EFFECTS OF THE HEAVY METALS COPPER AND ZINC ON ZOOXANTHELLAE CELLS IN CULTURE

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Abstract. The growth of symbiotic algal cells (zooxanthellae) isolated from the coral, *Montipora verrucosa* under the influence of the heavy metals copper (Cu) and Zinc (Zn) was investigated. Zooxanthellae cells cultured in f/2 enriched seawater medium were subjected to a maximum Cu level of $42 \mu g.l^{-1}$, Zn concentration of $509 \mu g.l^{-1}$, and various combinations of the two metals, in ecotoxicological bioassays lasting up to 28 days. A Cu level of $40 \mu g.l^{-1}$ caused significantly depressed specific growth rates of cell cultures obtained using a standard growth model. Low concentrations of the metals Cu and Zn in combination elicited synergistic effects of sublethal toxicity. The use of cultured zooxanthellae cells in bioassays investigating sublethal effects of heavy metal stress has relevant applications in the field of pollution monitoring.

1. Introduction

The micro-algal culture technique recommended by Stebbing *et al.* (1980) has been employed in many pollution studies, as well as in 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 use of marine plankton cultures in heavy metal pollution studies.

With respect to the study on the toxic effects that Cu and Zn have on cultures of marine algae, several kinds of algae have been employed. Algae used in heavy metal work include the blue-green algae, red algae, dinoflagellates, Haptophyta, diatoms, as well as mixed phytoplankton communities from the open ocean (Chipman *et al.*, 1958; Marvin *et al.*, 1961; Mandelli, 1969; Aleem, 1970; Erickson *et al.*, 1970; Steeman Nielson & Wium-Anderson, 1970; Erickson, 1972; Hannan & Patouillet, 1972; Davey *et al.*, 1973; Jensen *et al.*, 1974, 1976; Donnier, 1975; Rosko & Rachlin, 1975; Zingmark & Miller, 1975; Berland *et al.*, 1976; Kayser, 1976; Overnell, 1976; Saifullah, 1976; Sunda & Guillard, 1976; Bentley-Mowat & Reid, 1977; Davies & Sleep, 1979; Patin, 1982).

The dinoflagellate, *Symbiodinium (Gymnodinium) microadriaticum* commonly called zooxanthella, is symbiotic with reef building scleractinian corals, as well as other coelenterates and bivalves, and can be cultured successfully in enriched seawater media. Zooxanthellae are important to coral reefs, playing an essential role in the calcification and reef building process of hard corals (Goreau, 1963; Pearse & Muscatine, 1971; Vandermuelen & Muscatine, 1974). Owing to their importance in coral calcification, the use of zooxanthellae in heavy metal bioassays may increase our understanding of the possible impacts metal pollutants may have on coral reefs.

To date, the use of zooxanthellae cultures in a heavy metal bioassay has been employed only once (Goh & Chou, 1993) in a study of the effects of Zn on the growth rates of cell cultures. This study further explores the use of zooxanthellae cultures in heavy metal pollution

bioassays and looks into the toxic effects of Cu. It also examines the effects of combining the metals Cu and Zn on cell cultures, a situation that is environmentally realistic, where various pollutants are present in combination.

2. Materials and Methods

Axenic cultures of zooxanthellae *Symbiodinium microadriaticum* isolated from the coral, *Montipora verrucosa* were used in the static bioassays. Zooxanthellae cells were grown in glass culture tubes of 12.5cm length and 1.6cm diameter. All glassware was cleaned with non-phosphate detergent and 10% nitric acid, thoroughly rinsed with deionized water and autoclaved. The f/2 culture medium used for the bioassays was made up of membrane (0.2μm) filtered seawater enriched with various nutrients (Guillard & Ryther, 1962; McLachlan, 1973)

In the bioassays using only Cu as the toxic agent, two experiments were set up to determine if metal chelators present in the culture medium affected the level of toxicity of Cu to zooxanthellae cells. The first experiment was prepared using regular f/2 media and the other, a modified f/2 medium prepared by removing ingredients that were known to be able to chelate metals, namely Na_2EDTA and $NaSiO_3.9H_2O$. A preliminary experiment indicated that acute lethal effects on cultured zooxanthellae could be observed with a Cu concentration of 50 $\mu g.l^{-1}$. Treatments were therefore prepared with Cu concentrations of 20 $\mu g.l^{-1}$ and 40$\mu g.l^{-1}$, respectively, using $CuSO_4.5H_2O$ (AnalaR; Merck) added to the media. Four replicate culture tubes were observed for each treatment concentration and controls over 23 days.

In the bioassays combining both Cu and Zn (Cu + Zn), treatments were prepared to determine if the two metals acted synergistically or antagonistically, and the following combinations were tested: 10 $\mu g.l^{-1}$ Cu with 500 $\mu g.l^{-1}$ Zn, and 20 $\mu g.l^{-1}$ Cu with 100 $\mu g.l^{-1}$ Zn, respectively. In addition, a further bioassay using 500 $\mu g.l^{-1}$ Zn alone was also tested, as the growth rates of zooxanthellae cultures exposed to this treatment concentration has not previously been reported. Both $CuSO_4.5H_2O$ (AnalaR; Merck) and $ZnSO_4.7H_2O$ (Pro analysi; Merck) were used in the preparation of the treatments, together with regular f/2 media. Seven replicate treatments and controls were monitored in the 28 day bioassay.

All test media were sterilized separately by filtration through sterile 0.2μm Millipore filters before inoculation with algal cells from the stock culture, to give an initial algal density of > 2.0 x 10^4 zooxanthellae $cells.ml^{-1}$ of media (Goh & Chou, 1993). Aliquotes of 8ml were transferred into sterile culture tubes and lightly capped. After initial cell counts were made for each culture tube, experimental cultures were placed under continuous fluorescent lighting (Duro-test®, Power-twist®, 2500 lux) at a constant temperature of 24°C throughout the experiment (Goh & Chou, 1993).

Samples of seawater in all treatment and control bioassays were taken at the start and end of each experiment and analyzed for heavy metals using a Hitachi Atomic Absorption Spectrophotometer with Zeeman correction. This heavy metal analysis procedure was performed in order to determine the actual metal levels that the cell cultures were exposed to, as well as background metal levels in control cultures.

Zooxanthellae density in each culture tube was estimated on alternate days by haemocytometer counts under a phase contrast microscope. The specific growth rate (SGR) of each cell culture was calculated with the aid of a SYSTAT software package, using the

logistic growth formula given in Pielou (1977),

$$N_t = K/\{1 + \exp[-r(t-t_0)]\}$$

where N_t = cell density at time t, K = carrying capacity of culture medium and r = specific growth rate of cell culture.

SGRs obtained for all treatment cultures were expressed in terms of the percentage of control SGRs (relative SGR) to illustrate how growth rates of treatments differed from controls. SGRs of cell cultures under the different metal treatments were statistically analyzed using one-way analyses of variance (ANOVA) to determine significant treatment effects of metals on zooxanthellae growth. Statistically significant treatment effects were further analyzed using Duncan's new multiple range test (Duncan, 1955) to identify how sensitive the cultured zooxanthellae cells were to threshold concentrations of metals (Goh & Chou, 1993). All statistical tests were performed with Statistical Analysis System software.

3. Results

Analyses of seawater from control cultures in the Cu bioassays revealed that background levels of the metal were < 5 $\mu g.l^{-1}$ Cu throughout the experiment. Measured concentrations of Cu in treatment cultures are given in Table 1. At the end of the bioassays, a decrease in Cu concentration was observed in treatment cultures that did not contain metal chelators. In the Cu + Zn bioassays, control cultures were found to contain < 4 $\mu g.l^{-1}$ of Cu and < 23 $\mu g.l^{-1}$ of Zn, respectively (Table 1). Treatments of 11 $\mu g.l^{-1}$ Cu + 506 $\mu g.l^{-1}$ Zn and 21 $\mu g.l^{-1}$ Cu + 82 $\mu g.l^{-1}$ Zn were initially analyzed from solutions sampled from experimental assays testing the toxicity of Cu and Zn in combination (Table 1). There was also a decrease in the concentrations of Cu and Zn measured in all solutions at the end of the experiment.

TABLE 1

Initial and final metal concentrations measured from cultures ($\mu g.l^{-1}$)

Treatment		Metal measured	Metal concentration ($\mu g.l^{-1}$) Initial Mean	Initial S.D.	Final Mean	Final S.D.
Cu bioassays						
Control	(+)	Cu	3.2	0.6	4.4	2.8
$20\mu g.l^{-1}$	(+)	Cu	20.2	0.6	20.8	0.4
$40\mu g.l^{-1}$	(+)	Cu	40.0	---	40.9	---
Control	(-)	Cu	4.8	---	3.0	---
$20\mu g.l^{-1}$	(-)	Cu	20.1	---	16.1	---
$40\mu g.l^{-1}$	(-)	Cu	41.8	---	11.4	---
Cu + Zn bioassays						
Control		Cu	3.7	---	1.4	---
$10\mu g.l^{-1}$ Cu + $500\mu g.l^{-1}$ Zn		Cu	11.3	1.8	7.4	0.2
$20\mu g.l^{-1}$ Cu + $100\mu g.l^{-1}$ Zn		Cu	21.0	4.2	16.0	0.4
Control		Zn	22.8	0.3	26.2	2.1
$500\mu g.l^{-1}$ Zn		Zn	509.4	22.2	338.5	---
$10\mu g.l^{-1}$ Cu + $500\mu g.l^{-1}$ Zn		Zn	506.0	---	213.3	11.4
$20\mu g.l^{-1}$ Cu + $100\mu g.l^{-1}$ Zn		Zn	82.1	3.1	67.3	12.4

Note: (+) = cultured in the presence of metal chelators

(-) = cultured in the absence of metal chelators

--- = no replicate analyses carried out

In the bioassays testing Cu alone, a lag-phase of 5 days was observed in all cultures, during which cells did not increase beyond the initial density of 3.3×10^4 cells.ml^{-1} (Fig. 1). In cultures prepared with chelators, controls were seen to revert quickly to log-phase growth from day 5, followed by the 20 μg.l^{-1} Cu bioassay. A final mean density of > 10^6 cells.ml^{-1} was attained in control cultures with metal chelators (Fig. 1). Zooxanthellae cells cultured in the absence of chelators were not observed to increase to great numbers, only managing to reach an approximate density of 23.0×10^4 cells.ml^{-1} after 23 days (Fig. 1). From day 17, cells cultured in the presence of metal chelators were observed to approach the stationary phase of the growth curve, while bioassay cultures without metal chelators continued to increase at a very slow rate. The mean specific growth rate calculated from control cultures using the modified media excluding metal chelators was 0.0884, compared with the mean specific growth rate of 0.2253, in controls cultured in the presence of chelators (Table 2).

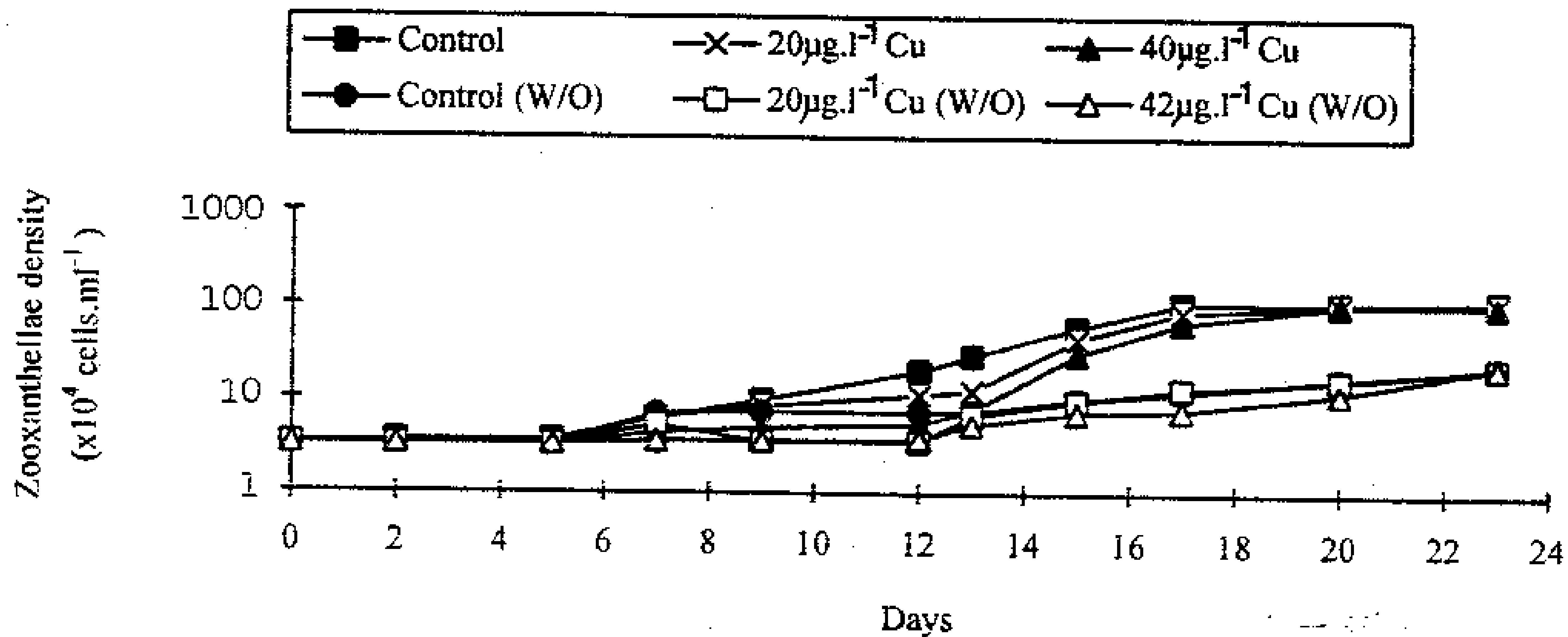

FIGURE 1

Zooxanthellae culture density versus days of exposure for the various Cu treatment concentrations (W/O indicates growth curves prepared in the absence of metal chelators. All other curves are from cultures prepared with metal chelators)

TABLE 2

Mean specific growth rates (and standard deviations) calculated from cell cultures subjected to Cu toxicity

Treatment	Specific growth rate Mean	S.D.
Control (+)	0.2253	0.0076
20μg.l^{-1} (+)	0.1698	0.0630
40ug.l^{-1} (+)	0.1224	0.0767
Control (-)	0.0884	0.0065
20ug.l^{-1} (-)	0.0826	0.0288
42ug.l^{-1} (-)	0.0652	0.0338

Note: (+) = cultured in the presence of metal chelators
(-) = cultured in the absence of metal chelators

TABLE 3

Mean specific growth rates (and standard deviations) calculated from cell cultures subjected to Cu and Zn toxicity

Treatment	Specific growth rate Mean	S.D.
Control	0.2337	0.0064
509μg.l^{-1} Zn	0.2253	0.0129
11μg.l^{-1} Cu+506μg.l^{-1} Zn	0.2150	0.0082
21μg.l^{-1} Cu - 82μg.l^{-1} Zn	0.2150	0.0196

Specific growth rates of control and treatment cultures estimated from the logistic model and analyzed using one - way ANOVA revealed significant treatment effects ($F = 6.71$; $df = 5, 18$; $p < 0.0006$). Bioassays carried out with media containing metal chelators yielded significant differences in specific growth rates between the 40 $\mu g.l^{-1}$ Cu treatment cultures and control cultures in Duncan's new multiple range test ($p < 0.05$) (Fig. 2). All cell cultures with media prepared excluding metal chelators had significantly lower specific growth rates as compared to cultures prepared in the presence of metal chelators ($p < 0.05$). Also, there was no statistical difference in the specific growth rates of controls and treatments without metal chelators ($p > 0.05$; Fig. 2). The threshold concentration at which zooxanthellae cell cultures become susceptible to the metal Cu is therefore between 20 $\mu g.l^{-1}$ and 40 $\mu g.l^{-1}$.

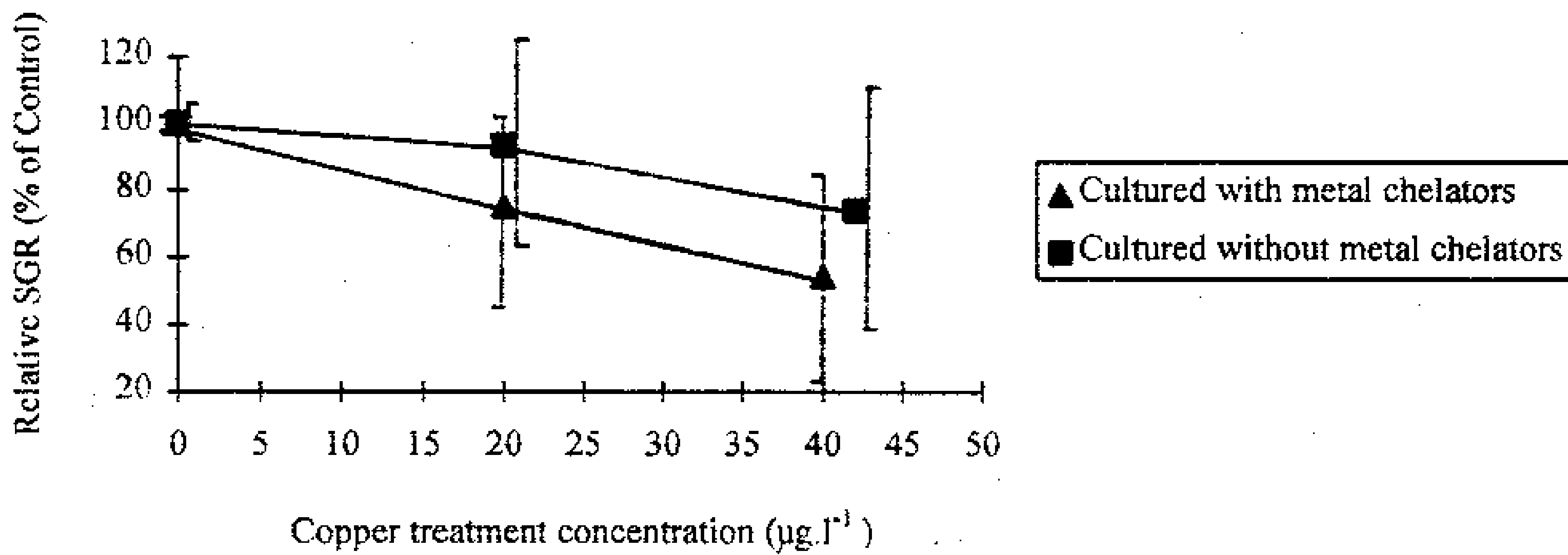

FIGURE 2
Mean specific growth rates (SGR) of zooxanthellae cultures (expressed as percentages of controls ± S.D.) for various Cu treatments

In the Cu + Zn bioassays, individual growth curves obtained from cultures exhibited a lag-phase of 5 days (Fig. 3). Control and treatment cell cultures began to approach the stationary phase of the logistic growth curve from day 21 onwards.

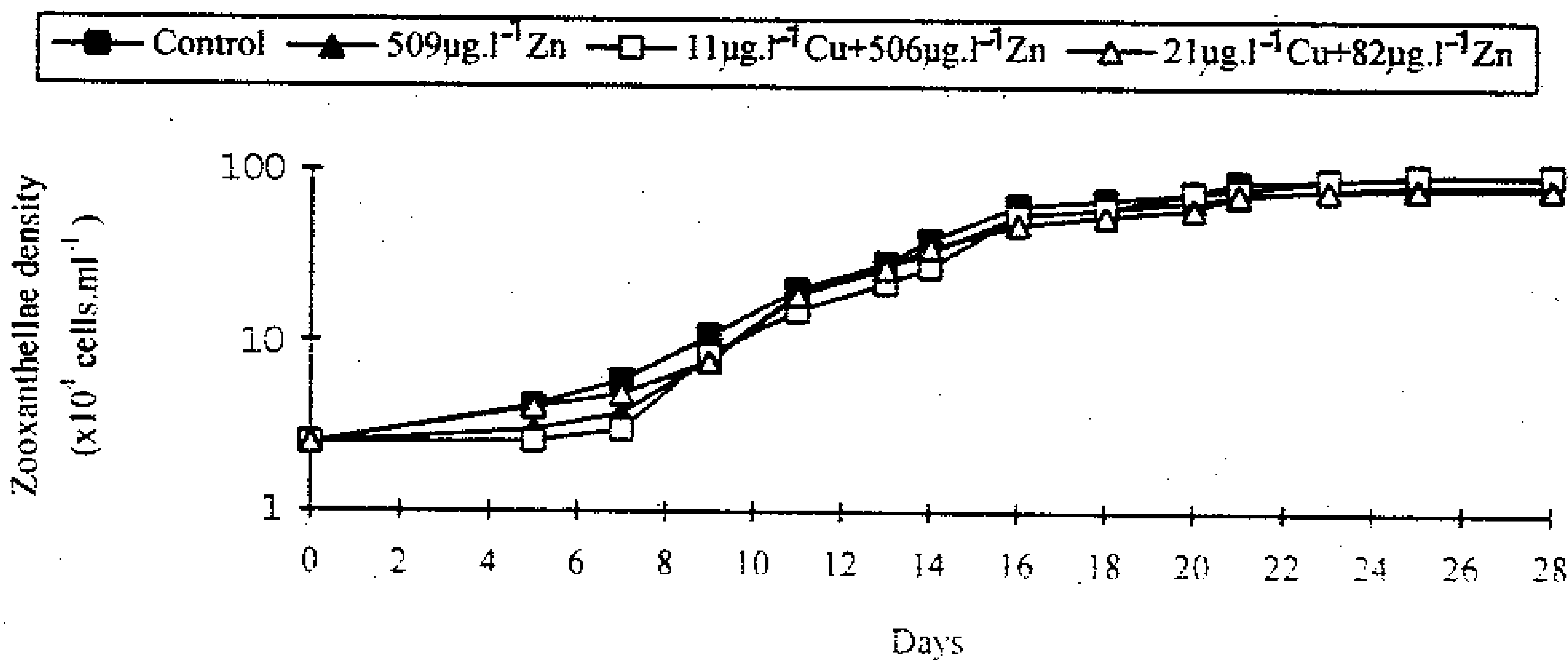

FIGURE 3
Zooxanthellae culture density versus days of exposure for the various treatment concentrations of Cu and Zn

One-way ANOVA on the specific growth rates of control and treatment cultures revealed significant treatment effects ($F = 3.08$; $df = 3, 24$; $p < 0.05$). Duncan's test indicated that specific growth rates of cells subjected to exposure to 509 μg.l^{-1} Zn were not significantly different from controls ($p > 0.05$; Table 3), but zooxanthellae cells grown in the two treatments employing a combination of both Cu and Zn had significantly lower specific growth rates compared to control cultures ($p < 0.05$) (Fig. 4).

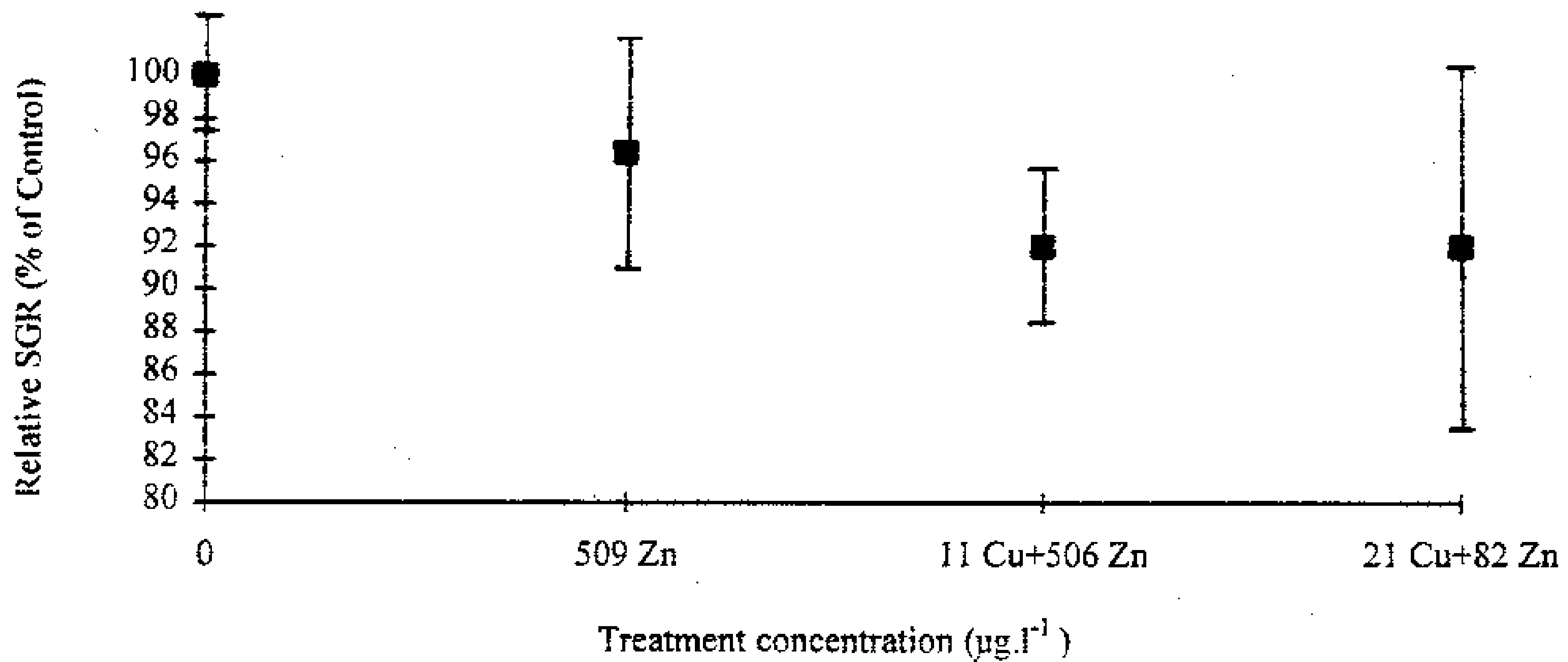

FIGURE 4
Mean specific growth rates (SGR) of zooxanthellae cultures (expressed as percentages of controls ± S.D.) for various treatments using both Cu and Zn

4. Discussion

Although chelators have been reported to reduce the toxicity of several heavy metals to phytoplankton (Spencer, 1957; Droop, 1960; Davey *et al.*, 1970; Steeman Nielsen & Wium-Anderson, 1971; Sunda & Guillard, 1976; Fisher & Frood, 1980), this study has shown that they cannot be excluded entirely from the culture medium. Stauber & Florence (1989) documented that the usage of Medium f containing metal complexing chelators like silicate and disodium EDTA caused a 10 to 20 fold reduction in the toxic effect of Zn on cultures of *Nitzschia closterium* as compared to unenriched seawater. The low nutrient medium used was sufficient to support algal growth in a 4-day assay (Stauber & Florence, 1989). It may be true that chelators serve to bind excess metals that may have toxic effects on cultured cells, and thus serve to regulate the metal concentrations in cultures. In this study, however, only Cu treatments with chelators present were shown to exhibit significantly reduced growth rates compared with controls.

The threshold level of 20 μg.l^{-1} to 40 μg.l^{-1} Cu to cultured zooxanthellae obtained here correspond with Cu thresholds reported by other researchers, for different species of cultured dinoflagellates. Mandelli (1969) documented toxic Cu limits of 30 μg.l^{-1} to 55 μg.l^{-1} to *Glenodinium* sp., 25 μg.l^{-1} to 45 μg.l^{-1} to *Exuviaella* sp., and 30 μg.l^{-1} to 35 μg.l^{-1} to

Glenodinium foliaceum. Similarly, Marvin *et al.* (1961) discovered that 30 $\mu g.l^{-1}$ Cu was toxic to the dinoflagellate, *Gymnodinium breve*, while Erickson *et al.* (1970) reported that 100 $\mu g.l^{-1}$ Cu reduced the cell population of *Amphidinium carterae* to 7% that of controls. Berland *et al.* (1976) also documented that 25 $\mu g.l^{-1}$ Cu was sufficient to cause growth inhibition in *Amphidinium carterae*, and 10 $\mu g.l^{-1}$ Cu caused growth inhibition in cultures of *Exuviaella mariaelebouriae*.

The two metals Cu and Zn were found to act synergistically in their toxicity to zooxanthellae cultures, where increased growth inhibition (as a percentage of the controls) was observed, compared to experiments using the metals individually. Research conducted on other marine phytoplankton species confirms this finding (Bræk *et al.*, 1976; 1980). Bræk *et al.* (1976) reported that Cu and Zn acted synergistically to inhibit the growth of the dinoflagellate, *Amphidinium carterae*, and the diatoms, *Skeletonema costatum* and *Thalassiosira pseudonana* cultured in enriched seawater media, while the metals acted antagonistically to each other in affecting the growth of the more resistant diatom, *Phaeodactylum tricornutum*. Zn and Cd were also documented to act synergistically to *Skeletonema costatum* and *Thalassiosira pseudonana* growth.

Increasing concentrations of Cu in this study did not elicit an obvious extension of the lag-phase in cell cultures reported in various publications (Steeman Nielsen & Wium-Anderson, 1970; Bartlett *et al.*, 1974; Zingmark & Miller, 1975; Metaxas & Lewis, 1991). This extended lag-phase has been attributed to a modification of the culture medium in the initial period of culture by chelating exudates produced by living cells, or leachates from dead cells (Metaxas & Lewis, 1991). The chelating agents apparently bind metals in the culture, making them less toxic to surviving cells (Bræk *et al.*, 1976). In this study, Cu toxicity was not observed to increase the lag-phase periods in cell cultures, but significant reductions in specific growth rates were obvious.

Treatment culture media analyzed at the end of the bioassays were observed to have reduced heavy metal concentrations (Table 1). This could most likely be attributed to the uptake of the elements by cells for growth (Davies, 1973, 1978). Adsorption of treatment metals onto culture tube surfaces (Robertson, 1968; Hennig & Greenwood, 1981) was unlikely in cultures using regular f/2 media with metal chelators, owing to the presence of EDTA in the medium (Davies, 1978). Some adsorption onto cell and culture tube surfaces might have taken place in Cu treatments prepared without metal chelators.

In conclusion, this study has established that the threshold level of sublethal stress of Cu to cultured zooxanthellae is between 20 $\mu g.l^{-1}$ and 40 $\mu g.l^{-1}$. The metals Cu and Zn elicit a synergistic effect in the reduction of the specific growth rates of zooxanthellae cultures. Metal chelators included in the ingredients for Medium f/2 are essential to normal cell division and culture growth. The specific growth rate of zooxanthellae cells in culture is a suitable index to be used in the study of sublethal stress due to heavy metals.

Acknowledgements

The first author would like to thank the Hawaii Institute of Marine Biology, Kaneohe, for the supply of stock cultures of zooxanthellae obtained from *Montipora verrucosa* corals, and Dr. Anthony Cheshire, University of Adelaide, Australia, for the formulation of the logistic growth equation on SYSTAT.

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