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Toxicity of arsenic on marine diatom, *Skeletonema costatum* and their enzyme activities (Greville, 1866)

P. Umamageswari, K. Manimaran and P. Sampathkumar*

Centre of Advanced Study in Marine Biology
Faculty of Marine Sciences
Annamalai University, Parangipettai-608 502, Tamil Nadu, India
*Corresponding author: *sampathcas@gmail.com*

Abstract

To investigate the acute and chronic toxicity effects of arsenic on marine diatom, *Skeletonema costatum*, in addition to their growth, chlorophyll 'a' concentrations, protein and enzyme activities viz. Superoxide dismutase (SOD) and Malondialdehyde (MDA) under the arsenic exposure. The acute and chronic tests were conducted with the concentration of arsenic *viz.* 15, 29, 54, 103, 195, 371 and 706 µg/l for 96 hours and 9, 16, 31, 58, 111, 211 and 401µg/l for 120 hours on *S. costatum* respectively.The enzyme activities *viz.* SOD and MDA were assayed in the LOEC, Chronic and IC_{50} (Acute) arsenic concentration of 9.8, 6.9 and 112.4 µg/l exposed for 96 hours and compared with control. The minimum cell density, growth rate, high growth inhibition and doubling time were observed at the concentration of 656 µg of Arsenic in L^{-1} with respect to control. The calculated $^{96h}IC_{50}$ value was112.4 µg As. L^{-1}. The NOEC of 5.0µg As. L^{-1}, LOEC of 9.8µg As. L^{-1} and Chronic Value of 6.9 µg As. L^{-1} were derived as mean concentration from chronic tests based on measured arsenic concentrations. The maximum chlorophyll 'a' content of 0.0154 ± 0.004 pg. $cell^{-1}$ was found in control and the minimum of 0.0072 ± 0.0016 pg.$cell^{-1}$ found in (IC_{50}) 50 % growth reduction concentration of 112.4 µg. L^{-1}. The maximum cell density of 238.3 ± 12.5 was recovered from control and minimum of 124.98 ± 4.6 mg fresh wt./l was recovered at the concentration of 112.4 µg As. L^{-1} after 96 hours of exposure. The highest protein content of 0.742 ± 0.089 in sub-chronic concentration 6.9 µg/l of arsenic and the lowest of 0.647 ± 0.047 pg. $cell^{-1}$ in control. The maximum SOD and MDA activity of 0.022 ± 0.003 U/mg and 1.494 ± 0.192 µmol/cell was observed at the concentration of arsenic 112.4 µg. L^{-1} respectively. From the present results, the growth inhibition toxicity tests are to be conducted on marine diatom to understand the toxicity differences among the species and also it is suggested that the antioxidant responses of marine diatom could be used as a tool to assess the oxidative stress due to metal pollution.

Keywords: *Skeletonema costatum,* Arsenic, Protein, Superoxide dismutase, Malondialdehyde, Chlorophyll 'a'

Introduction

The water contamination by toxic metal have become increasingly widespread, a point of large attention for researchers and posing a global threat (Lavoie *et al*., 2009). Pollution resulting from industrial activities poses a threat to marine ecosystems and species important to the quality of life of the local populations. Among the aquatic organisms, microalgae are most important and basic component in marine and freshwater ecosystems (Li *et al*., 2006). They are also the primary producers at the base of aquatic food chain, one of the first groups to be affected by metal pollution and also the bioindicator of various pollutions (Sampathkumar and Kannan, 1998; Stauber and Davies, 2000; Ashokprabu *et al.*, 2008; Karthikeyan *et al*., 2010). Therefore, it is essential that toxicity testing be undertaken using locally occurring aquatic organisms including phytoplankton in order to establish acceptable limits for the safe discharge of

potential toxicants into the environment and to aid in the establishment of local water quality criteria.

Despite published guidelines for toxicity tests employing marine microalgae (Walsh, 1988) and reviews on the toxicity of heavy metals to phytoplankton (Rai *et al.*, 1981). there is a dearth of information on tropical species (Ismail *et al*., 2002). Clearly, it would be more appropriate when determining local water quality criteria to generate data using organisms which are representative of the natural flora of that region rather than standard test species (Joy and Joseph, 1995). Though, numerous reports available on various aspects of different diatoms and are very little for Indian waters.

Arsenic is found in all living organisms, including those in aquatic systems. Little is known about the mechanisms of arsenic toxicity to aquatic organisms, however, arsenic readily forms stable bonds to sulfur and carbon in organic compounds. For example, the arsenic (III) reacts with sulfhydryl groups of proteins, enzyme inhibition by this mechanism may be the primary mode of toxicity but arsenic (V) does not react with sulfhydryl groups due to uncouple oxidative phosphorylation (Fowler, *et al*. 1977). Because of the variety of forms of inorganic arsenic (III & V) and lack of definitive information about their relative toxicities, no available analytical measurement is known to be ideal for expressing aquatic life criteria for arsenic. Previous aquatic life criteria for arsenic (U.S.EPA, 1980) were expressed in terms of total recoverable inorganic arsenic (III), but the total recoverable method cannot distinguish between inorganic arsenic (III) and arsenic (V).

- Although many heavy metals when in trace amounts are essential for various metabolic processes of organisms, they create physiological stress leading to generation of free radicals when in high concentration and stress in turn induces the production of reactive oxygen species (Choudhary *et al*., 2007). The plankton cells evolved reactive oxygen species (ROS) scavenging mechanisms that include enzymes and non-enzymatic compounds (Jin *et al*., 2008.
- SOD is generally accepted as a first defense line protector against ROS and it is responsible for catalyzing the dismutation of O_2^- to form O_2 and H_2O_2, which is decomposed by catalase and peroxidases in O_2 and H_2O (Torres *et al*., 2008).
- Malondialdehyde (MDA) is a cytotoxic product of lipid peroxidation and an indicator of free radical production and consequent tissue damage (Ohkawa *et al*., 1979). SOD is a metalloenzyme that catalyzes dismutation of superoxide anion into oxygen and hydrogen peroxide. Such enzymes provide a defense system for the survival of aerobic organisms (Beyer *et al*., 1991). There are many report concerning the response of the antioxidant systems in plants to mental stress (Mazhoudi et at., 1997), but studies on microalgae are very few (Elisabetta and Gioacchino, 2004).

Antioxidant activity can determined the toxicity stress in aquatic organisms. There are many reports concerning the response of the antioxidant systems in plants to metal stress (Mazhoudi *et al*., 1997), but studies on microalgae are very few (Elisabetta and Gioacchino, 2004). Teresa *et al.,* (2005) also analysed the activities of the antioxidant enzymes *viz*. superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), photosynthetic pigment contents and free malondialdehyde (MDA) in senescent batch cultures of *Tetraselmis gracilis* (Kylin) Butcher, under a cyclic light regime.

So, the present study was carried out to investigate the acute and chronic toxicity effects of arsenic on growth, Chlorophyll *a* concentrations, protein and enzyme activities *viz.* superoxide dismutase (SOD), Malondialdehyde (MDA content) of cosmopolitan species of marine diatom, *S. costatum.*

Materials and Methods

Marine diatom collection and maintenance

The marine diatom, *Skeletonema costatum* was collected from Vellar estuary, Southeast coast of India, Parangipettai (Lat.11'29^0 N; and Long.79'46^o E). The test species was identified under the light microscope using plankton identification manual (Tomas, 1997). The species were isolated and stock culture was maintained in Guillard's F/2 medium at Marine Algal Culture Laboratory, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai as per the methods described by Andersen (2005).

Experimental design for growth rate-inhibition bioassays

All the experiments were conducted in 250 ml conical flasks with 100 ml of 3-4 days old exponentially grown algal cultures. The standard growth inhibition test procedures were followed by OECD (2002) and EPA (2002). The range finding tests were conducted for 48 hours before the definitive test. The stock toxicant solution of arsenic was prepared in Milli-Q

Ultra pure water using its metallic salt of Arsenic trioxide (Merck, India (Pvt.) Ltd.).

The definitive tests were conducted in triplicate experiments using the concentrations of Arsenic *viz*. 15, 29, 54, 103, 195, 371 and 706 µg/l (each concentration were tested in triplicate; for 96 hours. The chronic tests were conducted in triplicate experiments using the concentrations of arsenic *viz*. 9, 16, 31, 58, 111, 211 and 401 µg/l (each concentration were tested in triplicate) for 120 hours. The cell density was estimated at every 24 hour intervals in all the experiments. Growth rate and percentage of growth inhibition were calculated using cell density and time (equations given below). Chlorophyll *a* and protein concentrations were estimated at the end of acute tests. The enzyme activities *viz*. Superoxide dismutase (SOD) and Malondialdehyde (MDA content) were assayed in the LOEC, Chronic and IC_{50} (Acute) concentration of arsenic (9.8, 6.9 and 112.4 µg/l) exposed for 96 hours and compared with control. During the experiment period, the physico-chemical parameters such as temperature of 25 ± 1º C, salinity of 30‰, pH of 8.0 ± 0.3 and the light intensity of 4500 ± 500 Lux were maintained.

Dissolved Arsenic Measurement

The dissolved arsenic concentrations in test chambers were analysed using Hydride Generation in Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) described by Thompson *et al*. (1978).

Cell Density

The cell density was calculated by conventional method with one hundred microlitre (100 µl) of *Skeletonema costatum* culture was made up to 1ml with the addition of lugol's iodine solution. 10 µl of diluted sample was placed on a glass slide exactly at the meeting point of plus mark. The slide was mounted on the binocular microscope and the cells were counted at 4x (ocular lens) and 10 x (stage lens) condition. The results were expressed as cells. ml^{-1} of culture.

The growth rate was calculated using the following formula (OECD, 2002)

Growth Rate (µ) = $N_x - N_0 / t_x - t_0$

Where, N_0 – Number of cells at time zero, N_x - Number of cells at time x, t_0 – starting time (0)
t_x – time X (in days)

The doubling time was calculated by the following formula

Doubling time = $(N_0 \times 2) / (N_t) \times t$

Where, N_0 – Number of cells in time zero, N_t – Number of cell in time t, t – Times in hours. The results were presented in doubling time (DT) in hours.

Percentage of growth inhibition = $(\mu_{control} - \mu_{concentration}) / (\mu_{control}) \times 100$

Chlorophyll *a* and Protein determination

Chlorophyll analyses were performed by the modified method of Strickland and Parsons (1972). To 2 ml of algal culture, 5 ml of acetone was added and vortexed for one minute and kept at 4ºC under dark in refrigerator for 24 hrs. Then the samples were centrifuged at 5000 rpm for 10 min and the supernatant was read at 630, 645 and 660 nm in UV-Vis. Spectrophotometer (Shimadzu UV-1800) and raw acetone was used as blank. The protein content was analyzed by Lowry et al. (1951).

Enzyme extraction procedure

After five days of exposure to arsenic, the algal biomass were collected by Millipore vaccum pump through plankton silk cloth and the biomass was homogenized in Mini Bead Beater with 1ml of 0.1M phosphate buffer (pH 7.0). The extracts were centrifuged at 12000 rpm for 15 min at 4 ºC. The centrifugation process was repeated for clear supernatant and it was used as enzyme extract for all assays.

Superoxide dismutase (SOD) and Melondialdehyde (MDA) Activity

The SOD activities were measured according to the method of Beauchamp and Fridovich (1971), and the content of MDA was measured by the Thiobarbituric acid (TBA) reactive substances test (Draper *et al*., 1993; Janero, 1990). One unit of SOD activity (U) was defined as the enzyme dosage used for inhibiting the reactive starting velocity to 50% and was calculated as

$$\text{SOD activity} = \frac{OD_b - OD_s}{\text{diluted aliquot of sample} \times 50\%\ OD_b}$$

where, OD_b is the optical density (OD) value of background, OD_s is the OD value of the sample.

Melondialdehyde (MDA) Activity

The MDA content was expressed as µmol/cell and calculated as follows,

$$\text{MDA content} = \frac{\text{OD}(532-600\text{ nm})/155 \times V_1 \times S/A}{Nt \times V_2}$$

where, $OD_{532-600nm}/155$ means ΔMDA (µmol/ml), V_1 (ml) is the volume of reaction mixture; V_2 (ml) is the volume of the algal culture, S (ml) is the extract volume, A (ml) is the measured volume and N_t (cells/ml) is the algal density at time t.

Results

Growth inhibition tests

The cell density and average specific daily growth rate (µ) were decreased while the percentage of growth inhibition and doubling time were increased with increasing concentration of arsenic after 96 hrs of exposure. Minimum cell density, growth rate, high growth inhibition and doubling time were observed at the concentration of 656 µg As. L^{-1} with respect to control. The calculated $^{96h}IC_{50}$ value was 112.4 µg As. L^{-1}. The NOEC (No Observable Effect Concentration) of 5.0µg As. L^{-1}, LOEC (Low Observable Effect Concentration) of 9.8µg As. L^{-1} and Chronic Value of 6.9 µg As. L^{-1} were derived as mean concentration from chronic tests based on measured Arsenic concentrations.

Chlorophyll *a* concentration

The chlorophyll *a* concentration reduced with increasing concentration of Arsenic from 6.9 µg. L^{-1} to 112.4 µg. L^{-1}. Maximum of 0.0154 ± 0.004 pg. $cell^{-1}$ was found in control and the minimum of 0.0072 ± 0.0016 $pg.cell^{-1}$ found in (IC_{50}) 50 % growth reduction concentration of 112.4 µg. L^{-1} **(Fig, 2).**

Total biomass

The maximum algal biomass were obtained from control after 96 hours of culture (238.3 ± 12.5 mg Fresh Wt./l) but it was significantly reduced (124.98 ± 4.6 mg Fresh Wt./l) with arsenic exposure at 112.4 µg As. L^{-1} **(Fig.3).**

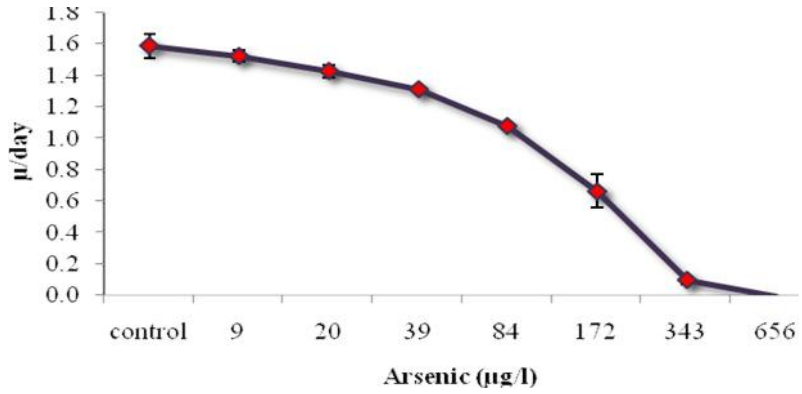

Fig 1. Average daily growth rate of *S. costatum* after 96 hours exposure with different concentrations of arsenic

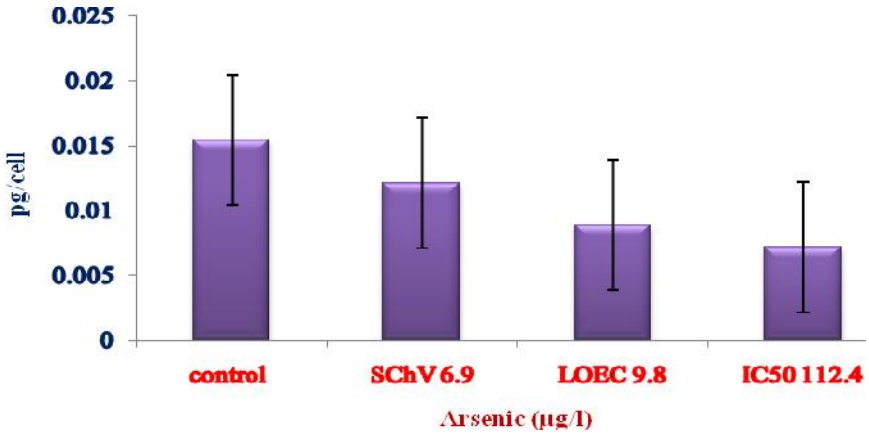

Fig 2. Chlorophyll *a* concentration of *S. costatum* after 96 hours exposure with different concentrations of arsenic

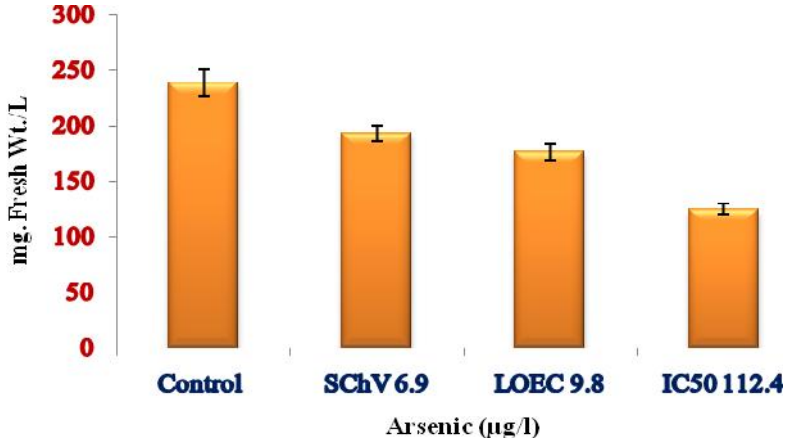

Fig 3. Total biomass production of *S. costatum* after 96 hours exposure with different concentration of arsenic

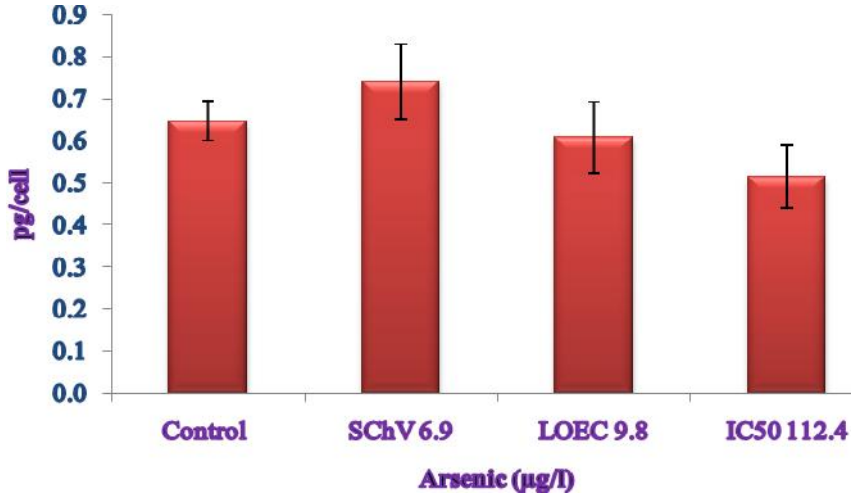

Fig 4. Total protein content of *S. costatum* after 96 hours exposure with different concentration of arsenic

Protein

Protein content of *Skeletonema costatum* was found higher (0.742 ± 0.089 pg. cell^{-1}) in sub-chronic concentration of 6.9 µg/l of arsenic than the control (0.647 ± 0.047 pg. cell^{-1}) (**Fig. 4**).

Enzyme activities

The MDA content was increased with increasing of arsenic concentrations compared to control. Maximum of 1.494 ± 0.192 µmol/cell was observed at the concentration of 112.4 µg. L^{-1} (IC_{50}) (**Fig. 5**). The SOD also increased with increasing of arsenic as MDA. The maximum activity of 0.022 ± 0.003 U/mg protein was observed at 112.4 µg. L^{-1} (**Fig. 6**).

Table 2. Nominal and Measured concentrations (Mean ± SD) of dissolved Arsenic (µg. L^{-1}) in test solution after the algal exposure

Acute Test		Chronic Test	
Nominal Concentrations	Measured Concentrations	Nominal Concentrations	Measured Concentrations
15	8.7 ± 0.58	9	5.0 ± 0.00
29	20.3 ± 3.21	16	9.7 ± 1.15
54	39.0 ± 1.00	31	21.3 ± 2.52
103	83.7 ± 1.53	58	42.3 ± 0.58
195	172.0 ± 3.00	111	86.7 ± 3.79
371	343.0 ± 4.36	211	170.0 ± 5.57
706	656.3 ±13.65	401	341.0 ± 3.51

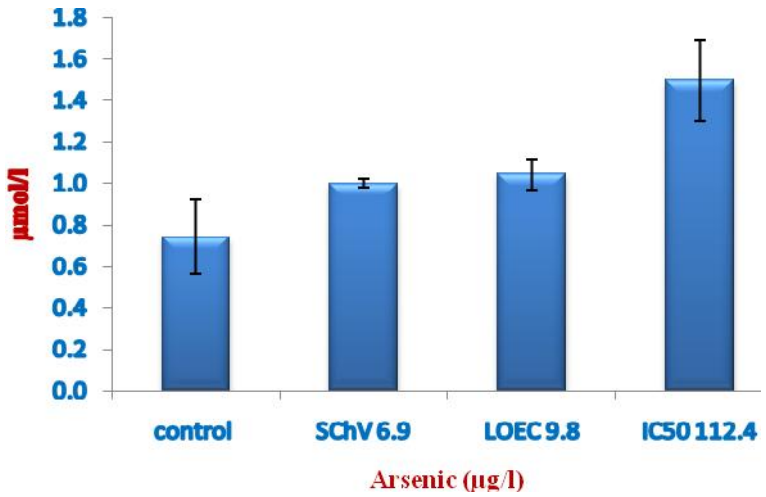

Fig 5. MDA activities of *S. costatum* after 96 hours exposure with different concentration of arsenic

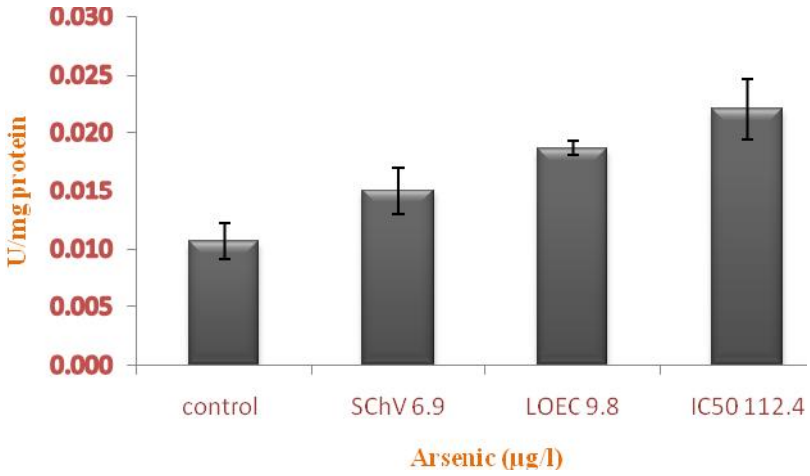

Fig 6. SOD activities of *S. costatum* after 96 hours exposure with different concentration of arsenic

Discussion

Effects on growth

The results of the present study showed the effects of arsenic on growth rate, photosynthetic pigment, protein and the antioxidant enzymatic responses of the marine diatom, *S. costatum*. The cell density and average specific daily growth rate (µ) decreased but the percentage of growth inhibition and doubling time increased with increasing concentration of arsenic after 96 hrs of exposure. The 50% growth reduction was found in 112.4 µg As/l based on measured concentration. Similarly, Sanders and Cibik (1985) have shown that 0.01 mg L^{-1} As (V) caused reduction in the growth rates of *Chaetoceros debile*. Also Sanders and Vermersch (1982) have observed depression of growth in *Tetraselmis contracta* at concentrations less than 0.1 mg L^{-1}. They also found the *Isochrysis galbana* and *C. pseudocrinitum* were far more sensitive to As (V), with growth ceasing at < 0.1 mg L^{-1}. In contrast, Ismail *et al*. (2002) reported the growth rates of micro flagellates, including *Isochrysis galbana* largely unaffected by arsenic exposure. Generally, direct comparisons of EC_{50} values are difficult because of the initial and final cell densities and laboratory set-ups in respect light illumination, temperature, composition of culture media and exposure time.

In general, the growth of algal culture is determined by the estimation of chlorophyll concentration. The chlorophyll *a* was significantly reduced with increasing concentrations of arsenic (Sub-Chronic concentration, LOEC and IC_{50}) in the present investigation. It is evidenced the reduced chlorophyll *a* of *S. costatum* reported by Hollbough *et al.* (1980) on the exposure to 22 µg/l of sodium arsenate.

In the present investigation, the algal biomass of 238.3 ± 12.5 mg. fresh wt./l after 96 hours culture was obtained from control but it was significantly reduced with arsenic exposure. In contrast, Coale (1991) reported Fe, Mn, Cu, and Zn treatments all showed increases in biomass, or productivity, or both. Fe had the most dramatic effect on productivity, yet Cu had a similarly dramatic effect on biomass. But the reports were not available on arsenic to compare the present results.

Effects on protein and enzymes

In the present study, protein content was slightly increased in arsenic treated cells than the control as reported earlier for *Spirulina* sp. and *Anabaena* sp. (Sultan and Fatma, 1999; Jetley *et al*., 2004; Kumar *et al*., 2004). Increase in protein content under heavy metal stress in *Spirulina platensis* was also reported by Choudhary *et al*. (2007). The increase in protein content against metal exposures may be due to the increase of metal chelating amino acid proline. Binding with metal ions due to the chelating ability of proline can also be a defense mechanism for survival.

Yang *et al*. (2002) reported the increased activities of antioxidant enzymes *viz*. SOD and MDA of *S. costatum* exposed to 2, 4-DCP for 96 h and suggested that there was an increase in the production of oxyradicals by the diatom. Roy and Hanninen (1994) reported the induction of POD and SOD in the aquatic plant, *Eichhornia crassipes*, after exposure to PCP. The similar observations were found in the present study that increased activities of SOD and MDA content. It is evidenced that the ROS formed by metal stress and triggered *S. costatum* to synthesis such enzymes for their survival.

Conclusion

The present study was suggested that the growth inhibition toxicity tests are to be conducted on other phytoplankton species to understand the toxicity differences among the species, strains of same species etc. Also this study suggests that the antioxidant responses could be used as a tool to assess oxidative stress caused by pollutants and healthiness of phytoplankton.

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