

Full Length Research Paper

## Effect of different dosages of zinc on the growth and biomass in five marine microalgae

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Accepted 10 December, 2013

Marine environment often restrain toxic heavy metals that may enter into the food web via uptake by microalgae and eventually cause severe poisoning problems at higher tropic levels. The effects of Zinc cations upon growth of five native microalgal species, *Chlorella marina*, *Isochrysis galbana*, *Tetraselmis* sp., *Nannochloropsis* sp., and *Dunaliella salina* were evaluated. Growth inhibition of the microalgal cells were determined by exposing them to different concentrations of aqueous solutions of zinc metal for 15 days. A major reduction in cell density was observed in all the five cultures in the concentration of 50 ppm. Among the five micro algal species tested, *Tetraselmis* sp. alone showed growth up to 250 ppm concentration of zinc metal till the final day (15<sup>th</sup> day) of experiment.

**Key words:** Zinc, microalgae, heavy metal, *Chlorella marina*, *Tetraselmis* sp.

### INTRODUCTION

Marine unicellular algae with towering metabolism rates fill the essential function in the primary production and focus of heavy metals, which are pollutants with multiple ingestion pathways to marine ecosystems. The hazard of heavy metal pollution is owing to its capability to move within marine and near-shore ecosystems for along-lasting length of time. By accumulating heavy metals in their cells, algae encourage further accumulation in the successive parts of the food chain, as well as in commercial aquatic organisms (Amiard et al., 1980). The responses to the occurrence of lethal concentrations of heavy metals vary considerably in different species. Among the marine organisms, phytoplankton resistant to heavy metals have been recommended as bioindicators for the assessment of marine pollution (Rijstenbeil et al., 1994; Kapkov and Belenikina, 2003, 2007). Based on the physical and chemical properties of phytoplankton, in

addition to its reaction patterns, a chain of lethal heavy metals for phytoplankton has been proposed, in which the following chemical elements are arranged in the following order: Hg > Cu > Cd > Zn > Pb (Eichenberger, 1993). Most of the artificial methods are currently famous for removing heavy metals from aqueous solutions such as precipitation, electrolysis, ionic exchange, filtration, evaporation and others. But the main disadvantages of these methods are high cost, low efficiency, especially during removing small amounts of heavy metals and a necessity of slag committal. In order to overcome these disadvantages an alternative other than the chemical is needed for detoxication and removal of metals from aqueous solutions (Nalimova et al., 2005). Microalgae have been found to be very effective in removing heavy metals from wastewater due to their large surface area and high binding affinity (Chong et al., 2000). The toxicity

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**Table 1.** Summary of protocol for marine microalgae species growth inhibition bioassay.

S/N	Task	Condition
1	Test type	Static
2	Temperature	23±2°C
3	Light quality	Cool white fluorescent lighting
4	Light intensity	5000 lux
5	Illumination	12:12 h light and dark cycle
6	Conical flask size	250 ml
7	Culture solution volume	100 ml
8	Renewal of test solutions	None
9	Age of test organisms	Every 3 days
10	Initial cell density in conical flasks	1.5×10 <sup>4</sup> cells ml <sup>-1</sup>
11	Number of replicate	3
12	Mixing rate	Constant aeration
13	Test medium	Walne medium
14	Metal concentrations	Minimum of six and a control
15	Test duration	15 days
16	Test endpoint	Growth rate

of heavy metals to different algal types varies (Fitzgerald and Faust, 1963). Some algal species demonstrated tolerance to different heavy metals possessing mechanisms to prevent their toxic effects on metabolism or damage of intracellular structures. Still now, most of the researchers studied the effect of heavy metal toxicity on fresh water algae cultivated under laboratory conditions. In this respect, study on the effects of the most dangerous metal zinc on the growth of algal species as an essential factor of natural phytocenoses seems enormously main, as it plays a determining role in near-shoremarine ecosystems. Identifying algae of different taxonomic ranks that are sensitive and resistant to heavy metals would also be needed for the development of biological markers of pollution in marine ecosystems. The aim of this study is to assess the effects of Zinc<sup>2+</sup> toxicity on growth, chlorophyll production and viability of the marine microalgae such as *Chlorella marina*, *Isochrysis galbana*, *Tetraselmis* sp., *Nannochloropsis* sp. and *Dunaliella salina*.

## MATERIALS AND METHODS

### Algal stock cultures

Experiments were performed with the algal cultures such as *C. marina*, *I. galbana*, *Tetraselmis* sp., *Nannochloropsis* sp., and *D. salina*. These strains were obtained from the Central Institute of Brackishwater Aquaculture, Chennai. The microalgae were grown in the Walne medium at pH 8 which was adjusted using 0.1 N NaHCO<sub>3</sub> and HCl (Walne, 1970). Stock cultures were incubated in 250 ml conical flasks containing 100 ml of sterilized seawater under a photon irradiance of approximately 5000 lux (using two cool white fluorescent tube lights) in 12:12 h light and dark cycle and temperature were maintained at 23±2°C.

### Toxicity analysis

Growth inhibition bioassay (Table 1) for microalgal species were conducted under laboratory conditions for fifteen days. To minimize the metal contamination, all laboratory wares contact with the culture medium were soaked in 1% HNO<sub>3</sub> for 24 h and rinsed with Milli-Q water. Zinc solution at a stock concentration of 1000 mg/L<sup>-1</sup> was prepared using analytical grade ZnCl<sub>2</sub>·2H<sub>2</sub>O (Dinesh Kumar et al., 2013). About 4.397 g of ZnSO<sub>4</sub>·7H<sub>2</sub>O salt was dissolved in 1000 ml of distilled water to make stock solution. From the stock solution, 0.1 ml was taken and dissolved in 100 ml of distilled water to make 1 ppm of zinc solution. The culture media with various zinc concentrations such as 5, 50, 250, 500, 1000 and 1500 ppm were prepared by diluting the stock according to Dinesh Kumar et al. (2013). Control medium was prepared in the same manner without adding the toxicant. A known cell density of the algal suspension with exponential growth phase was harvested from the stock cultures and inoculated in the test medium prepared with different concentrations of zinc and control fed with Walne culture medium, the initial cell density was approximately 1.5×10<sup>4</sup> cells ml<sup>-1</sup>. The growth rates of algal cultures were monitored carefully at regular intervals such as 3, 6, 9, 12 and 15 days of incubation by measuring the optical density of algal suspension at 650 nm (Wetherell, 1961). Triplicates were maintained for all the treatments and control.

At the end of experiment chlorophyll 'a' content of the microalgal cultures was estimated by following the method of Mantoura and Llewellyn (1983). As brief 10 ml of algal culture sample was filtered using Millipore filtering system fitted with a 4.5 cm diameter GF/C filter paper by applying low suction. Before filtering the sample, a thin bed of magnesium carbonate (2 ml) was poured over the GF/C filter paper for effective filtration. After filtration, the filtrate was removed and filter paper with algal cells ground with 90% of acetone using mortar and pestle. The resulting samples were transferred to screw cap test tubes covered with black cloth and incubated in the refrigerator for 24 h. After 24 h incubation, the contents were ground with 90% of acetone and centrifuged at 3000 rpm for 10 min. Then the clear supernatant was collected and optical density was measured at different wavelengths such as 630, 645 and 665 nm using UV- visible Spectrophotometer (1800

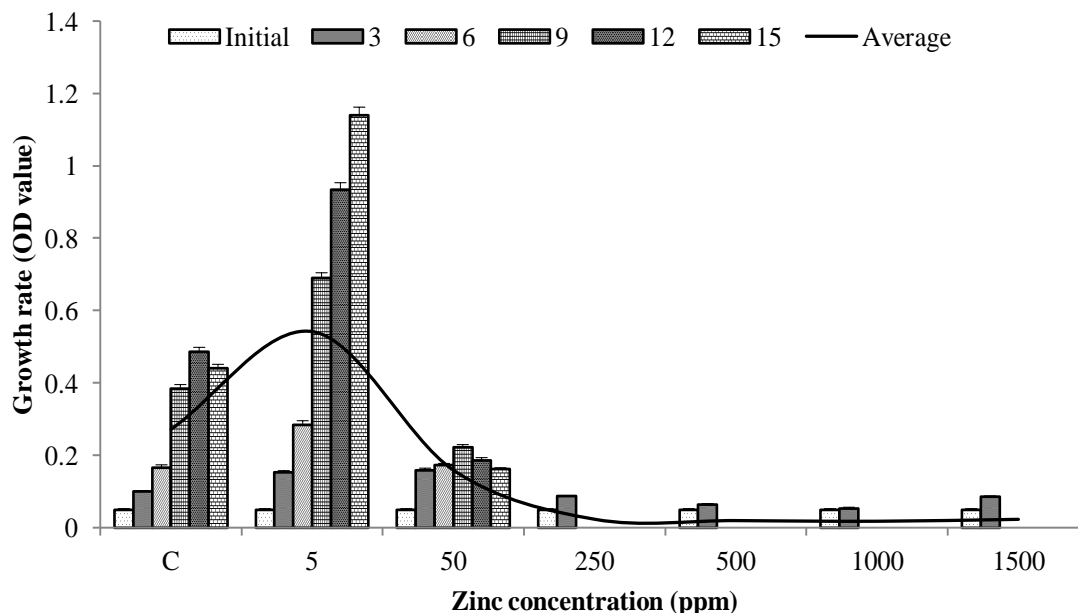


Figure 1. Effect of zinc on the growth of *C. marina*.

Shimadzu UV) for chlorophyll 'a' estimation.

## RESULTS AND DISCUSSION

### Effect of zinc on the growth of the microalgal cultures

In the present investigation, the effect of zinc on the growth of unicellular marine microalgae such as *C. marina*, *I. galbana*, *Tetraselmis* sp., *Nannochloropsis* sp., and *D. salina* were evaluated by using growth inhibition bioassay. The result indicates that the *Chlorella marina* showed good growth (average-  $0.101 \pm 0.003$  abs) in all the concentration of zinc up to 3 days of incubation. However, when the concentration of the zinc and incubation days increased, the growth of *C. marina* decreased drastically. The *Chlorella marina* displayed good growth in 5 and 50 ppm concentration of zinc compared to the control ( $0.153 \pm 0.005$  and  $0.158 \pm 0.007$  abs respectively). The growth rate was significantly increased in 5 ppm of zinc concentration up to 15 days of incubation. Though, the growth rate of *C. marina* was less in 50 ppm ( $0.158 \pm 0.005$  abs) when compared to the control ( $0.271 \pm 0.012$  abs), it shows steady growth up to the end of the experiment (Figure 1). These results were accordance with the previous published data on other algae (Kamp-Nielsen, 1971; Nazzi, 1972; Rachlin and Farran, 1974).

The inhibitory effect of zinc on the microalgae *I. galbana* demonstrated that their growth was decreased after 3 days of incubation as *C. marina*. In 50 ppm zinc concentration, the growth ( $0.113 \pm 0.019$  abs) was found to increase from the initial cell density ( $0.05 \pm 0.002$  abs)

till 6 days of incubation and after that a steady growth rate was observed till the end of the experiment. The increased optical density ( $0.278 \pm 0.035$  abs) in 5 ppm concentration of zinc up to 15 days of incubation showed that the *I. galbana* had good ability to tolerate at 5 ppm of zinc concentration. In addition, comparatively the cell density of *I. galbana* was high during 12 ( $0.257 \pm 0.029$  abs) and 15 days ( $0.278 \pm 0.02$  abs) of incubation than the control which denotes that they have grown well in the Walne medium with the presence of 5 ppm of zinc (Figure 2). It is clear that after 9<sup>th</sup> day the algal growth was found to be reduced with reference to metal concentration. It is understand that the metal concentration have made significant adverse impact on the algal growth as agreed earlier by Davies (1974). However, Fisher et al. (1984) have stated that the surface reactivity of the *I. galbana* did not showed any change for heavy metals over an incubation period. It is also suggested that the variability of metal accumulation in algae is probably the result of competition between the metals to bind with such polysaccharides (Kohn, 1975; Hurlburet et al., 1976; Veroy et al., 1980).

The optical density results of *Tetraselmis* sp. revealed that they were able to tolerate up to the concentration of 250 ppm zinc whereas in the highest concentrations such as 500, 1000 and 1500 ppm of zinc no growth was observed after 3 days of incubation. The maximum growth in *Tetraselmis* sp. was observed in 50 and 250 ppm concentrations as  $0.498 \pm 0.049$  and  $0.387 \pm 0.043$  abs respectively on 15<sup>th</sup> day of incubation (Figure 3). Zinc can easily be rationalized since it is a micronutrient required for microalgal metabolism (Bascik-Remisiewicz et al., 2009; Vallee and Auld, 1990) as it is a part of

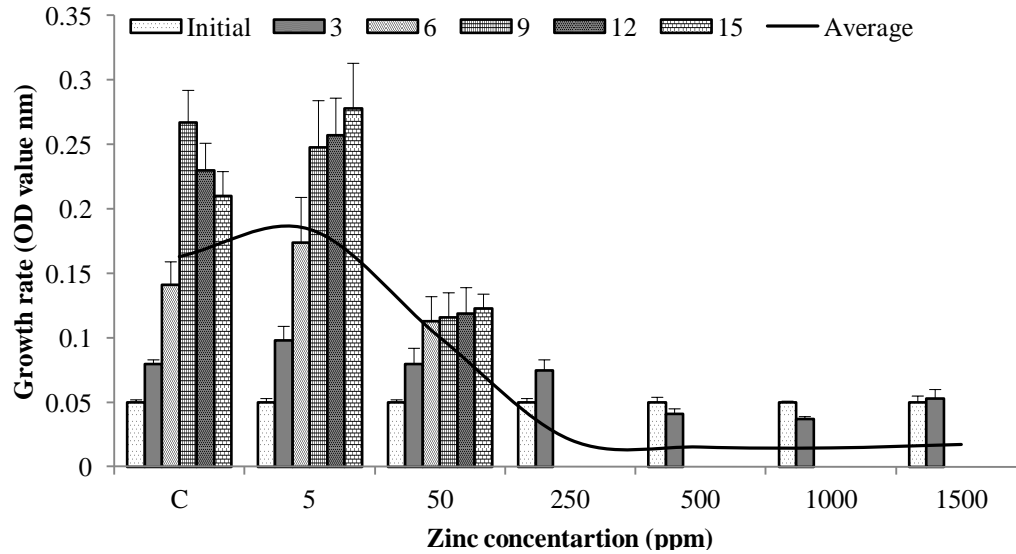


Figure 2. Effect of zinc on the growth of *I. galbana*.

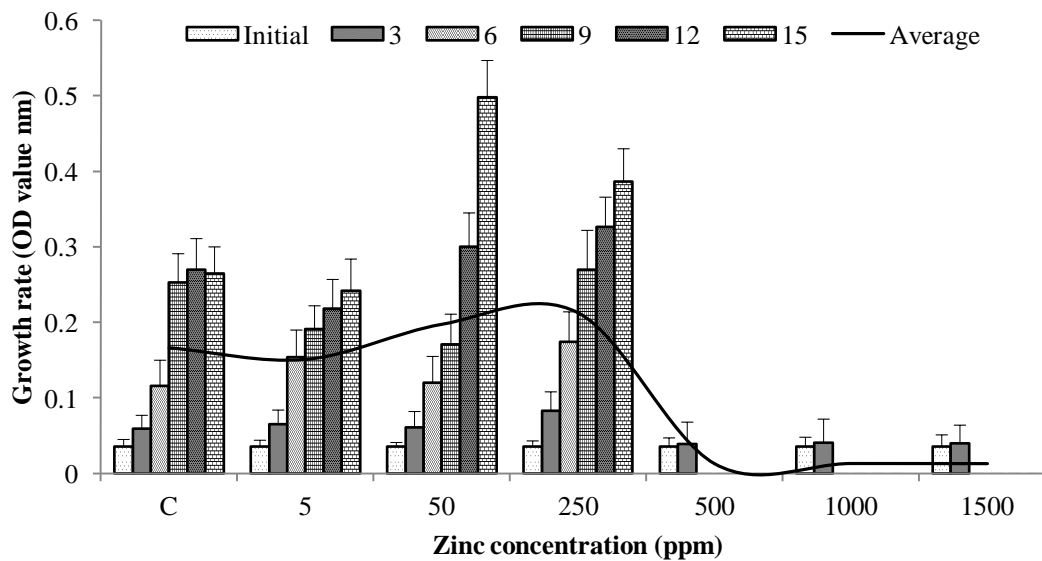


Figure 3. Effect of zinc on the growth of *Tetraselmis sp.*

prosthetic moieties of some of its relevant enzymes. In the present study, the toxicity effect of zinc was progressively decreased when the exposure time was increased which was judged by the increased growth rate of the cultures. This may be due to the accumulation of zinc onto algal cells in higher concentrations which significantly reduced the available zinc concentration and its toxicity to surviving cells in the final stage of experiment as agreed by Lim et al. (2006). Interestingly, the growth rate of the *Tetraselmis sp.*, was found higher in the zinc supplemented medium than the control medium (without heavy metal) which clearly defined that the zinc act as growth stimulant of the *Tetraselmis sp.*

and it can survive up to 250 ppm zinc concentration.

The effect of zinc on *Nannochloropsis sp.* was obtained by measuring the growth at 650 nm which showed that, they were relatively high in 5 ( $0.312 \pm 0.035$  abs) and 50 ppm ( $0.288 \pm 0.029$  abs) than the control ( $0.283 \pm 0.035$  abs) up to 6<sup>th</sup> day of incubation. At the 9<sup>th</sup> day of incubation, the growth rate was increased in 5 ppm and decreased at 50 ppm zinc concentration. After 9<sup>th</sup> day of incubation, growth rate was decreased from an initial value of  $0.36 \pm 0.037$  to  $0.268 \pm 0.031$  abs at 5 ppm concentration whereas in highest concentrations such as 250, 500, 1000 and 1500 ppm, no growth was noticed after 3 days of incubation, which denotes that the culture

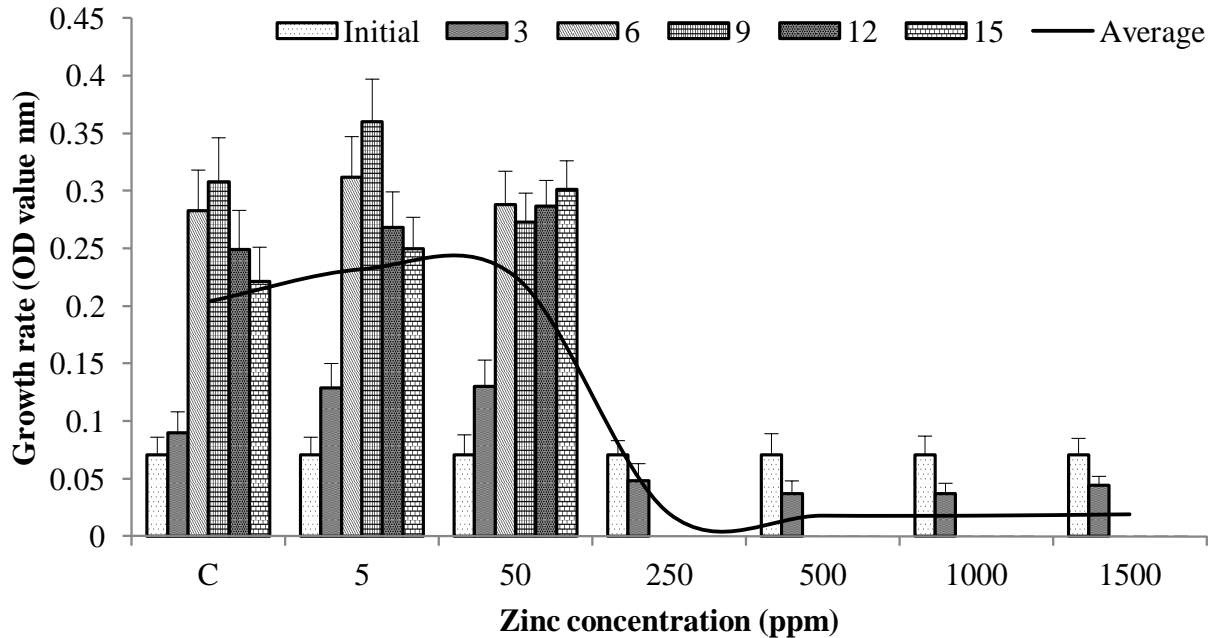


Figure 4. Effect of zinc on the growth of *Nannochloropsis* sp.

could not tolerate when the concentration of zinc increased (Figure 4). This occurs because the metal ion is interacted with the fat layer on the surface of microalgae cells (Darmono, 1995). The body surface of microalgae is covered by the cell membrane, therefore, the potential interaction between the membrane and the metal ion in the water is high (Fhencel, 1988). The surface of cells contains various functional groups, such as N-terminal of  $-NH_2$ , C-terminal of  $-COO^-$ , S-terminal of  $-SH$  and functional groups of side chains which are potential as metal binding sites. Binding of the metal ion by the active groups proceeds on the cell surface followed by the slow transport step into the membrane and then to the cytoplasm (Chu and Hashim, 2007). Accumulation of several metals (such as Zn, Co, Mo, Ca, and Mg) on algae occurs through the intracellular active biological transport. Toxic heavy metal ions can be isolated from the cytoplasm of the cell through three possible ways that is intracellular chelating by biological polymers; precipitation of heavy metals on the surface of cell wall; or surface adsorption through metal ion binding by chemical functional groups on the cell wall (Moreno-Garrido et al., 1998). The present results showed that the *Nannochloropsis* sp., can survive up to 50 ppm of zinc concentration as agreed by Hala et al. (2012).

In all the tested concentrations of zinc, the growth of *D. salina* was not completely inhibited. In high zinc concentration, slow growth was observed and maximum growth inhibition ( $0.329 \pm 0.039$  abs) was noticed after 6 days of exposure to 50, 250, 500, 1000 and 1500 ppm (Figure 5). In control, the growth was found good after 3 days of incubation and it was gradually decreased when

the incubation days prolonged. When compared to control ( $0.198 \pm 0.019$  abs) the growth of *D. salina* was found to be higher ( $0.226 \pm 0.039$  abs) at 5 ppm of zinc concentration till the end of the experiment. The present result suggests that *D. salina* can survive up to 5 ppm of zinc concentration could be attributed the metal binding affinity to the cell surface coat. This conclusion is in accordance with the suggestion made by Davies (1974) who stated that the cellular surface of microalgae consists of a mosaic of cationic and anionic exchange sites acting as ion exchangers for anionic and cationic species in the medium. The amount of metal bound to the cell surface coat at equilibrium should be a function of the affinities of the binding sites for the metal, the chemical form of the metal and its concentration in the medium. So, zinc can affect the microalgal growth during the growth period as agreed by previous researchers (Rebhun and Amotz, 1986; Imani et al., 2011; Shariati and Yahyaabadi, 2006).

On an average the relative growth rates of the algae tested decreased upon increasing zinc content in the sea water. Most of the trace metals are known to promote microalgal growth at low concentrations and inhibits at high concentrations, though, the impact may vary with type of metal and the microalgal species (Gao et al., 2004). In this study the tolerance limit of zinc was 50 ppm except *Tetraselmis* sp. (250 ppm) and *D. salina* (5 ppm). Among the microalgae tested *Tetraselmis* sp. alone tolerated Zn concentrations up to 250 ppm. Our species were more tolerant to Zn concentrations than others reported (Imani et al., 2011; Tripathi and Gaur, 2006).

This may be due to the fact that this metal was an

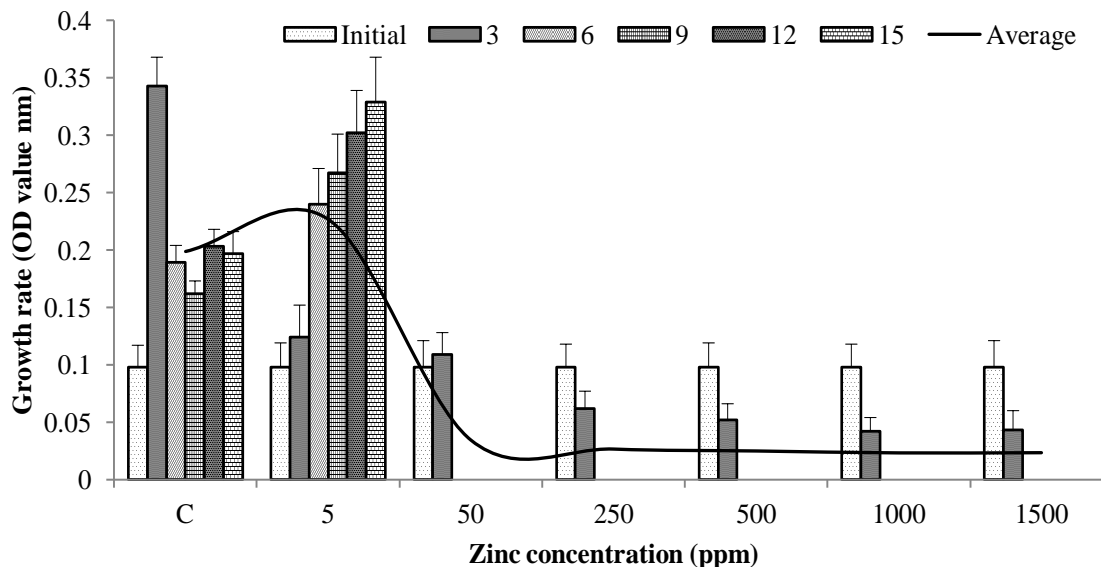


Figure 5. Effect of zinc on the growth of *D. salina*.

abundant contaminant in the original source site of our microalgae strains may account for our exceptional observations.

The effect of zinc on the algae becomes clearer when not only the growth rates in the early exponential phase, but also the total development of the cultures are considered. Many studies have proved non toxicity of zinc (in terms of cell division) at lower concentrations. Rasko and Rachin (1977) have also showed that  $7.5 \text{ mg l}^{-1}$  Zn does depress the concentration of chlorophyll a, suggesting that Zn, as well reduces algal cell division and is an aspect of its relatively mild toxicity. Thus long-term effect of the exposure to increased levels of zinc was, therefore, more serious than that expressed by the growth rate.

#### Effect of zinc in chlorophyll 'a' concentration

In this investigation chlorophyll 'a' content of the microalgal cultures were estimated on the final day of experiment (15<sup>th</sup> day) for zinc treated cultures as well as for the control. The results declared that the zinc significantly affects the chlorophyll 'a' concentration in all the tested marine microalgal cultures. Among the five microalgae tested, the *Tetraselmis* sp. showed maximum chlorophyll 'a' ( $8.21 \pm 0.97 \mu\text{g/l}$ ) content than rest of the algae. Likewise the lowest chlorophyll of  $0.19 \pm 0.11 \mu\text{g/l}$  was noticed in *Nannochloropsis* sp. at 50 ppm. The overall average chlorophyll 'a' concentration in all the tested zinc concentration was recorded to be higher in *Tetraselmis* sp. ( $4.81 \pm 0.52 \mu\text{g/l}$ ) followed by *Isochrysis galbana* ( $1.80 \pm 0.14 \mu\text{g/l}$ ) and *Chlorella marina* ( $1.11 \pm 0.22 \mu\text{g/l}$ ) as shown in Figure 6.

#### Conclusion

The present study provides clear evidence that the addition of zinc in various concentrations brings about significant differences in the growth of microalgal species due to the differences in their resistances. Our study determined the intervals between the threshold and lethal concentrations of the most dangerous heavy metals for bunch species of marine microalgae and identified the variations in resistance to this metal in various species of microalgae. From the results, we conclude that *Tetraselmis* sp., having the most metal tolerating ability which survives up to 250 ppm of zinc. Except *D. salina* other microalgal species such as *C. marina*, *I. galbana* and *Nannochloropsis* sp., shows resistance up to 50 ppm of zinc concentration. The *D. salina* can survive only 5 ppm zinc concentration. From the above results it could be understood that the marine phytoplankton can survive up to those limits. In view of the increasing contamination of aquatic ecosystems by toxic metals and given the placement of microalgae at the basis of the food chain, ecotoxicological studies encompassing wild strains are of greater importance towards the design of bioremediation strategies and integrated environmental monitoring.

#### ACKNOWLEDGEMENTS

Authors express their thanks to the Head, Department of Marine Science and authorities of Bharathidasan University for the facilities provided. Authors acknowledge Dr. A. R. Thirunavukkarasu, Principal Scientist, Fish Culture Division, Central Institute of Brackishwater Aquaculture, Chennai, for providing algal

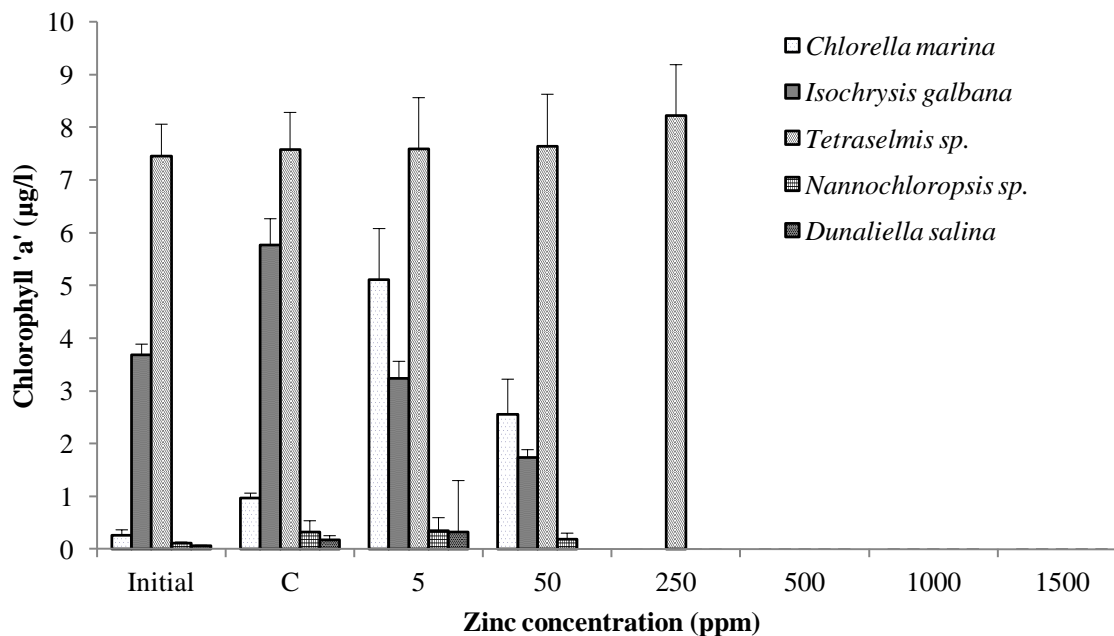


Figure 6. Effect of zinc on the chlorophyll 'a' content of five marine microalgae.

culture for our experiments.

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