t_1, t_2 and D have been defined previously. The number of replicates for controls and treatments were 6 and 3, respectively. Due to the limited amount of algal suspension that could be withdrawn from the incubator, the above experiments were divided into three sets of tests. Each set of tests contained two controls and five treatments with different toxicant concentrations. Two response parameters, i.e. growth rate based on cell density and growth rate based on TCV, were calculated to obtain EC50s at the end of the 24 h test period. A detailed description of the continuous testing technique could be found in the previously published work of Chen and Lin [14].

3. Results and discussion

Fig. 1 shows the time-related changes in cell densities and MCV values in batch cultures treated with different concentrations of nickel. Based on the cell density values, it could be found that exponential growth phase was maintained during the 96 h test period. Algal cells with 0.01 mg/l nickel had the same growth pattern as the control. Cultures exposed to nickel at concentrations ranging from 0.03 to 0.15 mg/l produced significant increase in MCV as compared to the control. Table 2 shows the inhibitory effects of nickel, as measured by different response parameters, observed at the end of the test. With increasing toxicant concentration, all the response parameters declined except for the mean cell volume (MCV) of algae.

The calculated EC50 values for various response parameters in batch tests are listed in Table 2. Using either cell density or TCV, the EC50s calculated based on growth rates are greater than those based on the 96 h final yields. This is in accordance with previous

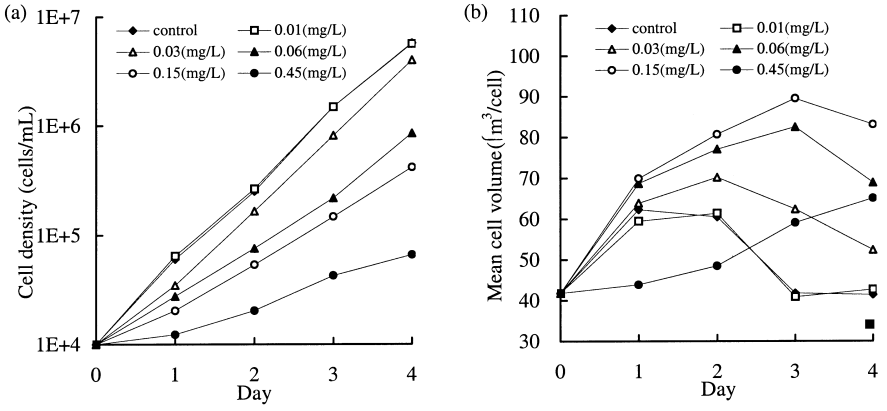

Fig. 1. Effect of nickel concentration on the growth of *Raphidocelis subcapitata* in batch tests: (a) cell density; (b) mean cell volume.

Table 2
Effects of nickel on *Raphidocelis subcapitata* from batch tests

Ni (mg/l)	96 h cell density × 10^4 (cells/ml)	GR[a] based on cell density (per day)	MCV[b] (μm^3 per cell)	96 h TCV[c] × 10^6 (μm^3/ml)	GR based on TCV (per day)
Control	562	1.58	40.5	228	1.57
0.01	559	1.58	42.5	238	1.58
0.03	380	1.49	51.6	196	1.53
0.06	90.6	1.12	63.0	57.1	1.23
0.15	45.1	0.95	79.9	36.0	1.10
0.45	8.1	0.52	76.8	5.3	0.64
EC50 (mg/l)	0.039	0.209	–	0.048	0.339

[a] Specific growth rate.
[b] Mean cell volume.
[c] Total cell volume.

comparisons drawn from the ISO's ring-tests which revealed that biomass is a more sensitive parameter than the growth rate [2,3]. Furthermore, parameters based on cell densities are obviously more sensitive than those related to TCV. At Ni = 0.03 mg/l, the inhibition rate based on 96 h cell density is equal to 32% (1 − (380/562)). However, based on 96 h TCV, the inhibition rate is only 14% (1 − (196/228)). As also evidenced by the corresponding MCV value, it may be concluded that the inhibitory effect at Ni = 0.03 mg/l is primarily on the binary fission mechanism rather than on the cellular photosynthesis of algae. Uninhibited cellular photosynthesis resulted in the accumulation of macromolecules and subsequent cell volume augmentation. The low sensitivities for parameters based on TCV are therefore due to the increase in the algal cell size, which results in lower growth reductions in terms of TCVs. When algal cells were exposed to 0.45 mg/l of nickel, MCV begins to decrease. This observation suggests that algal cells subjected to fairly high concentrations of metal toxicants be disturbed both in cell division and cellular photosynthesis.

Results of batch tests with four metal toxicants (Cd, Ni, Zn, Cu, and Pb) are listed in Table 3. In estimating the EC50 values, final yield measurements (96 h TCV and 96 h cell density) were consistently more sensitive than the growth rates (TCV and cell density). In addition, it could be observed that the parameters based on TCV were less sensitive than

Table 3
EC50 values derived from different response parameters in batch toxicity tests

Metal	96 h TCV[a] (a)	96 h cell density (b)	Ratio of (a)/(b)	GR[b] based on TCV (c)	GR based on cell density (d)	Ratio of (c)/(d)
Cd[c]	0.057	0.046	1.24	0.185	0.131	1.41
Ni[c]	0.044	0.037	1.19	0.382	0.190	2.01
Zn	0.070	0.060	1.17	0.243	0.187	1.30
Cu	0.024	0.023	1.04	0.059	0.050	1.18

[a] Total cell volume.
[b] Specific growth rate.
[c] Mean values from repeated tests ($n = 3$).

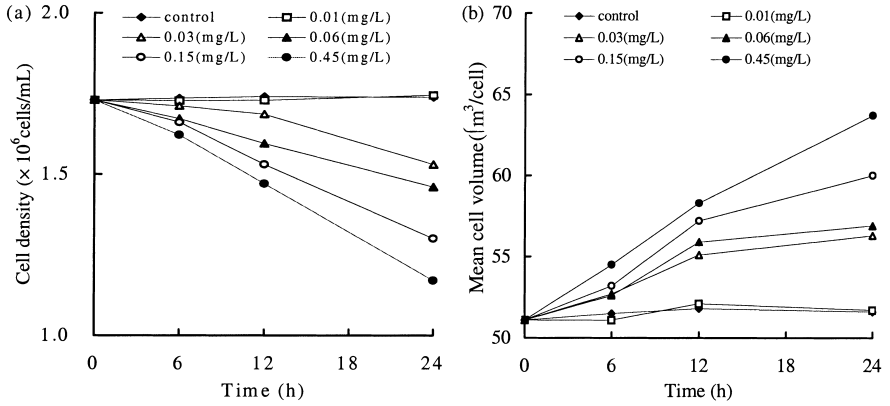

Fig. 2. Effect of nickel concentration on the growth of *Raphidocelis subcapitata* in continuous tests: (a) cell density; (b) mean cell volume.

those related to cell density for all metal toxicants. The distinct differences in the sensitivities of the TCV and cell density are manifested by the ratio of the corresponding EC50 values. The final yield measurements showed that the EC50s based on TCV were 1.04–1.24 times greater than these based on the cell densities. For the growth rate calculations, this ratio was in the range of 1.18–2.01. Table 3 depicts that the sensitivities for different response parameters are in the order of 96 h cell density > 96 h TCV > growth rate based on cell density > growth rate based on TCV. The conventional parameter, algal biomass, has been found to be directly correlated to the 96 h TCV [16]. Hence, it could conclude that 96 h cell density is a more sensitive response parameter than the conventional algal biomass (or dry weight).

Fig. 2 presents the changes in cell density and MCV in continuous cultures treated with different concentrations of nickel. Steady-state was maintained by the controls and, hence, both the cell density and MCV values remained constant during the 24 h test period. When algal growth was inhibited by the addition of metal toxicants, a decline in the cell density could be noticed due to the wash-out effect. The MCV of the algal culture augmented with increasing Ni concentrations.

Similar to the previous batch test results, the above observations are primarily due to the inhibitory effects on the cell division rather than on the cellular photosynthesis. Table 4 shows the inhibitory effects of nickel observed from the continuous tests. At a Ni concentration of 0.45 mg/l, inhibition based on the growth rate of cell density was 80% (1 − (0.1/0.5)). In marked contrast, the growth reduction based on the final yield of cell density was only 33% (1 − (1.15/1.71)). The above comparison indicates that, in a chemostat, the final yield measurements (24 h cell density or 24 h TCV) are not the ideal response parameters because the 24 h test duration is too short for the response parameters to display significant inhibitory effects. However, longer testing time for the continuous test technique, is not necessary because the growth rate parameters have already provided excellent sensitivities. The EC50 calculated according to the growth rate of TCV is about three times larger than the growth rate of the cell density (Table 4). The above comparison is in conformity with

Table 4
Effects of nickel on *Raphidocelis subcapitata* from continuous tests

Ni (mg/l)	24 h cell density × 10^6 (cells/ml)	GR[a] based on cell density (per day)	MCV[b] (μm^3)	24 h TCV[c] × 10^7 (μm^3/ml)	GR based on TCV (per day)
Control	1.71	0.50	51.8	8.85	0.50
0.01	1.69	0.49	51.7	8.78	0.49
0.03	1.50	0.37	55.9	8.39	0.45
0.06	1.45	0.34	56.2	8.16	0.42
0.15	1.30	0.20	58.4	7.35	0.31
0.45	1.15	0.10	59.8	6.87	0.25
EC50 (mg/l)	–	0.106	–	–	0.386

[a] Specific growth rate.
[b] Mean cell volume.
[c] Total cell volume.

the batch test results wherein the cell density is shown as a better response endpoint than TCV.

Both nickel and cadmium tests were repeated three times in order to derive mean EC50 values and test reproducibilities. Table 5 summarizes the results obtained from the batch and continuous tests. For the batch tests, CV of the parameters based on cell densities are not appreciably different from the CV values for the parameters related to TCV. However, it could be detected that parameters based on cell density are generally associated with smaller CV values than those based on TCV. The only exception being the comparison of the 96 h final yields for the toxicant Ni. In the continuous tests, CV values for the growth rates of cell density are apparently smaller compared to those based on TCV. Hence, parameters based on cell density are likely to provide better test reproducibility.

With respect to the test sensitivity, the data shown in Table 5 clearly shows that cell density is a more sensitive end point than TCV. In the batch tests, the EC50s based on cell density are generally lower and could differ by a factor of 2 compared to those based on

Table 5
Comparison of EC50 (mg/l) and CV (%) values based on different response parameters[a,b]

Metal	96 h TCV[c] (a)	96 h cell density (b)	Ratio of (a)/(b)	GR[d] based on TCV (c)	GR based on cell density (d)	Ratio of (c)/(d)
Batch tests						
Cd	0.057 (28)	0.046 (25)	1.24	0.185 (21)	0.131 (18)	1.41
Ni	0.044 (14)	0.037 (21)	1.19	0.382 (24)	0.190 (22)	2.01
Continuous tests						
Cd	–	–	–	0.082 (28)	0.013 (15)	6.31
Ni	–	–	–	0.493 (26)	0.112 (9)	4.40

[a] All EC50s are mean values from repeated tests ($n = 3$).
[b] Values in parenthesis are in %.
[c] Total cell volume.
[d] Specific growth rate.

TCV. In addition, an even more distinct difference (6.3 times) in EC50 values was detected in the continuous tests when different parameters were applied. The above comparisons show that obvious differences in test sensitivity and reproducibility exist when response parameters are defined by cell density or TCV, respectively.

4. Conclusion

This study compares the test sensitivities and reproducibilities revealed by different response parameters, in algal toxicity tests. Parameters related to algal cell density are found to be more sensitive and reproducible than the total cell volume which in turn is directly correlated to the algal biomass or dry weight. The differences in median effective concentration values can range up to six folds in magnitude. The main cause for the above discrepancies is because the cell density and total cell volume are two response endpoints reflecting the degree of inhibition on the binary fission mechanism and overall photosynthesis, respectively. At low toxicant concentrations, the inhibitory effects are primarily on the cell division rather than on the cellular photosynthesis. Thus, more severe toxic effects are expected to be observed based on the cell density than the total cell volume. The results of this study clearly demonstrate that cell density is a more sensitive and consistent parameter than algal biomass or total cell volume.

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References

[1] N. Nyholm, Water Res. 19 (1985) 273.
[2] A.O. Hanstveit, H. Oldersma, Evaluation of the results of the second ISO-interlaboratory study with an algal toxicity test, ISO/TC147/SC5/WG5, Nederlands Normalisatie Instituut, Delft, The Netherlands, 1981.
[3] A.O. Hanstveit, Evaluation of the results of the third ISO-interlaboratory study with an algal toxicity test, ISO/TC147/SC5/WG5, Nederlands Normalisatie Institute, Delft, The Netherlands, 1982.
[4] N. Nyholm, Arch. Environ. Contam. Toxicol. 19 (1990) 518.
[5] M.I. Abdel-Hamid, S.A. Shaaban-Dessouki, O.M. Skulberg, Environ. Toxicol. Water Qual. 8 (1993) 239.
[6] J. Hamada, S.G. Kim, S. Matsui, Water Sci. Tech. 33 (1996) 305.
[7] D. Mares, A. Bonora, G. Sacchetti, M. Rubini, C. Romagnoli, Cell Biol. Int. 21 (1997) 397.
[8] Y.P. Ting, F. Lawson, I.G. Prince, Chem. Eng. J. Biochem. Eng. J. 47 (1991) B23.
[9] O. Gotsis-Skretas, Environ. Toxicol. Chem. 33 (1991) 261.
[10] J. Abalde, A. Cid, S. Reiriz, E. Torres, C. Herrero, Bull. Environ. Contam. Toxicol. 54 (1995) 317.
[11] G. Fernandez-Leborans, A. Novillo, Water Res. 30 (1996) 57.
[12] B. Sosak-Swiderska, D. Tyrawska, U. Mazurek, A. Wilczok, Pol. Arch. Hydrobiol. 41 (1994) 133.
[13] S.G. Lawrence, M. Holoka, R.D. Hamilton, Sci. Total Environ. 87/88 (1989) 381.
[14] C.Y. Chen, K.C. Lin, Environ. Toxicol. Chem. 16 (1997) 1337.
[15] US EPA, Freshwater algae acute toxicity test, Fed. Reg. 50, 39, 323-39, 333, 1985.
[16] M.R. Chao, C.Y. Chen, Environ. Toxicol. Chem. 19 (2000) 1589.