Ecotoxicology (2010) 19:1095–1101
DOI 10.1007/s10646-010-0491-2

A study of the effects of chromium exposure on the growth of *Pseudokirchneriella subcapitata* (Korshikov) hindak evaluated by Central Composite Design and Response Surface Methodology

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Accepted: 22 March 2010 / Published online: 3 April 2010

Abstract The aim of this study was to evaluate the effects of chromium exposure on the growth of *P. subcapitata* using the Central Composite Design (CCD) and Response Surface Methodology (RSM). The highest values for algal density and biomass were obtained in the longest exposure times and for the lowest chromium concentrations. The CCD used for the analysis of treatment combinations showed that a second order polynomial regression model was in good agreement with experimental results, with $R^2 = 81.50$ and 89.90; for algal density and biomass ($p < 0.05$), respectively. Only the exposure time was significant for algal density. For chlorophyll, in contrast, the exposure time, chromium concentration and their interaction significantly affected the growth of *P. subcapitata*. The findings confirmed the sensitivity of *P. subcapitata* to chromium (VI), which makes it a suitable bioindicator of environmental contamination for this metal.

Keywords Metal · Algae · *Selenastrum capricornutum* · RSM · CCD · Toxicity

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Introduction

Environmental contamination with heavy metals may cause direct and/or indirect effects on terrestrial and aquatic ecosystems Fleeger et al. (2003). The toxicity of a substance (e.g. metals) to algae is usually assessed by standard growth inhibition tests using conventional species Pereira et al. (2005). The most frequently used freshwater alga is *Selenastrum capricornutum* Printz, renamed *Pseudokirchneriella subcapitata* (Printz) Korshikov 1990 Pardos et al. (1998), and studies have mostly focused on measuring endpoints resulting from chronic exposures of usually 3–4 days Lewis (1995).

In traditional experiments, most test designs are generated in such a way that one parameter is varied while the others are kept constant. The disadvantage of this type of design is two fold: the effect of only one parameter is studied, and possible interactions of different parameters cannot be assessed Heijerick et al. (2003). In contrast, the advantage of the CCD is that it can generate a maximum amount of information on the direct effect of test variables and their interactions while testing a minimum number of combinations De Schamphelaere et al. (2003).

Thus, a way to develop, improve and optimize processes consists of applying the CCD and the Response Surface Methodology (RSM), which can be very useful and advantageous for both the evaluation and optimization of some performance parameters. More specifically, the experimental design helps the researcher to verify if changes in the independent variables produce a statistically significant variation of the observed response, and this approach can be used each time this type of information is required Furlanetto et al. (2003).

The aim of this study was to evaluate the effects of chromium concentration and exposure time on the growth

of *P. subcapitata* using the CCD and the response surface analysis methodology. This experimental design generated mathematical models to predict chromium (Cr^{6+}) toxicity in *P. subcapitata.* The second-order polynomial model was used in the simulation to provide a better understanding of the effect of chromium concentration and exposure time on algal growth.

Materials and methods

Algal culture

The green algae *P. subcapitata* were obtained from cultures kept at the Ecophysiology Laboratory for Aquatic Organisms, Center of Water Resources and Applied Ecology (CRHEA), University São Paulo, and were cultivated in L.C. Oligo medium AFNOR (1980), which was first autoclaved (121°C) for 15 min in 2-l (litre) Erlenmeyer flasks containing 1 l of the medium (ABNT 2005). The composition of the synthetic culture medium per liter was the following: 0.17 M $Ca(NO_3)_2{\cdot}4H_2O$, 0.99 M KNO_3, 0.12 M $MgSO_4{\cdot}7H_2O$, 0.29 M KH_2PO_4, 0.00012 M $CuSO_4{\cdot}5H_2O$, 0.00009 M $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.0002 M $ZnSO_4{\cdot}7H_2O$, 0.0001 $CoCl_2$, 0.0002 M $Mn(NO_3)_2{\cdot}4H_2O$, 0.0005 M $C_6H_8O_2{\cdot}H_2O$, 0.001 M H_3BO_3, 0.005 M $C_6H_5FeO_7$, 0.002 M $FeSO_4{\cdot}7H_2O$, 0.002 M $FeCl_3{\cdot}6H_2O$, 0.2 M $NaHCO_3$.

An algal inoculum was prepared for each sample from fresh culture stocks sampled during the exponential growth phase, and the culture was kept at a temperature of 23 ± 2°C and under a constant irradiance of 1500 lux, provided by cool-white fluorescent lamp and constant aeration (ABNT 2005).

Toxicity tests

Tests were performed in 250 ml Erlenmeyer flasks with 100 ml of test medium and cells of *P. subcapitata* in the exponential growth phase. At the beginning of each test, each flask was inoculated with a concentration of approximately 10^4 cells/ml (ABNT 2005). The chromium ($K_2Cr_2O_7$) concentrations were 40.0, 41.5, 45.0, 48.5 and 50.0 µg/l, and the exposure times ranged from 81 to 183 h. Static toxicity tests were conducted in the same conditions described above for the algal culture maintenance procedure. Initial and final densities were verified by cell counts in an Improved Neubauer Bright-Line hemocytometer under an optical microscope (Carl Zeiss), standard model 25. All the aliquots were counts in triplicates. The mean number of cells produced at each concentration, after the exposure period, was expressed as a perceptual growth reduction with respect to the control Rodgher and Espíndola (2008). These percentages were used to calculate the IC50 chromium value (effective metal concentration causing 50% inhibition of algal growth after 96 h exposure) for the algae was determined by the Trimmed Spearman-Karber method Hamilton et al. (1977).

Algal biomass

The algal biomass was estimated from analysis of chlorophyll *a*. Samples (5–10 ml) from each test flask were filtered through a 0.45 µm membrane filter. Boiling ethanol (80%) was poured over the filter into a beaker and, after a few minutes of cooling, the filter was grinded with mortar and pestle to facilitate extraction. The filter slurry was rinsed with ethanol and passed through a hard paper filter into a calibrated tube. The extraction process was performed in the dark for 6–24 h. Measurements were at 665–750 nm in a spectrophotometer (0.2–0.4 mm slit width) (F600, FEMTO, USA) against a reference cuvette filled with 90% ethanol Nusch (1980). All samples were analyzed in triplicates.

Experimental design and statistical analysis

Algal toxicity tests were conducted in 10 experiments for the study of two parameters (see matrix in Table 1). The model studied is a 2^2 experimental design, where selected time of exposure (X1) and chromium concentration (X2) were treated as independent variables that affected density and algal biomass. Each of the parameters was coded at five levels: −1.41, −1, 0, 1, and 1.41. The range and levels of the variables in this study are shown in Table 1. RSM consists of a group of empirical techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and the measured responses

Table 1 Process variables used in the CCD showing the treatment combinations between chromium concentration and exposure time

Treatment	Coded setting levels X1 = time; X2 = [Cr]		Actual levels X1 = time (h); X2 = [Cr] (µg/l)	
	X1	X2	X1	X2
1	−1	−1	96	41.5
2	−1	1	96	48.5
3	1	−1	168	41.5
4	1	1	168	48.5
5	0	0	132	45.0
6	0	0	132	45.0
7	−1.41	0	81	45.0
8	0	−1.41	132	40.0
9	1.41	0	183	45.0
10	0	1.41	132	50.0

according to one or more selected criteria Bayraktar (2001). According to this design, the total number of treatment combinations was $2^k + 2k + n_0$, where 'k' is the number of independent variables and 'n_0' is the number of repetitions of the experiments at the center point. Based on the parameter estimates, the application of RSM provided an empirical relationship between the response variable and the test variables Qiao et al. (2009). By performing multiple regression analyses of the experimental data, the predicted response Y for density and algal biomass can be obtained through the second-order polynomial equation:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2 + \varepsilon \quad (1)$$

b_0, b_1, b_2, b_{11}, b_{22}, b_{12} are constant coefficients, and x_1, x_2 are the coded independent variables or factors. The test factors were coded according to the following regression equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i}\right) \quad (2)$$

where x_i is the *coded* value and X_i is the *actual* value of the ith independent variable, X_0 is the actual value at the center point, and ΔX_i is the step change value. In this case, $X_1 =$ (time -132)/36; $X_2 =$ ([Cr]–45.0)/3.5 were used. The linear, quadratic and interactive effects of parameters on metal toxicities were analyzed with Statistica 7.0 software (Statsoft, USA). In order to develop a mathematical prediction model, a backward regression analysis ($p < 0.05$) was also applied to the toxicity data of exposure time and Cr (VI). Based on this parameter estimate, the model can be statistically validated if it is able to reproduce the observed behavior Faller et al. (2003).

CCD was used to determinate the toxic effect of chromium on algal density and chlorophyll *a* concentration or algal biomass. Analysis of variance (ANOVA) was employed to determine significant parameters and to estimate algal density and biomass as a function of exposure time and chromium concentration. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test (ANOVA). The significance of the regression coefficients was tested by a t-test.

Results

Table 2 shows the results of the experimental design for density and algal biomass that was used to investigate the influence of chromium on *P. subcapitata* for different exposure times.

The results suggest that both algal density (Fig. 1) and algal biomass (Fig. 2) presented the smallest growth under the longest exposure time and the highest chromium concentration. The longest exposure time and lowest chromium concentration provided the best conditions for algal growth (Fig. 1).

Table 2 The mean experimental and standard errors in parentheses from design responses with the results obtained from algal density and algal biomass

Treatment	Algal density ($\times 10^4$ cells/ml)	Biomass (μg/l)
1	143 (8)	78.1 (7)
2	57 (2)	136.7 (16)
3	776 (44)	505.0 (73)
4	78 (9)	20.9 (1)
5	489 (15)	263.7 (15)
6	404 (9)	284.6 (25)
7	71 (2)	9.8 (0)
8	176 (9)	443.6 (51)
9	741 (42)	502.2 (52)
10	267 (22)	132.5 (10)

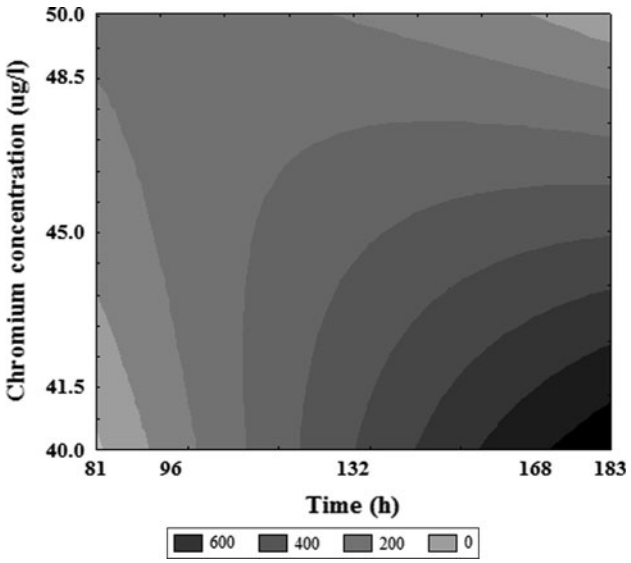

Fig. 1 Contour plot of algal density as a function of chromium concentration (40.0–50.0 μg/l) and exposure time (81–183 h) for *P. subcapitata*

Contour plot indicates that maximum density and algal biomass were attained for the longest exposure time range (168–183 h). However, the ranges of chromium concentration were narrower 40–41.3 μg Cr/l for algal biomass, than 40–44.3 μg Cr/l for algal density.

The first step in the design is therefore to take the declared objective of the experiment and translate it into some quantitative measurement that can be estimated, such as an EC50 (the 'effective concentration', or concentration of test chemical that affects 50% of the organisms tested) Chapman et al. (1996). The half maximal inhibitory

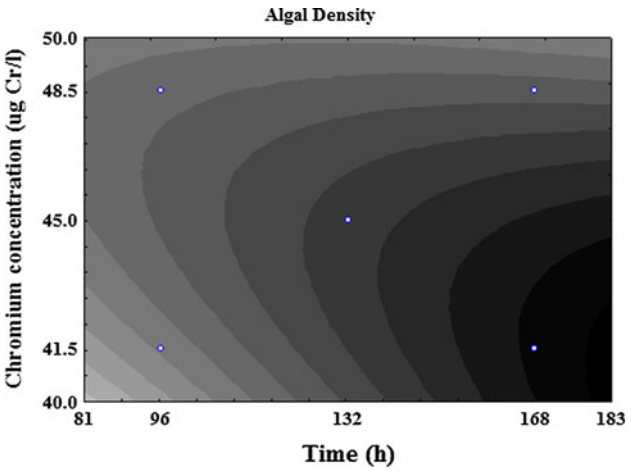

Fig. 2 Response surface of algal biomass as a function of chromium concentration (40.0–50.0 µg/l) and exposure time (81–183 h) for *P. subcapitata*

concentration (IC50) was estimated in order to assess the sensitivity of *P. subcapitata* to different chromium concentrations in different exposure times. In addition, based on the percentages of growth reduction and IC50 values obtained by the Trimmed Spearman-Karber method, the CCD coded models made it possible to generate data simulation to obtain IC50 values for different exposure times. Thus, it was possible to calculate IC50 at 96, 132, 168 and 183 h with 95% reliability. The values obtained were: 42.78 and 43.54 (limits not defined), 47.58 (47.26–47.90) and 45.62 (44.88–46.37) µg Cr/l, respectively.

A model fitting was performed for the experimental design, and the ANOVA (analysis of variance) was used to evaluate the adequacy of the fitted model. Table 3 shows the resulting model coefficients estimated by regression analysis. These findings suggest that the models were significant, and the coefficient of determination estimate indicate that 81.5%, for algal density, and 89.9%, for algal biomass, of the variability in these responses could be accounted for by the model, which is indicative that the model provides adequate representation.

There was significance for coefficients determined by Student's *t*-test (Table 3). In this study, only the mean and the linear time were significant (p *time-t* < 0.05) for algal density. Considering the second-order model of algal biomass, various coefficients were significant: the mean, the time and linear chromium concentration, and the interaction between them ($p < 0.05$). These findings indicate that they can act as limiting factors and their values might change responses in algal density and algal biomass to a considerable extent.

In addition, the CCD coded models permitted data simulation to IC50. The generated models were used to run the simulations of density and algal biomass (Fig. 3a, b).

The data simulation works similarly for algal density and biomass. At the initial exposure time (96 h) there is a slight, proportional increase as the chromium concentration rises, and later there is a drop. The difference between the parameters is the chromium concentration at which the curve inflection occurs: 46.0 µg Cr/l for algal density and 48.0 µg Cr/l for biomass. For the subsequent exposure times (132 a 183 h), there was a reduction in algal biomass while chromium concentration increased.

Discussion

Consistent algal growth inhibition was observed in this study, especially for the highest chromium concentrations and the longest exposure time (Figs. 1 and 2). However, Labra et al. (2007) and Pereira et al. (2005) also observed growth inhibition, though for a range of very low chromium concentrations (1.0–7.5 µg Cr/l) and soon after 24 h of treatment.

Table 3 Obtained model and regression coefficients to Eq. (1) and analysis of variance (ANOVA) for the experiments

Term	Algal density ($\times 10^4$ cells/ml)		Biomass (µg/l)	
	Coefficients and standard error (±)	*p* Value	Coefficients and standard error (±)	*p* Value
Mean/interc b_0	**446.50 (124.12)**	0.02	**279.35 (64.92)**	0.01
t (L) b_1	**200.13 (62.06)**	0.03	**125.94 (32.46)**	0.02
t (Q) b_2	−32.67 (82.10)	0.71	−33.39 (42.94)	0.48
c (L) b_{11}	−82.03 (62.06)	0.26	**−108.18 (32.46)**	0.03
c (Q) b_{22}	−125.00 (82.10)	0.20	−17.35 (42.94)	0.71
t*c b_{12}	−153.00 (87.77)	0.16	**−135.66 (45.91)**	0.04
R^2	81.50	–	89.90	–
R^2 adjusted	58.30	–	77.20	–
F value	8.79	–	17.75	–
df	1/9	–	3/9	–
F cal/Ftab	1.72	–	4.60	–

The values in bold are significant at $p < 0.05$, with confidence level of 95%

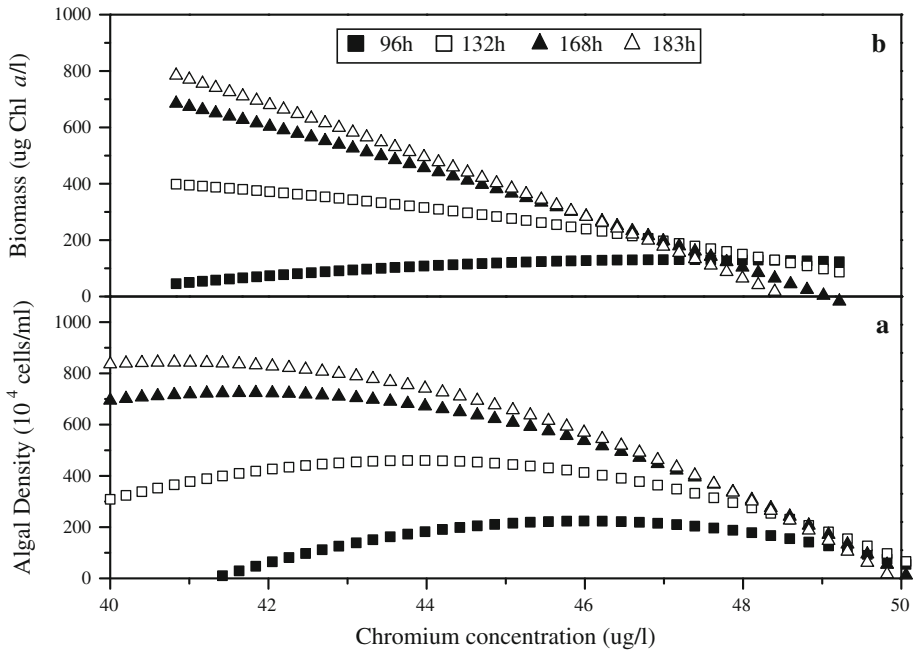

Fig. 3 Simulation of algal density (**a**), and algal biomass (**b**), as a function of chromium concentration (40.0–50.0 μg/l) in different exposure time for *P.subcapitata*. *filled square*: 96 h; *square*: 132 h; *filled triangle*: 168 h; *triangle*: 183 h

Rodgher et al. (2008) studied algal cells of *P. subcapitata* as routes for copper exposure and toxicity to cladocerans. Reductions were found in algal density and in chlorophyll *a* content for the algae subjected to the treatment with copper. The correlation analysis between algal cell densities and chlorophyll *a* content confirmed that chlorophyll-a reduction was a function of reduced algal cell density. These results are consistent with our data and can be also visualized on data simulation (Fig. 3).

A decrease in total chlorophyll, chlorophyll *a* and *b*, and carotenoids has been well documented in plants, moss and algae under Cr stress Chaudhury and Panda (2005); Panda (2003); Panda et al. (2003); Panda and Choudhury (2005). Experiments performed with *P. subcapitata* subjected to higher copper concentrations (1.0, 1.5 mg/l) induced chlorophyll degradation Cvetkovic et al. (1991); Rodgher et al. (2008).

Furthermore, the IC50 (half maximal inhibitory concentration) is a measurement of the effectiveness of a compound in inhibiting biological or biochemical functions. Although most standard algal assays used for regulatory purposes appear to have similar designs and operating procedures, subtle differences in test design may lead to a large variability in results. Nevertheless, it is unclear whether the differences in sensitivity among various algal taxas and within individual species reported in the current literature are caused by differences among the various biotic and abiotic factors in standard operating procedures Janssen and Heijerick (2003). Such variability can be verified in data obtained by different authors Masutti (2004); Rodgher and Espíndola (2008); Rojícková and Maršálek (1999); Turbak et al. (1986) (Table 4).

Table 4 Different IC50 values of chromium (VI) reported in the literature for *P. subcapitata*

IC50 [Cr^{6+}] (μg/l)	Exposure time (h)	Reference
542.71	96	Rodgher and Espíndola (2008)
420.0	96	Masutti (2004)
396.1	96	Rojíčková and Maršálek (1999)
238.0	96	Turbak et al. (1986)
42.78	96	In this work

The reduction in viable cell numbers was observed by Labra et al. (2007) and Pereira et al. (2005), suggesting that potassium dichromate is a strong algal cell pollutant and *P. subcapitata* is a suitable sensitive organism to monitor the presence of chromium in water. In addition, a direct relationship between Cr content and cell mortality was found only when the amount of Cr was related to protein content in *S. acutus* treated with Cr (VI) Gorbi et al. (2001). Corradi et al. (1998)) suggested that the ability of *S. acutus* to detoxify chromium was related to the higher production of carbohydrates and proteins in response to metal exposure.

In another study, the higher tolerance of *Chlorella kessleri* was accounted for by differences in production of extracellular organic substances under stressful conditions in comparison with *P. subcapitata* and *Scenedesmus quadricauda* (*P. subcapitata* was considered the most sensitive) Maršálek and Rojíčková (1996).

Considering that *P. subcapitata* responses to contaminants, such as heavy metals, are typically measured in terms of biomass, cell density, growth rate, etc. Labra et al.

(2007), the use of bioassays provides a direct and integrated estimate of the heavy metal's toxicity. Recently, environmental agencies have focused on optimizing methods, endpoints and test organism selection. Statistical advice in current ecotoxicity test guidelines is in need of improvement. More advice should be given on experimental design, statistical analysis and ways of reporting results Chapman et al. (1996).

Conclusion

The results from this study confirmed the sensitivity of *P. subcapitata* to chromium (VI), which makes it a suitable bioindicator of environmental contamination by this metal. The Central Composite Design represents a valuable tool to determine mathematical relationships to predict toxicity, which allows the simulation of any response (dependent variables) around a range of tested factors (independent variables). The use of CCD in aquatic environmental toxicology is a powerful technique for investigating multivariate systems (because many factors may interact simultaneously in the environment). It reduces the number of experiments and repetitions without loss of statistical reliability (since it is possible to calculate the experimental error). Besides, it increases the predictions and efficiency of data sets and reduces experimental residual volume.

Acknowledgments We are grateful for the financial support from the São Carlos Engineering School, the Water Resources and Applied Ecology Center, and the University of São Paulo, and for the assistance from the University State of West Center. We wish to thank Dr. Liane Biehl Printes for the valuable comments and suggestions on the manuscript.

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