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Carotenoids and pH of the culture medium play an important role in displaying metal stress in batch and semi-continuous cultures of *Anabaena doliolum*

Bhumi Nath Tripathi[1]*, Rohini Kasana[1], Vijeta Singh[1], Indu Bhatt[1], Ashutosh Singh[1], Vinay Sharma[1] and Jai Prakash Gaur[2]

[1] Department of Bioscience and Biotechnology, Banasthali University, Banasthali, 304022, Rajasthan, India
[2] Department of Botany, Banaras Hindu University, Varanasi, 221005, India

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Abstract – We analysed the responses of *Anabaena doliolum* to elevated levels of copper and zinc in batch and semi-continuous cultures. Approximately 10, 4 and 8 and 5-times greater inhibition in final yield of *A. doliolum* occurred at 1, 2 μM Cu and 2.5 and 5 μM of Zn, respectively, in semi-continuous culture in comparison to batch culture. Protein, chlorophyl *a* and carotenoid contents of *A. doliolum* showed significantly ($P < 0.05$) higher inhibition by test metals in semi-continuous culture than in batch culture. The greater sensitivity of different parameters of the test organism was related to the high metal content of the cells grown in semi-continuous system. Moreover, enhancement of pH of the culture suspension in batch culture showed a negative relationship with metal accumulation, and therefore with toxicity. This was due to decrease in free ionic concentrations of test metals. Carotenoids acted as a metal detoxifying agent by minimizing metal-induced inhibition in batch culture as was evident from its negative relationship with metal toxicity.

Key words: *Anabaena doliolum* / batch culture / carotenoids / copper / pH / semi-continuous culture / zinc

Introduction

The potential hazards of heavy metals to algae have been generally evaluated using the batch culture technique (Genter, 1996) due to its simplicity and low cost. The batch culture is run in a closed vessel with a single input of materials, nutrients as well as the test metal (Wong *et al.*, 1983). In this system the test organism follows the characteristic pattern of the growth cycle, depicting lag, exponential, stationary and death phases in a sequential manner (Fogg and Thake, 1987). But the regular cell division results in substantial decline in nutrient as well as metal level in the culture medium during the experimental period (Stauber and Davies, 2000). During the growth cycle, the algal cells display alterations in metabolic activities and cellular biochemical status with time. Thus algal metabolism over the experimental period in a batch culture results in increased concentration of some biomolecules, such as carotenoids (Fogg and Thake, 1987), and also cause a drift in pH (Nyholm and Kallquist, 1989) and subsequent chemical alteration of the culture medium with the passage of time (Meador, 1991). Therefore metal toxicity to algae in a batch culture may be influenced by pH of the culture medium and carotenoid content of the cells.

The pH of the culture medium is an important factor that might affect metal toxicity to algae, although it is difficult to establish a relationship between the two. Some authors have reported an increased toxicity of Cu, Zn and other metals with decreasing pH (Staurodub *et al.*, 1987) due to predominance of the free ionic forms of metals (Staurodub *et al.*, 1987). Conversely, others observed decreased toxicity of metals, including Cu and Zn, with a decline in pH (Franklin *et al.*, 2000), a consequence of reduced metal uptake due to competition between H^+ and metal ions for binding on the cell surface (Mehta *et al.*, 2000). However, most of the previous studies aimed at finding out the influence of pH on metal toxicity to algae were based on short-term batch culture run at different pH.

It is widely acclaimed that carotenoids may provide protection to the photosynthetic apparatus from oxidative

*Corresponding author: bhuminathtripathi@hotmail.com

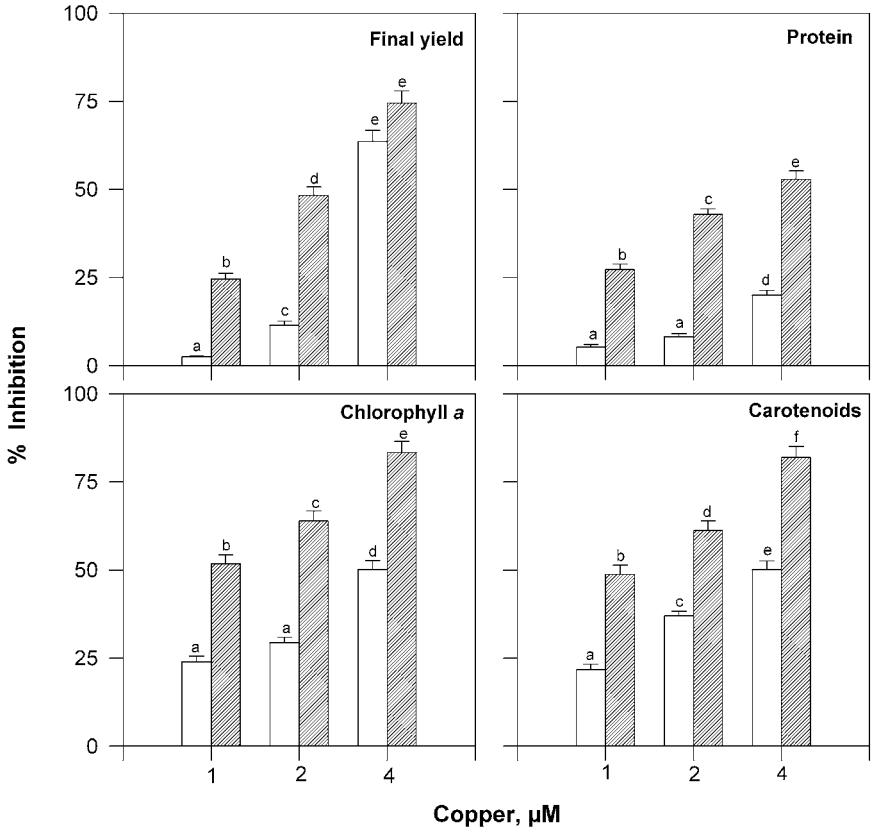

Fig. 1. Responses of different parameters of *Anabaena doliolum* to elevated levels of copper in batch (empty bars) and semi-continuous cultures (filled bars). Bars marked with same letters are not significantly different ($P > 0.05$).

stress (Mallick and Mohn, 2000). Although a significant rise in the carotenoid level of algae was previously noticed under metal stress (Okamoto *et al.*, 2001), their possible role in mitigating metal toxicity to algae has not been well explored. The present study was planned to compare the metal toxicity to *Anabaena doliolum* in batch and semi-continuous cultures taking pH of the culture media and carotenoid levels of the test organism in the consideration. Unlike the batch culture, semi-continuous cultures maintain a nearly constant cell population, cells remain physiologically active throughout and do not experience any ageing effect which might enhance carotenoid level, and regular input and output of culture medium does not allow fluctuation in pH of the culture medium.

Material and methods

The test cyanobacterium *Anabaena doliolum* (locally isolated) was cultivated in Allen and Arnon's (1955) medium (4 times diluted). The cultures were incubated in an air-conditioned culture room (temperature $24 \pm 1\,^{\circ}C$) receiving 72 $\mu mol.m^{-2}.s^{-1}$ PAR for 12 h daily. The cultures were hand shaken 2–3 times daily.

An initial cell density of about 10^4 $cells.mL^{-1}$ was adjusted in 250-mL Erlenmeyer's flasks containing 100 mL sterilized culture medium and kept undisturbed for 4–5 days at standard growth conditions. After this period, the batch culture was dosed with different concentrations of copper and zinc from freshly prepared stocks of $CuCl_2.2H_2O$ and $ZnCl_2$, to obtain desired metal concentrations (1.0, 2.0 and 4.0 µM for Cu and 2.5, 5.0 and 10.0 µM for Zn) in the culture medium. Whereas, after 4–5 days in semi-continuous culture, the culture suspension was diluted everyday in the morning hours with addition of 20-mL of sterilized culture medium and same volume was removed from the culture as catabolic waste. When semi-continuous cultures reached to the steady state (less than 5% fluctuation in cell number), it was treated with different test metal concentrations to give desired metal concentration in the culture medium as in batch culture. These metal concentrations were selected on the basis of a preliminary trial which showed about 10, 50 and 75% inhibition of growth of *A. doliolum* at 1 µM Cu and 2.5 µM Zn, 2 µM Cu and 5 µM Zn, and 4 µM Cu and 10 µM Zn, respectively.

At the final day of experiment (after 15 days of metal treatment), 15 mL algal culture suspension was withdrawn from the control as well as metal-treated batch and semi-continuous cultures. Different parameters such as cell number, protein, pigments, pH, phosphate and metal content in the culture were analysed by following standard methods. The cell counts of *A. doliolum* were made microscopically by using a Spencer's brightline haemocytometer. The protein content of the cell was measured using the method given by Lowry *et al.* (1951). The pigments

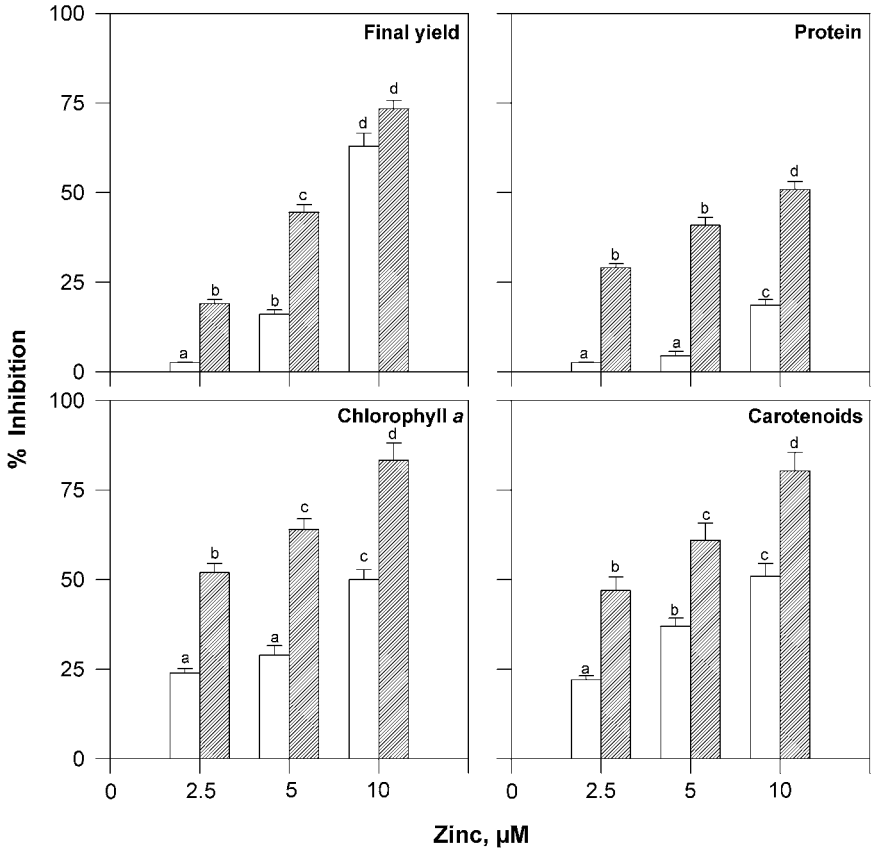

Fig. 2. Responses of different parameters of *Anabaena doliolum* to elevated levels of zinc in batch (empty bars) and semi-continuous cultures (filled bars). Bars marked with same letters are not significantly different ($P > 0.05$).

were extracted in 80% acetone and chlorophyll *a* and carotenoid contents were estimated as per Mackinney (1941) and Myers and Kratz (1955) respectively. A known volume of algal suspension was centrifuged and the supernatent was used to analyse the pH, phosphate and metal content in the medium. The pH was measured by the help of a digital pH meter (Control Dynamics APX 175). The level of phosphate in the culture was estimated by the method of stannous chloride (APHA, 1995). The metal content of the medium was analysed by an atomic absorption spectrophotometer (Perkin Elmer, 2380). The chemical equilibrium computer model, MINEQL (ver. 3.01) (Westall *et al.*, 1976) was used to calculate the ionic concentrations of Cu and Zn in the culture medium. All experiments were conducted in triplicate.

All the data were statistically analyzed. Student's *t*-test and regression analysis were used for the test of significance.

Results

Figures 1 and 2 show the Cu and Zn induced reduction in final yield, protein, chlorophyll *a* and carotenoids of *A. doliolum* in batch and semi-continuous cultures. Approximately, 10, 4 and 8 and 5-times greater inhibition in final yield of *A. doliolum* occurred at 1, 2 µM Cu and 2.5 and 5 µM of Zn, respectively, in semi-continuous culture in comparison to batch culture. At 4 µM of Cu and 10 µM of Zn, the inhibition of final yield of *A. doliolum* was also higher in semi-continuous culture than in batch culture. The final yield of *A. doliolum* in the control cultures of batch and semi-continuous system were 51.7 ($\pm$ 2.5) and 24 ($\pm$ 1.4), respectively. Similarly, the inhibition of protein content, chlorophyll *a* and carotenoid contents of *A. doliolum* was significantly ($P < 0.05$) greater at all the tested concentrations of Cu and Zn in semi-continuous culture in comparison to the batch culture (Figs. 1 and 2). The control cultures of *A. doliolum* showed 183 ($\pm$ 6.4), 169.3 ($\pm$ 1.2) fg.cell^{-1} protein, 77 ($\pm$ 2.26), 57 ($\pm$ 2.9) fg.cell^{-1} chl *a* and 86 ($\pm$ 2.0) and 66 ($\pm$ 0.8) fg.cell^{-1} carotenoid contents in of batch and semi-continuous system, respectively.

The depletion of Cu and Zn from the culture medium were noticed in batch and semi-continuous cultures, but the depletion was higher in batch culture than that of semi-continuous culture (Fig. 3). The amount of Cu and Zn accumulated in the cells of test organism was greater in semi-continuous culture in comparison to the batch culture. The amounts of Cu and Zn were approximately, 2–3 times higher in semi-continuously grown cells than in those cells from the batch culture (Fig. 3).

Table 1 shows the changes of pH of culture and depletion of phosphate from the culture medium as

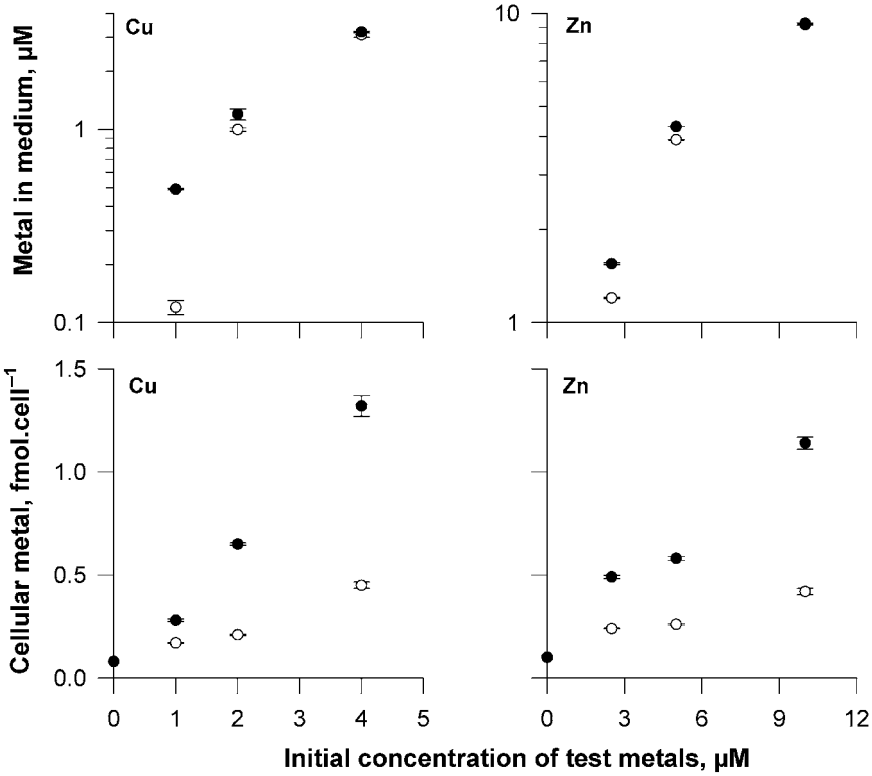

Fig. 3. Changes in cellular metal content and metal content in the medium as observed in batch (open symbol) and semi-continuous culture (filled symbol) at different levels of Cu and Zn.

Table 1. Copper- and zinc-induced changes in pH and phosphate level as well as percentage of phosphate depletion from the culture medium. B = batch culture, S = semi-continuous culture. Values are mean (± SD in parentheses) of three replicates.

Test metal	Concentration (µM)	pH		Phosphate (mM)		Phosphate depletion (%)	
		B	S	B	S	B	S
	Control	7.89 (0.02)	7.21 (0.04)	0.62 (0.06)	1.52 (0.01)	70 (5)	25 (3)
Cu	1	7.86 (0.03)	7.19 (0.02)	0.79 (0.01)	1.55 (0.04)	60 (4)	22 (2.4)
	2	7.83 (0.06)	7.19 (0.04)	1.0 (0.08)	1.70 (0.02)	50 (2.9)	15 (1.8)
	4	7.61 (0.03)	7.14 (0.03)	1.51 (0.09)	1.76 (0.07)	25 (2.2)	12 (.89)
Zn	2.5	7.93 (0.02)	7.20 (0.01)	0.75 (0.01)	1.53 (0.02)	62 (8)	23 (3.2)
	5	7.91 (0.03)	7.17 (0.03)	1.0 (0.06)	1.69 (0.08)	50 (3)	16 (2)
	10	7.68 (0.05)	7.12 (0.02)	1.50 (0.1)	1.72 (0.13)	25 (1.1)	14 (1.6)

induced by Cu and Zn in batch and semi-continuous cultures. The pH of the batch culture showed an increase from the beginning of the experiment (from 7 to 7.9) whereas, in semi-continuous there was no considerable increase.

Although the depletion of phosphate from culture medium was noticed in both culture systems, the depletion of phosphate was greater in batch culture than semi-continuous culture. The percentage of phosphate depletion from their initial concentration (2 mM) was 60, 50, 25 and 62, 50 and 25% from the culture medium dosed with 1, 2 and 4 µM of Cu and 2.5, 5 and 10 µM of Zn, respectively. Only 22, 15, 12 and 23, 16 and 14% phosphate was depleted from the medium containing 1, 2 and 4 µM Cu and 2.5, 5 and 10 µM Zn, respectively in semi-continuous culture (Table 1). The control cultures of batch and semi-continuous systems showed 70 and 25% depletion of phosphate, respectively.

Figure 4 showed the relationship between the pH, rate of metal accumulation and percent inhibition of different tested parameters of *A. doliolum*. A negative relationship was observed between pH and metal accumulation and/or percent inhibition. With the increase of the pH the rate of metal accumulation and consequently percent inhibition of different parameters were decreased. The ionic concentrations of test metals in the culture medium were

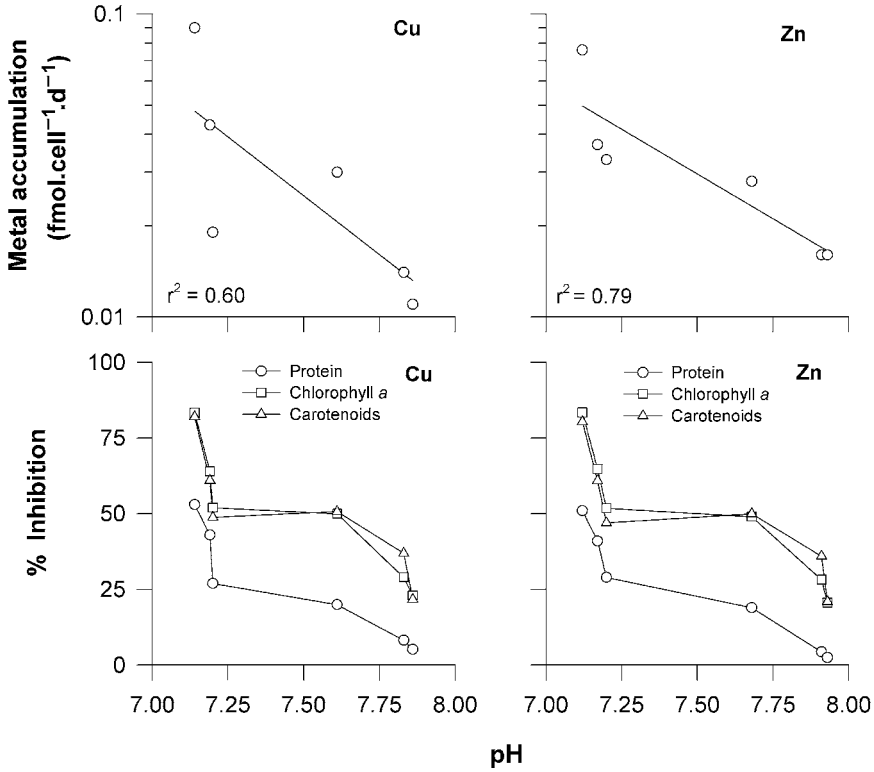

Fig. 4. Relationship between pH of the culture medium and metal accumulation and percent inhibition of different parameters of *Anabaena doliolum*.

determined at different pH using the programme MINEQL. At pH 7.0, more than 90% of test metals were present in ionic forms in the culture medium at all the tested concentration of test metals. Whereas, at pH 7.9, the total ionic Cu and Zn were reduced to 55, 30, 30, 35 and 30% from their initial concentrations 2 and 4 μM Cu and 2.5, 5 and 10 μM Zn, respectively.

The contents of carotenoids of batch grown *A. doliolum* was significantly high ($P < 0.05$) in comparison to the cells of the semi-continuous culture at each tested concentrations of metals as well as control (Fig. 5). Additionally, an inverse relationship between the carotenoid contents of the cell and metal induced inhibition of different tested parameters of *A. doliolum* was observed.

Discussion

The higher toxicity of Cu and Zn to *A. doliolum* in semi-continuous than in batch culture was associated with greater metal content of cells in the former culture. As the metal accumulated inside the cell is responsible for toxicity (Mehta and Gaur, 1999), difference in cellular levels of metals must be the prime cause behind differences in metal toxicity to *A. doliolum* in batch and semi-continuous cultures.

In semi-continuous culture, the higher metal content of the test organism was due to higher metal uptake by the cells. The higher metal uptake in semi-continuous culture was due to the continuous renewal of Cu and Zn in the medium, which maintained a high level of metal in the surroundings of the cells as it was noticed in semi-continuous culture. Continuous renewal of metal also prolonged the duration of exposure of the test organism to high level of toxic metals. This presumption is supported by the previous reports suggesting that extent of exposure and levels of metal in the surrounding affect the metal uptake (Nyholm and Kallquist, 1989; Pascucci and Sneddon, 1993). A negative relationship between pH and metal accumulation in the present study also suggest that increased pH of the culture medium might be one possible reason for the lowered metal content of cells in batch culture. This is also supported by the MINEQL calculation, which showed reduction in free ionic concentrations of Cu and Zn in culture medium from pH 7 to 7.9. Moreover, higher culture density of batch culture may cause substantial reduction of test metals to cells and also from the culture medium (Stauber and Davies, 2000). Higher cell density was also responsible for greater depletion of phosphate from the batch culture.

The high amount of carotenoids in batch grown *Anabaena* in comparison to semi-continuous culture as well as its negative relationship with metal-induced inhibition strongly suggests the involvement of carotenoids in amelioration of metal toxicity. The previous literature has well documented the role of carotenoids in protecting cells from stress-induced oxidative damage (Mallick and Mohn, 2000). The differential toxicity of

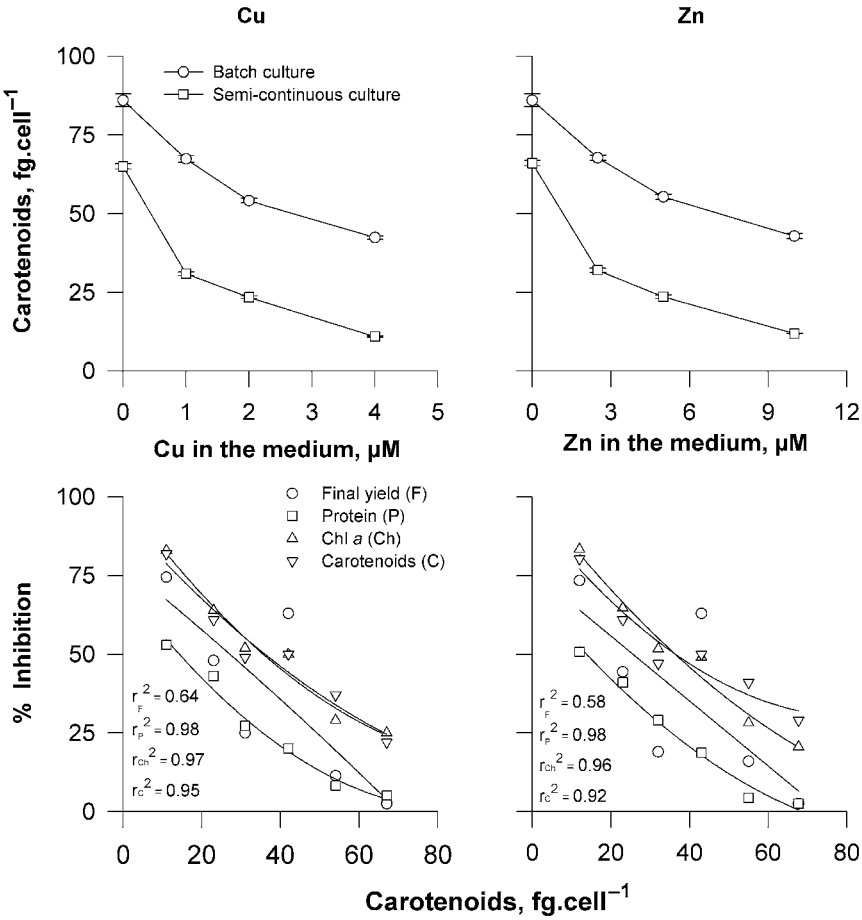

Fig. 5. Carotenoid content of *Anabaena doliolum* at different concentrations of test metals and their relationship with metal induced inhibition of different parameters in batch and semi-continuous cultures.

metals to *Anabaena* in batch and semi-continuous cultures might also be due to different cellular status of carotenoids. But the mechanism of amelioration of metal toxicity by carotenoids warrants further research.

Our results question the use of batch cultures for the long-term evaluation of metal toxicity. The results derived from a batch culture experiment may actually underestimate the toxicity of metals to algae, and therefore appear misleading. The present study recommends the use of semi-continuous culture as it approximates natural systems for predicting the actual events and for a better understanding of algal responses to metals in metal-contaminated environments.

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