Proceedings of the Seventh International Coral Reef Symposium, Guam, 1992, Vol. 1

Effect of Low Levels of Zinc on Zooxanthellae Cells in Culture

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Abstract. The effect of low levels of Zn^{++} on the growth of *Montipora verrucosa* zooxanthellae in culture was investigated. Cells cultured in f/2 enriched seawater media were subjected to Zn^{++} concentrations of up to 4.2 ppm for 20 days. Mean cell densities recorded in controls were significantly higher than the densities of cells under all treatments throughout the experiment. Zinc concentrations of 0.1 ppm and 1.0 ppm were sufficient to cause significantly lowered rates of growth in cultured zooxanthellae up to 4 and 8 days, respectively. Pooled results showed that the treatment concentration of 1.2 ppm Zn^{++} caused significantly lowered rates of growth of cells throughout the experiment. The threshold level at which zooxanthellae cells cultured in f/2 media become sensitive to the heavy metal is between 0.1 ppm and 1.0 ppm up to a period of 8 days. The presence of metal chelators in the growth media used may have rendered the cells a higher threshold tolerance after 8 days.

Introduction

The use of cell cultures in pollution studies is not new. The micro-algal static bioassay has been established as a suitable method for monitoring pollution (Stebbing et al. 1980) and has been employed for the establishment of water quality standards for heavy metals in both marine and freshwater systems (North et al. 1972; Van Coillie et al. 1983). Davies (1978) provides an excellent review of the various types of marine plankton used in heavy metal pollution studies.

Experiments that have been performed on the effects of zinc on cultures of marine algae include the Haptophyceae, diatoms, Chlorophyceae and dinoflagellates (Bernhard and Zattera 1970; Jensen et al. 1974; Rosko and Rachlin 1975; Overnell 1976; Patin 1982). Although zinc is essential in the growth of plants and animals, as a necessary cofactor in many biological processes (Anderson et al. 1978), it is detrimental to cells in higher concentrations, as most toxicity bioassays have shown.

Dinoflagellates (Dinophyceae) have been used fairly extensively in heavy metal toxicity tests (Mandelli 1969; Erickson et al. 1970; Tkachenko et al. 1974; Zingmark and Miller 1975; Kayser 1976; Saifullah 1976; Patin 1982). The symbiotic dinoflagellate (*Symbiodinium* (*Gymnodinium*) *microadriaticum*, a zooxanthellae) is of interest here as it is important in the calcification and reef building process of hard corals (Goreau 1963; Pearse and Muscatine 1971; Vandermeulen and Muscatine 1974).

Heavy metal pollution from industrial effluents and sewage is a real threat to the marine environment, and is receiving increasing attention. Increased industrial development in tropical countries is a pollution threat to their coastal ecosystems including coral reefs (Johannes 1975). Methods of studying the effect of pollutants on coral reefs and of predicting potential impacts include ecosystem community observations, the use of biological (growth rate, cell numbers) and physiological indices (Brown and Howard 1985). The use of zooxanthellae cultures in a heavy metal bioassay has not been employed previously and is explored here. Studying the reaction of zooxanthellae under heavy metal stress may help us better understand the possible impacts metals may have on coral reef ecosystems.

This study investigates the toxic effects of zinc on the growth rates of cultures of zooxanthellae. It

Table 1. Initial and final Zn^{++} concentrations measured from cultures (in ppm)

Treatment	Initial		Final	
	Mean	S.D.	Mean	S.D.
Control	0.05	0.001	0.05	0.005
0.1 ppm	na	na	na	na
1.0 ppm	na	na	na	na
1.25 ppm	1.2	0.1	0.9	0.05
2.5 ppm	2.2	0.04	1.6	0.1
5.0 ppm	4.2	0.1	3.1	0.1

na = not analysed (samples contaminated during analyses)

also attempts to determine the threshold limit of Zn^{++} that zooxanthellae cell cultures can withstand.

Materials and Methods

Axenic cultures of *Symbiodinium microadriaticum* isolated from *Montipora verrucosa* were used in the experiments. Stock cultures were obtained from the Hawaii Institute of Marine Biology, Kaneohe. Cultures were grown in glass culture tubes of 12.5 cm length and 1.6 cm diameter. Experimental glassware were washed in non-phosphate detergent, soaked overnight in 10% nitric acid, thoroughly rinsed with distilled and deionized water and autoclaved before use.

Bioassay nutrient medium consisted of Millipore (0.2 μm) filtered seawater enriched to make f/2 (McLachlan 1973) medium, and was modified from Medium f (Guillard and Ryther 1962). Test solutions were made by adding zinc chloride ($ZnCl_2$) to the medium in various amounts. A preliminary experiment found that acute lethal effects on zooxanthellae cultures were observable between 1.0 ppm and 5.0 ppm of Zn^{++}, therefore test solutions of 0.1 ppm, 1.0 ppm, 1.25 ppm, 2.5 ppm and 5.0 ppm Zn^{++} were used for this study.

All test media were sterilized separately by filtration through sterile 0.2 μm Millipore filters before inoculation with algal cells from the stock culture, to give an initial algal density of approximately 20,000 cells/ml. Aliquots of 8 ml were then placed in sterile culture tubes and lightly capped. Twelve replicate culture tubes were filled for each test concentration and controls, and initial counts were made for each culture tube. All experimental cultures were placed under continuous fluorescent lighting (Duro-test® Power-twist®, 2500 lux) and kept at a constant temperature of 24° C throughout the experiment. Zooxanthellae density in each culture tube was estimated on alternate days.

Seawater samples from the bioassays were taken at the start and end of the experiments and analysed for Zn^{++} using atomic absorption spectrophotometry. This was done to determine actual levels of the metal that the algal cells were exposed to, and background levels in the controls.

Zooxanthellae cell growth was measured in cultures with a haemocytometer. Specific growth rates (SGR) were calculated using the formula given in Greenberg et al. (1981):

$$SGR = \ln(\text{density at day b}/\text{density at day a})/(b - a)$$

SGRs obtained for all treatment cultures were expressed as percentages of control growth rates (or relative specific growth rates) graphically, to illustrate how much the growth of treatment cultures differed from controls.

Cell counts and SGRs obtained were separately analysed using one-way analysis of variance (ANOVA) to determine significant differences in growth between the controls and various treatments. Significant treatment effects were further analysed using Duncan's new multiple range test, an a posteriori test (Duncan 1955) to identify Zn^{++} concentrations that were significantly detrimental to cell growth. Statistical tests were performed with Statistical Analysis System software.

Results

Measured amounts of Zn^{++} in the 1.25 ppm, 2.5 ppm and 5.0 ppm treatment cultures were found to be 1.2 ppm, 2.2 ppm and 4.2 ppm, respectively (Table 1). Control cultures had a mean background concentration of 0.05 ppm Zn^{++}. Treatments of 0.1 ppm and 1.0 ppm were not successfully analysed due to contamination during the atomic absorption analysis process. There was a decrease in the amounts of inorganic Zn^{++} measured at the end of the experiments for all treatments.

Mean algal densities monitored over 20 days are presented in Table 2. Log-phase growth was ob-

Table 2. Mean cell densities and standard deviations obtained from cultures (×10000 cells/ml)

Treatment		Days								
		0	2	4	8	11	13	15	18	20
Control	Mean	est	2.56	3.54	23.42	59.13	79.81	94.47	103.15	105.69
	S.D.	—	0.48	0.72	4.77	11.19	16.49	11.66	9.61	7.83
0.1 ppm	Mean	est	2.04	2.71	16.59	33.48	60.13	75.40	82.52	83.58
	S.D.	—	0.14	0.65	3.28	8.14	17.89	9.60	8.47	7.90
1.0 ppm	Mean	est	est	2.40	12.13	24.90	52.65	70.98	79.04	83.38
	S.D.	—	—	0.34	1.55	4.71	15.94	10.64	7.57	7.14
1.2 ppm	Mean	est	est	est	2.83	3.42	5.46	8.21	20.52	26.82
	S.D.	—	—	—	0.97	0.94	1.56	1.35	4.58	5.12
2.2 ppm	Mean	est	est	est	est	3.94	2.06	2.19	2.50	2.89
	S.D.	—	—	—	—	1.94	0.16	0.44	1.11	1.02
4.2 ppm	Mean	est	est	est	est	est	est	est	est	est
	S.D.	—	—	—	—	—	—	—	—	—

est = estimated density (< 20000 cells/ml) (culture densities not increased from initial inoculation)

served from the fourth day of the experiment in controls, 0.1 ppm and 1.0 ppm cultures, and 1.2 ppm treatment (Fig. 1). At 2.2 ppm Zn^{++}, growth in cultures began at day 8, decreased abruptly at day 11 and seemed to recover again at day 13, whilst at 4.2 ppm Zn^{++}, no growth was observed in cultured zooxanthellae over the entire duration of the experiment. One-way analysis of variance (ANOVA) of density data revealed significant differences in the densities of cells between controls and all treatment regimes from day 2 of the experiment ($F = 14.74$; $df = 4, 55$; $P < 0.0001$). Duncan's new multiple range test indicated that mean cell densities recorded in controls were significantly higher than the densities of cells under the 0.1 ppm and 1.0 ppm treatments, and the 1.2 ppm and 2.2 ppm treatments, respectively ($p<0.05$).

Specific growth rates of cultures were calculated for time intervals of 0–4 days, 4–8 days, 8–11 days, 11–15 days and 15–20 days (Table 3). Maximum specific growth rates were obtained from control, 0.1 ppm, and 1.0 ppm treatment cultures at 4–8 days, 1.2 ppm cultures at 15–20 days and 2.2 ppm cultures at 8–11 days (Table 3). One-way ANOVA on the pooled specific growth rate data of control and treatment cultures observed at all five abovementioned time intervals revealed significant treatment effects ($F = 17.33$; $df = 4, 294$; $p < 0.0001$). Further analysis using Duncan's test indicated that specific growth rates of control, 0.1 ppm and 1.0 ppm treatment cultures did not differ significantly over the duration of the experiment, whilst all three had significantly higher rates of growth as compared to the 1.2 ppm and 2.2 ppm treatment cultures, respectively ($p < 0.05$).

At 0–4 days and 4–8 days, specific growth rates of control cultures were significantly larger than

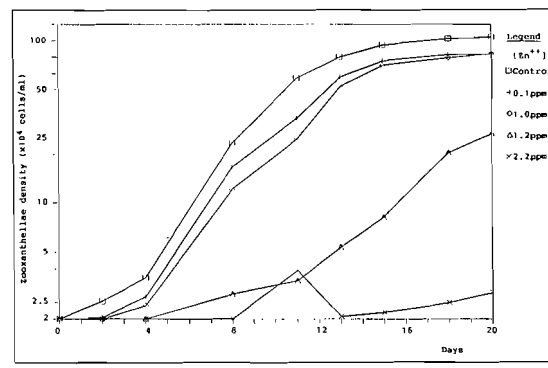

Fig. 1. Zooxanthellae culture density versus days of exposure for various Zn^{++} treatment concentrations

treatments (one-way ANOVA, $F > 27.52$; $df = 4, 55$; $p < 0.0001$). The 0–4 day interval, in particular had cultures under all treatment regimes exhibiting growth rates of less than 50% of control cultures (Fig. 2). Duncan's test on the 4–8 day data indicated that the growth rates of 0.1 ppm cultures had caught up with the controls (i.e. not significantly different) but the growth rates of cultures under the 1.0 ppm. 1.2 ppm and 2.2 ppm regimes were still significantly less than controls ($p<0.05$) (Fig. 3). At the 8–11 day interval, specific growth rates of control, 0.1 ppm and 1.0 ppm treatment cultures were beginning to decrease, while 1.2 ppm and 2.2 ppm treatments were only beginning to go into log-phase growth (Fig. 4). However, growth rates of 1.2 ppm and 2.2 ppm treatment cultures were still significantly lower than controls (Duncan's test, $p<0.05$).

After 11 days, growth rates of control and 0.1 ppm cultures decreased even further as they approached the stationary phase of the growth curve, whilst the growth rate of cells cultured under the

Table 3. Mean specific growth rates and standard errors calculated from cell cultures

Treatment		Days 0–4	4–8	8–11	11–15	15–20
Control	Mean	0.14	0.47	0.31	0.12	0.02
	S.E.	0.01	0.02	0.02	0.01	0.01
0.1 ppm	Mean	0.07	0.46	0.23	0.21	0.02
	S.E.	0.02	0.02	0.02	0.01	0.01
1.0 ppm	Mean	0.04	0.41	0.24	0.26	0.03
	S.E.	0.01	0.01	0.02	0.02	0.01
1.2 ppm	Mean	nc	0.07	0.07	0.23	0.23
	S.E.	—	0.02	0.03	0.02	0.01
2.2 ppm	Mean	nc	nc	0.19	−0.12	0.05
	S.E.	—	—	0.05	0.04	0.02
4.2 ppm	Mean	nc	nc	nc	nc	nc
	S.E.	—	—	—	—	—

nc = not calculated

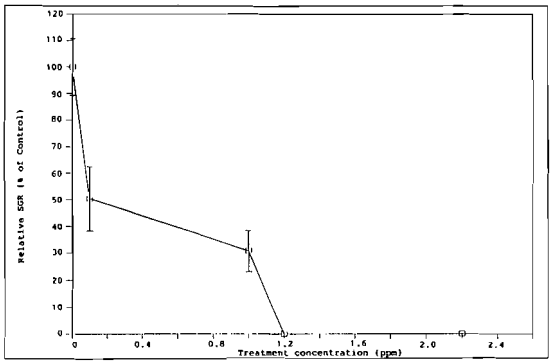

Fig. 2. Mean specific growth rates (SGR) of zooxanthellae cultures (expressed as percentages of controls ± S.E.) for various Zn^{++} treatments during the 0–4 day interval

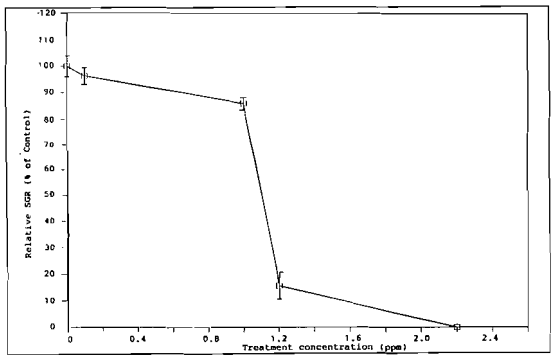

Fig. 3. Mean specific growth rates (SGR) of zooxanthellae cultures (expressed as percentages of controls ± S.E.) for various Zn^{++} treatments during the 4–8 day interval

1.2 ppm treatment regime slowly increased. At this point it became meaningless to carry out ANOVA tests on the specific growth rates as control cultures were already on the decline.

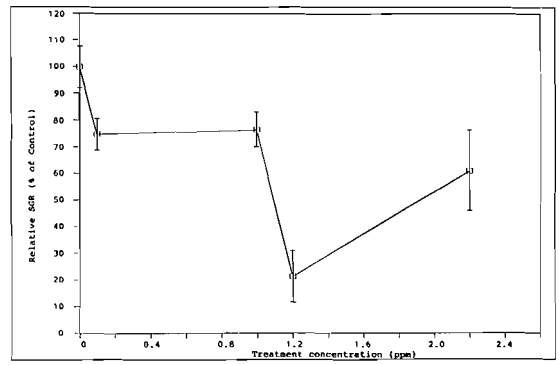

Fig. 4. Mean specific growth rates (SGR) of zooxanthellae cultures (expressed as percentages of controls ± S.E.) for various Zn^{++} treatments during the 8–11 day interval

Discussion

Anderson et al. (1978) noted that growth limitation observed in cultured algae may be due to low zinc activity in the media rather than toxic effects of the metal. They computed that Medium f/2 had a zinc ion activity of $10^{-10.7}$M ($\simeq$ 6.5 x 10^{-7} ppm) which was not growth limiting. The depressed specific growth rates in all treatments observed in our study therefore indicate direct toxic effects.

In this study, Zn^{++} concentrations of 0.1 ppm and 1.0 ppm caused significantly depressed zooxanthellae growth during early log-phase growth. Pooled data also showed that a treatment concentration of 1.2 ppm significantly lowered growth rates throughout the duration (20 days) of the experiment. This indicates that the threshold level at which zooxanthellae cells cultured in f/2 media become sensitive to the heavy metal is between 0.1 ppm and 1.0 ppm up to a period of 8 days. The results here are in direct agreement with results ob-

tained from work on the effect of zinc on the dinoflagellate *Gyrodinium fissum*. Patin (1982) reported toxic Zn^{++} levels of 1 to 10 ppm and threshold levels of 0.1 to 1.0 ppm for the dinoflagellate.

The results of this study also correspond to research investigating the effects of zinc on other marine algae. Chipman et al. (1958) obtained reduced growth rates of *Nitzschia* sp. at a concentration of 0.25 ppm. Jensen et al. (1974) using dialysis cultures found large differences in the zinc tolerance of three species of diatoms. The relative growth rates of *Skeletonema costatum, Thalassiosira pseudonana* and *Phaeodactylum tricornutum* were found to decrease at and above zinc concentrations of 0.05 ppm, 0.25 ppm and 25 ppm, respectively. Rosko and Rachlin (1975) reported a 50% decrease (EC_{50}) in the growth rate of the diatom *Nitzschia closterium* exposed to 0.271 ppm Zn^{++} for 96 hr. Similarly, Subramanian et al. (1980) documented tolerance levels of up to 0.3 ppm zinc for short term experiments on *Nitzschia longissima*, and 0.2 ppm zinc for *Skeletonema costatum*. Hollibaugh et al. (1980) reported that concentrations of Zn^{++} above 300nM ($\simeq$0.02 ppm) were toxic to phytoplankton communities in their experiments.

Chelators reduce the toxicity of several heavy metals (Spencer 1957; Droop 1960; Steemann Nielsen and Wium-Andersen 1971). Stauber and Florence (1989) documented that Medium f containing metal complexing chelators like silicate, iron and disodium ethylenediaminetetraacetate (EDTA) reduced the toxic effect of zinc on cultures of *Nitzschia closterium* 10 to 20 fold compared to unenriched seawater. This study used Medium f/2 in the presence of EDTA, and results obtained here may not be fully representative of the actual sensitivity of cultured zooxanthellae. We observed a slight drop in treatment concentrations when the cultures were analysed at the end of the experiment (Table 1). This is most likely due to the uptake of the metal by cells for growth (Davies 1973, 1978). Adsorption of the metal onto culture tube surfaces (Robertson 1968; Hennig and Greenwood 1981) is unlikely owing to the presence of the EDTA in the medium (Davies 1978). Our observation that the toxic effects of 0.1 and 1.0 ppm treatments only affected the growth rate of the cells for 4 and 8 days respectively, may be attributed to the presence of EDTA, rendering the metal less available to the cultured cells. It may well be that the the actual threshold level of sublethal stress to zooxanthellae in the sea is lower than the 0.1 to 1.0 ppm Zn^{++} range obtained here.

Analysis of estuarine and coastal seawater in Singapore has revealed Zn^{++} concentrations of 0.022 ppm (Sin et al. 1991) to 0.14 ppm (pers. observation). Published average concentrations of zinc in near-shore surface waters that are relatively free of pollution range from 0.0012 ppm to 0.004 ppm (Chester and Stoner 1974). In areas affected by pollution and terrestrial runoff, zinc levels of up to 0.05 ppm (open oceans) and 3.56 ppm (estuarine) have also been encountered (Phillips 1977). Zinc measured in the waters of Singapore may possibly be from terrestrial inputs and other forms of marine pollution. These concentrations that our coral reefs are subjected to are not at levels that could be detrimental to zooxanthellae growth.

Overnell (1976) studied the effect of $ZnSO_4$ on the rate of photosynthesis of several marine algae and discovered that levels of up to 10^{-3}M ($\simeq$65.4 ppm) of the metal did not significantly reduce photosynthesis in the algae, concluding that the toxic effect of zinc was not exerted on photosynthesis but on some other part of cell metabolism, for example cell division. Similarly, Stauber and Florence (1990) found that photosynthesis and respiration in *Nitzschia closterium* were unaffected by zinc concentrations up to 0.5 ppm, but a mere concentration of 0.065 ppm halved cell division rates. They postulated that chemical energy from the algal cells was channelled to the zinc-thiol (SH) detoxification process triggered by elevated concentrations of the heavy metal, at the expense of cell division or growth.

In conclusion, this study has established that the threshold level of sublethal stress of Zn^{++} to cultured cells of zooxanthellae is between 0.1 and 1.0 ppm up to 8 days of culture. Chelators present in the growth media may render cultured cells more resistant to the metal over a longer exposure time. Zinc levels in the waters surrounding coral reefs in Singapore are within safe limits to the survival of the symbiotic coral zooxanthellae. This study has also shown that the growth rate of zooxanthellae cells in culture is a suitable index to be used in pollution studies to monitor effects of sublethal stress on marine algae.

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