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Influence of Cellular Density on Determination of EC_{50} in Microalgal Growth Inhibition Tests

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Growth inhibition tests for copper were carried out on four marine microalgal species: *Chlorella autotrophyca*, *Nannochloris atomus* (Chlorophyceae), *Phaeodactylum tricornutum* (Bacillariophyceae), and *Isochrysis* aff. *galbana* (Primnesiophyceae). The test initial cellular densities were reduced to 50 and 10% from the recommended initial cellular density in most of standardized assays. OECD test protocol (originally described for freshwater) was adapted for seawater. The EC_{50} values were reduced when initial cellular density decreased. The green algae used in this study exhibited lower sensitivity than *P. tricornutum* and quite lower than *I.* aff. *galbana*. The latter species was found to be very sensitive to copper. The concept of cellular toxic quote (amount of toxic per cell) is defined in order to improve the results of toxicity tests. © 2000 Academic Press

Key Words: **toxicity test; marine microalgae; EC_{50}; heavy metals.**

INTRODUCTION

Several heavy metals are essential for living beings at very low concentrations, but at higher doses most of them are toxic for organisms belonging to different levels of the trophic chain (Warnau *et al*., 1995). Presence of heavy metals normally increases near shorelines (de Filippis and Pallaghy, 1994), and in some cases ecological disasters, as occurred at Aznalcóllar mine near Doñana natural park southwest of Spain, induce quite high levels of these toxicants in coastal waters. Mine acid waste waters containing very high concentrations of heavy metals were accidentally dropped to the Guadiamar river, a tributary of the Guadalquivir river. Part of the toxic sludge was removed, but an increase of heavy metal levels in Guadalquivir estuary is unavoidable. The primary producers in seawater are microalgae, and heavy metals affect these organisms by enzyme inactivation, cellular transport interference, and/or interference with major nutrient assimilation (Price and Morel, 1994). Copper is especially toxic to aquatic biota (Sadiq, 1992), mainly phytoplankton (Riisgård *et al*., 1980; Barón *et al*., 1995). Recently, some authors presented evidence of the biomagnification processes for copper in trophic chains (Edding and Tala, 1996).

Freshwater microalgae are used more frequently in laboratory toxicity tests than any other type of aquatic plant (Nalewajko and Olaveson, 1988), but there are few tests that can be described as "standard" in marine environments, although some of them have been used more than others (Walsh, 1993). Green algae and diatoms are the most commonly used microalgae in marine toxicity tests (Walsh, 1993). *Phaeodactylum tricornutum* has been proposed by some authors as a standard organism for seawater toxicity tests (Adema *et al*., 1980). On the other hand, Walsh (1993) report many tests using *Thalassiosira pseudonana* or *Skeletonema costatum*, but he recognized that *Minutocellus polimorphus* was much more sensitive to 19 organic and inorganic toxicants than the two species mentioned above. In addition, *M. polimorphus* grow faster, allowing a reduction of the assay time from 72 to 48 h. OECD (1984), recommends, as do many other microalgal tests, an initial cellular concentration for growth inhibition tests of 10^4 cells mL^{-1}. Wong and Couture (1986) recommend an algal inoculum of approximately 10^5 cells mL^{-1}. There is evidence that the sensitivity of a toxicity test increases if initial cellular density decreases (Moreno-Garrido, 1997). Therefore, a maximum of 10^4 cells mL^{-1} was used in this experiment. This is the lowest cellular concentration able to be counted with the Neubauer cell count. But electronic particle counters, such as the Coulter counter, allow the possibility to count lower cellular densities. This allows designing an experiment involving cellular concentrations of 5×10^3 and 10^3 cells mL^{-1} and comparison with OECD recommended initial cellular density. Diatoms such as *S. costatum* or *T. pseudonana* are not good target organisms if electronic

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counts are performed, due to the possibility of the occurrence of cellular chains.

Calculation of EC_{50} for biomass (cellular density) was chosen instead calculation of EC_{50} for growth rate because in the latter only data corresponding to the exponential phase of the curves can be used. Thus, calculation of EC_{50} for biomass presents fewer sources of variation.

In the present work, four microalgal species belonging to three different taxonomic classes have been used in order to test the influence of initial cellular density on copper toxicity in phylogenetically separated species.

MATERIAL AND METHODS

Culture media. Artificial seawater was used in these experiments. The formulation was modified from the ASTM ocean water formulation. This modified artificial seawater presents a salinity value near 30. All cultured species used in this experience normally grow in natural seawater at a salinity value of 36. Therefore, a balanced increase of all components of ASTM ocean water was developed in order to reach this salinity value. Formulation is presented in Table 1. All chemicals used were reagent grade, solved in ultrapure Milli-Ro water. Obtained artificial seawater was enriched with a modification of f/2 medium (Guillard and Ryther, 1962), lacking EDTA. It has been demonstrated that EDTA greatly decreases the toxicity of heavy metals (Sunda and Guillard, 1976; Moreno-Garrido *et al.*, 1997) due the chelating properties of the molecules. After stirring all components, obtained artificial seawater was filtered through 0.45-μm-mesh membrane filters (Millipore) and used immediately. This medium presents a pH value of 8.0 ± 0.1.

Microalgal cultures. All species used in this experience [*Chlorella autotrophyca*, *Nannochloris atomus* (Chlorophyceae), *Phaeodactylum tricornutum* (Bacillariophyceae), and *Isochrysis* aff. *galbana* (Primnesiophyceae)] were obtained from the Marine Microalgal Culture Collection in the Instituto de Ciencias Marinas de Andalucía (Lubián and Yúfera, 1989) and precultured for a minimum of 1 month in artificial seawater supplemented with complete enriched f/2 medium. Inocula were obtained from exponentially growing cultures, centrifuged slightly, and resuspended in test medium at the needed initial cellular densities. Counting of desired initial cellular concentrations and daily counting during the tests were performed using a Coulter counter with a 50-μm-hole tube. A preliminary experiment was also carried out counting cells by means of a Neubauer chamber (hematocytometer).

TABLE 1
Composition of Artificial Seawater Used

Reagent	g L^{-1}
NaCl	27.068
$MgCl\ 6H_2O$	12.252
$NaSO_4$	4.513
$CaCl_2\ 2H_2O$	1.695
KCl	0.767
$NaHCO_3$	0.222
KBr	0.111
$SrCl_2$	0.046
H_3BO_3	0.029
NaF	0.004

Test conditions. Tests were carried out in a culture chamber (Koxka), at $24 \pm 0.1°C$, under continuous white light ($300\ \mu E^{-2}s^{-1}$). Spherical flasks of 250-mL volume were used for keeping 100 mL of test medium volume, and topped with transpirable tops. The OECD algal growth inhibition test guideline for testing chemicals was closely followed (OECD, 1984), although medium composition and recommended species were necessarily changed in order to adapt this test to seawater. A wide range concentrations in a previous test was developed in order to find the adequate range of toxicity for each microalgal species at the recommended initial cellular concentration (10^4 cells mL^{-1}). Then, similar test doses were tested with initial cellular concentrations of 10^4, 0.5×10^4, and 10^3 cells mL^{-1}. All experiments were performed in triplicate. Two types of control were disposed: flasks with microalgae but no metals (growing controls) and flasks without algae or metals (background controls). The latter were established in order to determinate whether a significant number of particles appeared in the media with time. Flasks were alternately disposed in the culture chamber and shaken two times each day by hand. All glassware was cleaned with nitric acid and rinsed several times with ultrapure Milli-Ro water before experiments. Metal was added as pentahydrated copper II sulfate (reagent grade).

RESULTS

Following OECD guidelines, a preliminary wide-range copper concentration experiment was performed for each microalgae, in order to select the toxic doses for the growth experiment. After 3 days, the cellular concentrations of all species were checked by two methods: electronic counting and microscope counting. No significant differences between counting methods could be observed.

After this, growth inhibition toxicity tests were carried out for 72 h in order to calculate the EC_{50} for biomass. Measures of background flasks did not vary significantly with time. Growth curves for each microalgae are presented in Figs. 1–4. By comparing areas under growth curves for each copper dose with area under control curve, inhibition average was calculated. Semilogarithmic plotting of inhibition average data fits to straight line (Fig. 5) and from

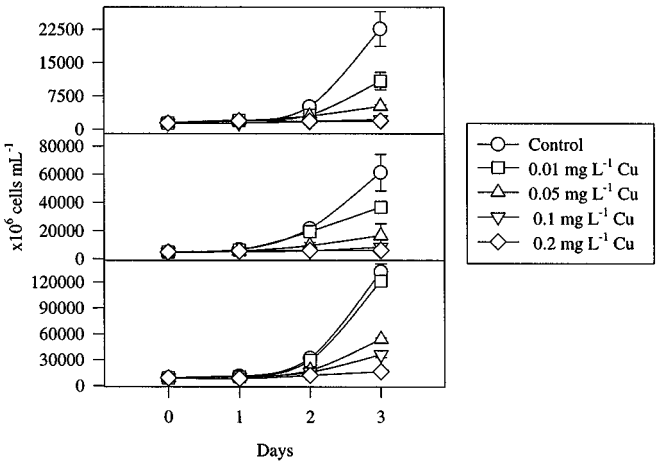

FIG. 1. Growth curves for *Chlorella autotrophyca* for 3 days when exposed to selected doses of copper. Error bars mean standard deviation between replicates.

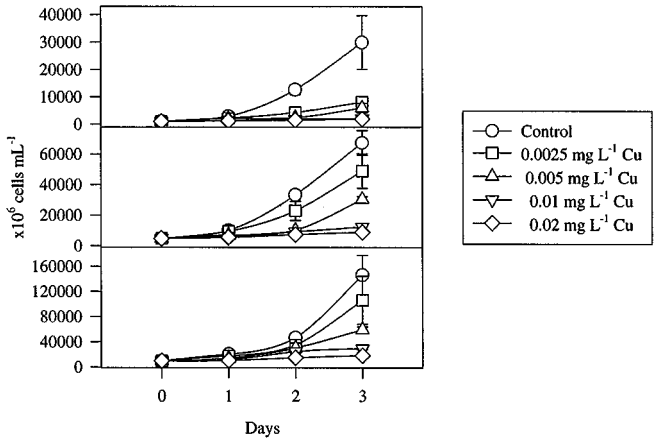

FIG. 3. Growth curves for *Isochrysis* aff. *galbana* for 3 days when exposed to selected doses of copper. Error bars mean standard deviation between replicates.

equation of fitted line EC_{50} values are calculated. Data for calculated EC_{50} values for copper on used microalgae for each initial cellular density are provided in Table 2. An easier way of viewing the relation between these two parameters is to calculate the amount of toxic given for each cell at the beginning of the test (this is defined as "toxic cellular quote") and plot these data versus the inhibition average in relation to the control provoked by these doses (Moreno-Garrido, 1997). Results can be seen in Fig. 6. The *x*-axis is plotted in logarithmic scale in order to fit all points to a straight line. From the equation of this line an EC_{50} related to the metal quote can be calculated. These data, in addition to the slope of fitted lines, are presented in Table 3.

DISCUSSION

The concept of toxic cellular quote may be quite important in toxicity tests developed with microorganisms, especially in tests on heavy metals. It is demonstrated that there are two phases in metal adsorption by microalgae: a first phase, nondependent on cellular metabolism, where metal binds to the cellular surface (adsorption), and a second, slower phase dependent on metabolism, where metal is accumulated in the interior of the cell (absorption) (Garnham *et al.*, 1992; Moreno-Garrido *et al.*, 1998). The amount of metal adsorbed seems to be much higher than metal absorbed in living cells (Sakaguchi *et al.*, 1979; Moreno-Garrido *et al.*, 1998). Metal accumulation greatly depends on metal concentration in media (Tobar *et al.*, 1993), but cells at lower cellular densities accumulate a higher amount of

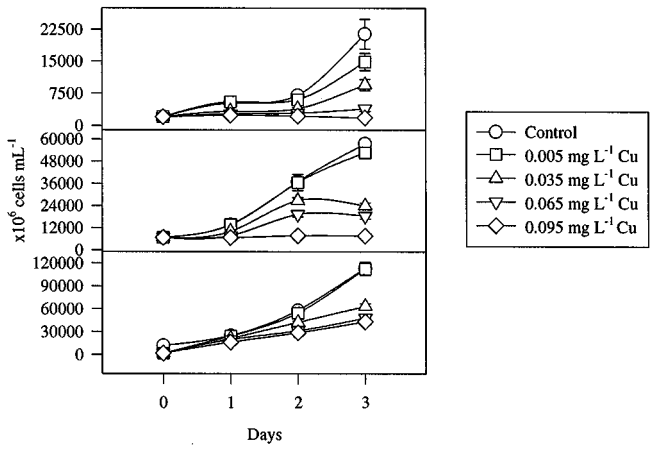

FIG. 2. Growth curves for *Nannochloris atomus* for 3 days when exposed to selected doses of copper. Error bars mean standard deviation between replicates.

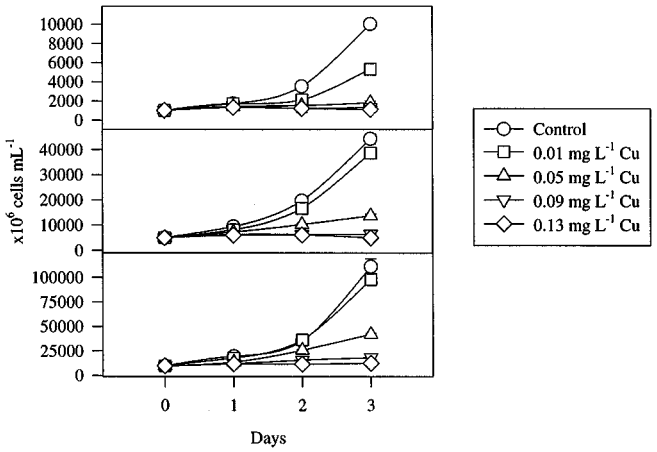

FIG. 4. Growth curves for *Phaeodactylum tricornutum* for 3 days when exposed to selected doses of copper. Error bars mean standard deviation between replicates.

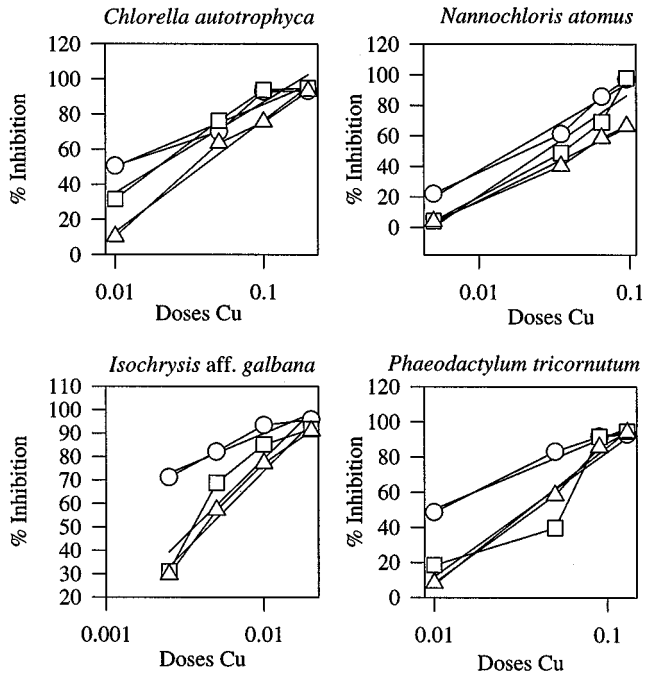

FIG. 5. Semilogarithmic plotting of inhibition average versus copper doses in 72-h experiment. Fitted straight lines have also been plotted. Circles, 10^3 cells mL^{-1}; squares, 5×10^3 cells mL^{-1}; triangles, 10^4 cells mL^{-1}.

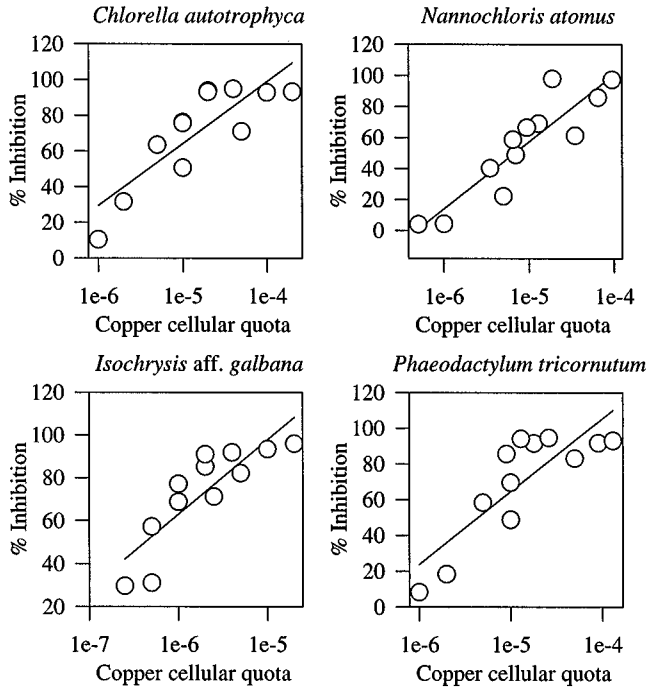

FIG. 6. Semilogarithmic plot of inhibition average versus copper cellular quota in 72-h experiment. Fitted stright lines have also been plotted.

metal at the same metal concentration in the media (Moreno-Garrido, 1997). Some microalgal species, such as *Nannochloropsis gaditana* (Eustigmatophyceae), are able to remove 100% of copper or zinc after 24 h in accumulation experiments when exposed to concentrations of these metals of 0.5 mg L^{-1} (Moreno-Garrido, 1997). It is accepted that toxicity of copper to microoganisms depends on the concentration of ionic copper and not on total copper concentration (Sunda and Guillard, 1976), but it now is known that hydroxides are also toxic for photosynthetic organisms (Riisgård *et al.*, 1980). Copper solubility in water decreases when pH increases, and photosynthetic organisms create a local zone near cells with elevated values of pH. This would be responsible for rapid binding of divalent cations as copper to cell walls (Lüeritz and Nicklisch, 1989). It is possible that adsorbed metal would be available for cells, and this would explain an increase of toxicity when initial cellular density decreases. Other possible explanations could be given if specific or unspecific substances capable of chelating metals were dropped to the media from cells. Chelation of metals by cellular exudates have been reported by González Dávila *et al.* (1995) for *Dunaliella tertiolecta.* The chelating properties of extracellular polisaccharides from *Chlorella* spp. have been pointed out by Kaplan *et al.* (1987). There are many reports on the capacity of some algal species to produce phytochelatins as a response to the presence of metals in media (Ahner and Morel, 1995, Ahner *et al.*, 1995; Gekeler *et al.*, 1988; Lee *et al.*, 1996). As toxicity

TABLE 2
EC_{50}% 72-h Values for Each Microalgal Species at Different Initial Celluar Densities

	10^3 cells mL^{-1}	5×10^3 cells mL^{-1}	10^4 cells mL^{-1}
Ch. autotrophyca	9.6	19.3	38.3
N. atomus	16.7	27.3	46.2
I. aff. *galbana*	0.4	3.6	4.4
P. tricornutum	9.8	34.4	35.0

Note. Values expressed as μg L^{-1}.

TABLE 3
EC_{50}% 72-h Values Related to Toxic Cellular Quota

	EC_{50} %72-h quota	Slope
Ch. autotrophyca	3.9×10^{-6}	34.7
N. atomus	6.8×10^{-6}	40.8
I. aff. *galbana*	4.2×10^{-7}	34.7
P. tricornutum	4.4×10^{-6}	43.2

Note. Values are expressed as mg L^{-1} $cell^{-1}$. Slopes of stright lines fitted to semilogarithmic plot of growth inhibition average versus copper cellular quota have also been showed.

of heavy metals depends on the amount of nonchelating dissolved ions (Davey *et al*., 1973), higher cellular densities could drop higher amounts of substances to aquatic media, and thus reduce toxicity of metals by complexation.

As can be seen in Table 2, EC_{50} values increase by one order of magnitude when initial cellular densities increase by one order of magnitude in the case of *I*. aff. *galbana*, and around three or four times in the case of the other species used. The slope of fitted lines to plots of inhibition averages versus toxic cellular quote could give an idea of the range of toxicity of certain species (smaller slope, wider range between non-effect and lethal doses), but in this experiment these data should be carefully revised, as many points near 100% inhibition were used.

CONCLUSIONS

Initial cellular density is a very important parameter in toxicity tests developed using microalgae. In this work, an inverse proportion between initial cellular density and sensitivity of tests for calculating EC_{50} for biomass is demonstrated on the microalgal species used. The toxic cellular quote can improve results obtained in toxicity tests, as values of EC_{50} related to this parameter can be calculated.

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