Pergamon

Chemosphere, Vol. 35, No. 9, pp. 1959–1965, 1997

0045-6535/97 $17.00+0.00

PII: S0045-6535(97)00270-1

COMPARISON OF THE RELATIVE TOXICITY RELATIONSHIPS BASED ON BATCH AND CONTINUOUS ALGAL TOXICITY TESTS

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(Received in USA 27 January 1997; accepted 2 May 1997)

ABSTRACT

In this study, we compare individual and combined responses of metal toxicants, on *Selenastrum capricornutum*, using batch and continuous techniques. 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. The two test methods depict completely different relationships in relative toxicity. In addition, the batch tests seriously underestimate the toxicity of metal mixtures containing lead. The above phenomena can be attributed to the surplus amounts of a limiting nutrient (phosphorus) and chelators in the growth medium. Since algae in the field are grown under low concentrations of nutrients and chelators, the results presented herein suggest that the significance and adequacy of current algal toxicity test protocols should be carefully reviewed. © 1997 Elsevier Science Ltd

INTRODUCTION

Batch technique is conventionally adopted by most standard algal test protocols for assessing the relative toxicity of chemicals and/or waste discharges (ASTM, 1994; ISO, 1987; US EPA, 1985; OECD; 1984). Those methods, although employing different medium compositions, are quite similar in one respect; the growth mediums 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. On the other hand, our previous study indicated that the general tolerance of an algal culture decreases as the nutrient status becomes limiting (Chen, 1994). Our study involving the toxic responses of *Escherichia coli.* at different nutrient concentrations also noted a similar phenomenon (Lin and Chen, 1996). These findings suggest that microorganisms' sensitivity to a toxicant varies under different physiological conditions, as regulated by the nutrient status. Based on the above conclusions, a continuous algal toxicity test method was developed, whereby algae were grown and tested under nutrient-limited conditions. Toxicity tests employing six different metal toxicants revealed that algae cultured in a continuous reactor are more sensitive than those cultured by the batch technique (Chen and Lin, 1997).

Establishing a standardized method (protocol) attempts to resolve problems related to relative toxicity and sensitivity of tests. Data generated by standard test methods, although incapable of accurately predicting the effects of chemicals in the field, should at least be able to identify the most toxic chemical or the most harmful industrial effluent, according to the determined relative toxicity. However, the relative toxicity relationship might change due to alternations of algal sensitivities at different nutrient levels. Therefore, this paper compares the relative toxicity relationships revealed by two different algal toxicity test methods: (a) the continuous technique (chemostat) where algae are grown with a deficiency of nutrients and, (b) the batch method where nutrients are abundant. Data involving individual toxicity, as obtained from our previous study, are used (Chen and Lin, 1997). Experiments are also performed to examine the combined effects of binary mixtures of metal toxicants.

MATERIALS AND METHODS

(1) Batch tests

The alga *Selenastrum capricornutum* (UTEX 1648) was obtained from the University of Texas, Austin. Batch tests were conducted following the EPA method (US EPA, 1985). Algal growth rate was selected as the response parameter. For each toxicant concentration (treatment) and the control, three flasks were used for the batch tests. The initial inoculum cell density was approximately 15,000 cells/ml. The specific growth rate and the degree of inhibition can be defined as

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

$$\text{Inhibition rate} = 1 - (\mu / \mu_m) \quad (2)$$

where x_1 and x_2 denote densities, in terms of cells/ml, at time t_1 and t_2, respectively. μ denotes the specific growth rate for treatments, and, μ_m represents the control's growth rate.

(2) Continuous tests

Alga (*Selenastrum capricornutum*) was grown in a transparent chemostat reactor with the volume of the culture medium maintained at 400 ml. 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±1 ℃. Light intensity was set at 4304 lux (±10%). Growth medium composition was basically the same as that described by the EPA bottle technique (US EPA, 1985), but with contents of phosphorus, nitrogen, and EDTA revised. The dilution rate (D) for the chemostat was set at 0.5/day to insure 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 (Chen and Lin, 1997). Samples were taken directly from the effluent pore. The algal population was determined on a 24-h basis using an electronic particle counter. Growth rate was selected as the response parameter. The system was first allowed to reach a steady state with no toxicant involved. In a chemostat at steady state, the specific growth rate (μ) is equal to the dilution rate (D) and the population density remains constant. Next, toxicants were added both to the reactor and the medium influent until the required concentration levels were reached. This addition created a nonsteady state in the reactor. The new growth rate μ (disturbed growth rate) can be calculated from the rate of population decline according to the following equation.

$$\mu = \ln(\frac{x_2}{x_1}) / (t_2 - t_1) + D \qquad (3)$$

where x_1 and x_2 denote densities (cells/ml) at time t_1 and t_2, respectively. The exposure time for the nonsteady state test was set at 24 hours. The degree of inhibition is calculated as

$$\text{Inhibition rate} = 1 - (\mu / D) \qquad (4)$$

All tests were performed in duplicates. The continuous method applied in this study is a very stable technique: Results from repeated tests (n equals 3 or 5) indicated that the coefficient of variation (CV) for median effective concentrations (EC50) varied from 8.5% to 22.6%. In addition, the maximum deviation in inhibition rates observed from duplicated tests was only 6%. Quality assurance (QA) procedures, e.g., cell density, pH, nutrient concentrations, and negative controls using a reference toxicant (Cd), were routinely conducted. A detailed description regarding the above continuous testing technique and the related QA procedures can be found in the author's previous works (Chen and Lin, 1997).

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 millipore membrane.

RESULTS AND DISCUSSION

Table 1 presents the median effective concentrations at 50% growth inhibition (EC50) for various metal toxicants applied individually. These tests were performed with five treatments (differing in toxicant concentrations) and one control. EC50 based on nominal toxicant concentrations was determined by the probit analysis (Finney, 1971). The continuous tests were conducted with a EDTA concentration equal to 30 μg/l because, according to Bergers and De Groot(1994), EDTA content for the majority of surface waters ranges from 10 to 30 μg/l. The EDTA concentration for the batch tests, however, was set at 300μg/L

Table 1. EC50 values obtained by continuous and batch tests

	Continuous test		Batch test	
Metal	EC50	95% C. I.	EC50	95% C. I.
Cd	0.013*	0.012-0.017	0.341	0.110-1.672
Cu	0.021	0.018-0.031	0.038	0.031-0.054
Zn	0.015	0.011-0.026	0.178	0.110-0.271
Pb	0.256	0.285-0.356	2.655	1.983-4.539
Ni	0.125	0.106-0.173	0.233	0.174-0.311
Hg	0.027	0.011-0.085	0.009	0.002-0.034

* Data from Chen and Lin (1997), (Unit : mg/l)
95% C. I. = 95% confidence intervals.

following the standard EPA bottle test. The continuous test is generally more sensitive than the batch test, except for the case of Hg. The 95% confidence intervals derived by the two tests did not overlap with each other and, hence, these EC50s were statistically different at 5% level of significance.

The relationships of the relative toxicity (toxicity order), as depicted by the two test methods, are given below:

Continuous test : Cd > Zn > Cu > Hg > Ni > Pb
Batch test : Hg > Cu > Zn > Ni > Cd > Pb

The toxicity orders displayed by the two tests are obviously different. The above changes can be attributed to the different degrees of sensitivitiy revealed by the two test methods and the variations of the EDTA content in the growth medium. (Chen and Lin, 1997).

Six binary mixtures of metal toxicants were prepared to compare the relative toxicity relationships, using batch and continuous methods. The metal concentrations were set to be equal to twice the amount of their individual EC50 values as obtained by our continuous tests (refer to Table 1). Tests were performed at two different EDTA concentrations (i.e., 300 and 30 μg/l) in order to distinguish the effects of nutrient status and EDTA content. The toxicity orders for these mixtures were determined according to their corresponding inhibition rates. Table 2 illustrates the combined effects of Zn and Pb on algal growth, observed from batch and continuous tests. It is obvious that, throughout the entire test period, substantial amounts of algal growth can still be found from batch tests. The corresponding growth rate varies from 1.36 to 0.943/day, at two different EDTA concentrations. For the continuous tests, however, no growth was observed and the cell density decreased owing to the system's wash-out effect. The marked differences in EC50 values produced by the two test methods (Table 1) also provide an explanation for the above observation. At a specific EDTA concentration, the difference in inhibition rates between the two test methods indicates changes in the sensitivity of the two algal cultures. The batch test's inhibition rates were calculated based on 96-hr cell densities. However, similar conclusion can be reached using the 24-hr cell density data.

Table 2. Inhibitory effect of Zn-Pb mixture observed from batch and continuous test

	Cell density (10^4cells/ml)			Unperturbed growth rate	Disturbed growth rate	$\mu/\mu m$	Inhibition rate
	0 hr	24 hr	96 hr	(μm or D)	μ	(or μ/D)	(%)
Batch test							
EDTA=300μg/l	1.5	5.77	350.	1.51	1.36	0.90	10
EDTA=30μg/l	1.5	4.11	65.2	1.52	0.943	0.62	38
Continuous test							
EDTA=300μg/l	178	108	--	0.5	0	0	100
EDTA=30μg/l	176	106	--	0.5	0	0	100

In order to provide more convincing discussions, batch and continuous results from mixture toxicity tests are shown in Fig. 1 and Table 3, respectively. Corresponding growth rates and inhibition rates can be calculated using equations (1) through (4).

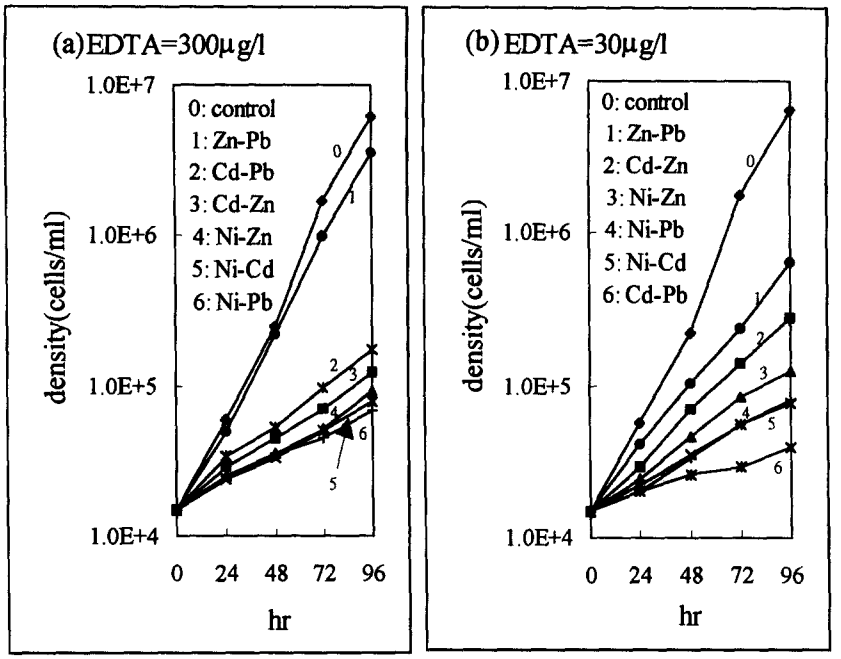

Figure 1. Observed growth conditions from batch toxicity tests.

Table 3. Cell densities (10^6 cells/ml) obtained from continuous algal toxicity tests

Mixture	Cd-Zn	Ni-Zn	Ni-Cd	Cd-Pb	Zn-Pb	Ni-Pb†
EDTA=300 μg/l						
0 hour	1.785	1.785	1.785	1.785	1.785	1.785
24 hour	1.420	1.148	1.136	1.134	1.082	1.209
Inhibition rate (%)	46	88	90	91	100	78
EDTA=30 μg/l						
0 hour	1.760	1.760	1.760	1.760	1.760	1.760
24 hour	1.229	1.220	1.284	1.109	1.063	1.130
Inhibition rate (%)	72	73	63	92	100	89

† Cd = 0.026 mg/l , Zn = 0.030 mg/l , Ni = 0.25 mg/l , Pb = 0.512 mg/l.

Table 4 lists the inhibition rates of six binary mixtures for metal toxicants, as revealed by the continuous and batch tests. The continuous tests produced markedly higher inhibition rates than the batch tests, except for the Cd-Zn (at EDTA = 300 μg/l) and Ni-Cd (at EDTA = 30 μg/l) mixtures. Hence, algae cultured in a chemostat (under limiting nutrient status) are generally more sensitive than those cultured by batch technique. This observation corresponds to the results in Table 1, where metal toxicants are individually applied. Furthermore, Table 4 indicates that a high EDTA content does not necessarily imply a lower degree of inhibition, as evidenced by the toxic responses of Cd-Zn (batch tests) and Ni-Cd (continuous tests) mixtures. Such an observation can be related to a previous study in which certain metal-EDTA complexes are also quite toxic (Starodub et al., 1987).

Table 4. Inhibition rates (%) of various metal mixtures

Mixture	Cd-Zn	Ni-Zn	Ni-Cd	Cd-Pb	Zn-Pb	Ni-Pb†
EDTA=300 μg/l						
Continuous test	41*	85*	88*	91	100	78
Batch test	55*	70	72	59	9*	75
EDTA=30 μg/l						
Continuous test	75*	76*	68*	92	100	89
Batch test	45*	65	73	84	36*	72

* Average values from repeated tests.

†C_d = 0.026 mg/l，Zn = 0.030 mg/l，Ni = 0.25 mg/l，Pb = 0.512 mg/l.

The toxicity orders displayed by the standard algal bottle tests (EDTA = 300 μg/l) and our continuous tests (EDTA = 30 μg/l) are given below:

Continuous test : Zn-Pb > Cd-Pb > Ni-Pb > Ni-Zn > Cd-Zn > Ni-Cd
Batch test : Ni-Pb > Ni-Cd > Ni-Zn > Cd-Pb > Cd-Zn > Zn-Pb

Again, the toxicity orders depicted by the two test methods are entirely different. In the continuous test, the Zn-Pb mixture was rated the most toxic solution among all. Yet, in the batch test, this mixture was shown as the least toxic one. Moreover, its inhibition rate was 100% in the continuous test; whereas in the batch test, it was only 9%. The same phenomenon can be found for the case involving Cd-Pb mixture. We can thus conclude that the batch test significantly underestimates the toxicity of metal mixtures containing lead. In fact, different toxicity orders were obviously produced by the four sets of tests in Table 4. Hence, changes in either nutrient status or EDTA concentration redefine the relationship of the relative toxicity. The consistency of the above relationships in relative toxicity has been verified by repeating some of the selected tests (Table 4). This is why data in Table 4 do not match entirely with those presented in Table 3 and Fig. 1.

Stumm and Stumm-Zollinger (1972) reported that the orthophosphorus content in an unpolluted lake ranges from 0.001 to 0.012 mg/l. This suggests that, as phosphorus is the major limiting nutrient for algal growth, algae in the field are grown under nutrient-limited conditions. In addition, the EDTA content in natural aquatic environments are generally low as reported by Bergers and De Groot(1994). Hence, the standard bottle technique for algal toxicity testing has a phosphorus concentration (0.186mg/l as P) around 15 to 186 times higher than field conditions. Its EDTA concentration (300 μg/l) is approximately 10 to 30 times of that in the field. As a comparison, the equilibrium phosphorus and EDTA concentrations in our chemostat tests are only 0.007 mg/l and 30 μg/l, respectively (Chen and Lin, 1997). The above discussion reveals that the culture conditions in batch tests significantly deviate away from the field conditions. In contrast, the continuous method applied herein more closely simulates general field conditions than the batch test. As both nutrient status and the chelator concentration are crucial factors in determining the toxicity order, it stands to reason that the relative toxicity relationships derived by the continuous test should be closer to reality than that obtained from the batch technique.

CONCLUSIONS

We have compared the relative toxicity relationships of six metals and six binary metal solutions using batch and continuous algal toxicity testing techniques. The two test methods have revealed completely different relationships in relative toxicity. In addition, the batch tests seriously underestimate the toxicity of metal mixtures containing lead. The above phenomena can be attributed to the surplus amounts of a limiting nutrient (phosphorus) and chelators in the growth medium. Since the objective of a standard test protocol is not to determine the field impact of various toxicants and/or effluents, underestimating the toxicity of certain metal solutions (for example, the Zn-Pb mixture case) may not be an important consideration here. However, the possibility that batch method may yield different relative toxicity relationship, as compared to that in the field conditions, should be of major concern to ecotoxicologists. The results presented herein suggest that the significance and adequacy of current algal toxicity test protocols should be carefully reviewed.

ACKNOWLEDGEMENTS

This research was supported in part by grants from the National Science Council, Taiwan, Republic of China (NSC 86-2211-E009-005). The authors would like to thank Mr M. L. Choa for his help in carrying out part of the experiments.

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