Cah. Biol. Mar. (1997) 38 : 201-206

Disorders provoked by copper in the marine diatom *Phaeodactylum tricornutum* in short-time exposure assays.

Angeles CID, Enrique TORRES, Concepción HERRERO & Julio E. ABALDE
Laboratorio de Microbioloxía. Facultade de Ciencias. Universidade da Coruña.
Campus da Zapateira s/n. 15071 A Coruña. Spain.
Fax: 34 81 167065 - E-mail: abaldej@udc. es

Abstract: Copper toxicity on the marine diatom *Phaeodactylum tricornutum* was assessed after 24 hours of exposure, using different parameters: growth, ^{14}C-bicarbonate assimilation, ^{3}H-thymidine incorporation, photosynthetic pigment content, and others assayed by flow cytometry, such as cellular volume, chlorophyll *a* autofluorescence and cell viability. Different degrees of sensitivity were observed for the different analysis. Cell viability assayed by flow cytometry was the less sensitive parameter.

Résumé : *La toxicité du cuivre sur la diatomée marine* Phaeodactylum tricornutum. Cette toxicité a été étudiée après 24 heures d'exposition, par la mesure de différents paramètres : croissance, assimilation de ^{14}C-bicarbonate, incorporation de ^{3}H-thymidine, teneur en pigment photosynthétique, et d'autres paramètres évalués par cytométrie de flux, tels que le volume cellulaire, l'autofluorescence de la chlorophylle *a* et la viabilité cellulaire. Différents degrés de sensibilité ont été observés pour les différentes analyses. La viabilité cellulaire, étudiée par cytométrie de flux, s'est avérée comme le paramètre le moins sensible.

Keywords: copper, marine diatom, photosynthesis, pigments, viability.

Introduction

The toxicity of heavy metal ions in solution to microalgae is well known. The essentiality and toxicity of heavy metals have been the subject of extensive research. Essential heavy metals have been reported to be toxic at high concentrations (Poole & Gadd, 1989). Many of these metals have a direct influence on various physiological and biochemical microalgal processes including reduction in growth, photosynthesis, and chlorophyll content, inhibition of enzyme activities and degeneration of chloroplasts and mitochondria (Reddy & Prasad, 1990).

Tolerance to heavy metals is considered highly specific (Visviki & Rachlin, 1991). Algae, in general, show a great sensitivity to environmental variations (Hörnström, 1990), and toxicity tests using these organisms have thus a clear justification with regard to environmental protection; among them, diatoms are considered the most sensitive microorganisms to heavy metals toxicity (Hörnström, 1990).

The test species chosen for this study was the marine diatom *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae). Different parameters for microalgal activity show widely different sensitivities to pollutants. The present work examines the effect of copper in short-time exposures on parameters commonly used in microalgal toxicity assays, such as growth, photosynthetic pigments composition and ^{14}C-bicarbonate incorporation. Since the incorporation of ^{3}H-thymidine was assayed as a possible indicator of DNA synthesis (Karl, 1982), and flow cytometry could be considered useful in the monitoring of the physiological state of microalgal cells (Cid *et al.*, 1996), ^{3}H-thymidine incorporation and other parameters assayed by flow

Reçu le 23 juillet 1997 ; accepté après révision le 9 octobre 1997.
Received 23 July 1997 ; accepted in revised form 9 October 1997.

cytometry, such as cellular volume, viability and chlorophyll *a* fluorescence, were examined.

Materials and methods

Algae cultures

Phaeodactylum tricornutum (isolated from Ria de Arousa waters by Dr. J. Fábregas, University of Santiago, Spain) was cultured in axenic batch conditions in natural seawater filtered through a 0.45 µm Millipore filter; after that, this filtered seawater was filtered again through an active carbon filter, in order to eliminate organic chelating substances, and autoclaved at 120°C for 60 min. The assays were carried out in this raw, unenriched seawater, with no inorganic nutrients added. Initial cell density was 24 $x10^4$ cells ml^{-1}. Copper concentrations assayed were 0.05, 0.10, 0.25, 0.50 and 1 mg Cu l^{-1}, added as copper chloride; control cultures without copper were also included. The free copper ion concentration in each culture was quantified (Abalde *et al.*, 1995), showing that in this range of concentrations, all copper added to the cultures appeared as free copper ion. Salinity of seawater was 35 PSU and the initial pH of the cultures was 7.6. Cultures were grown in KIMAX test tubes, containing 40 ml of medium. The tubes were previously rinsed with nitric acid and washed several times with redistilled water. Cultures were maintained at 18 ± 1°C and 140 αmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12 h. All experiments were carried out in triplicate.

Measurement of growth

Growth was measured by counting culture aliquots in a Neubauer hemocytometer, and the growth rates are expressed as doublings d^{-1}.

Thymidine incorporation

The effect of copper on thymidine incorporation by this microalga was determined after 12 hr of copper exposure. The incorporation of ^{3}H-thymidine (Amersham, 40-60 µCi $µmol^{-1}$) into trichloroacetic acid-insoluble material was measured by the method of Fuhrman & Azam (1982), as described by Tubbing & Admiraal (1991a), and modified for marine microalgae cultures. Cells were exposed to the different copper concentrations during the light period (12 hr). Just before the dark period, ^{3}H-thymidine was added to a final concentration in the medium of 1 pM. After 15, 30, 45 and 60 min, 20 ml subsamples were filtered through 1.2 µm MF-Millipore filters; 10 ml of an ice-cold 10% solution of trichloracetic acid (TCA) were poured on filtered cells. After 15 min the 10% solution of TCA was filtered. Each filter, with the TCA-insoluble material, was washed twice with 5 ml of an ice-cold 5% solution of TCA and subsequently put into a scintillation vial. The ^{3}H-thymidine radioactivity in microalgal cells was counted in a LKB (Pharmacia) scintilliation counter with 10 ml of Readysafe (Beckman) as scintillator.

Measurement of photosynthesis

The effect of copper on photosynthesis was determined by estimation of carbon fixation, recording the incorporation of ^{14}C from $NaH^{14}CO_3$. ^{14}C-bicarbonate (Amersham, 674 µCi mg^{-1}) was added to the microalgal suspensions in culture tubes to give an activity of 0.01 µCi ml^{-1}. Incubation periods tested were 0.5, 1, 2 and 4 hr, and the photosynthetically fixed radioactivity in microalgal cells was counted in a LKB (Pharmacia) scintillation counter with Readysafe (Beckman) as scintillator.

Photosynthetic pigments analysis

The analysis of photosynthetic pigments was carried out after 24 hours of copper exposure. Pigments were extracted in acetone 90% at 4°C, in dark conditions, and concentrated by evaporation of the solvent. Dry extracts of pigments were resuspended in 100 µl of methanol (HPLC grade). The HPLC analysis of photosynthetic pigments was carried out as Wright *et al.* (1991).

Flow cytometry determinations

Cellular volume, viability and chlorophyll *a* fluorescence were determined after 24 hours of copper exposure by flow cytometry (FCM), using a FACScan flow cytometer (Becton Dickinson Instruments), equipped with an argon-ion excitation laser (488 nm). Forward scatter (FSC), which can be correlated with the size of the cell, and the fluorescence of the propidium iodide (PI), which can be correlated with the viability of the cells, were used to set gating levels.

In the study of chlorophyll *a* fluorescence, aliquots of microalgal cultures were directly analysed in the flow cytometer. The red fluorescent emission of chlorophyll *a* was collected in the FL3 (mean 660 nm) channel.

Propidium iodide (final concentration: 60 µM) was used to counterstain the DNA of non viable cells: the fluorescence of cells stained with PI was measured to study cell viability. PI is a fluorescent dye that intercalates with doubled stranded nucleic acids to produce red fluorescence when excited by blue light. It is unable to pass through intact cell membranes; however, when the cell dies the integrity of the cell membrane fails and PI is able to enter and stain the nucleic acids (Ormerod, 1990). In this way PI can be used to discriminate between live non-fluorescent cells and dead fluorescent cells; the red fluorescent emission of this compound was collected in the FL2 channel.

Data analysis

Data were statistically analysed by an one-way analysis of variance (ANOVA) and, when differences observed were significant, means were compared by the multiple range Duncan test, at a level of significance of 0.05.

The most common parameter used in toxicity assays is the EC_{50}, i.e., the concentration of the tested substance which decreases the studied parameter (growth,

photosynthesis) by 50%. In order to obtain a confidence interval for the EC_{50} value, a statistical method such as probit analysis should be used. Probit analysis of growth data was carried out using the SPSS-PC+ software.

For each cytometric parameter investigated, 10^4 cells were analysed per condition and fluorescence measurements were in the logarithmic scale. Data collection was performed using the list mode. The mean fluorescence for any given population was provided by the instrument software (LYSIS II program; Becton Dickinson Instruments).

Data on chlorophyll fluorescence and cellular volume of *P. tricornutum* cells were expressed as a percentage (%) of the signal of the control cells according to the equation of Reader *et al.* (1993):

$$\%P=100-\{100(Pc-Pt)/Pc\}$$

where % P is the percentage of signal of the *P. tricornutum* cells; Pc the mean signal of control cells; and Pt mean signal of copper-treated cells.

Results

Growth rates and cellular densities reached after 24 hours of copper exposure of *Phaeodactylum tricornutum* cultures are shown in Table 1. Growth rate was function of copper concentration in the medium (Table 1), being maximum in control cultures (0.95 doublings d^{-1}), while in that cultures with the maximum copper concentration assayed this growth rate is close to 0. The EC_{50} of growth after 24 hours of copper exposure is 0.667 mg l^{-1}.

Table 1. Cell densities (expressed as $x10^4$ cells ml^{-1}) and growth rates (μ) (expressed as day^{-1}) of *Phaeodactylum tricornutum* cultures with different copper concentrations (expressed as mg l^{-1}).

Table 1. Densité cellulaire ($x10^4$ cellules ml^{-1}) et taux de croissance (μ) ($jour^{-1}$) de cultures de *Phaeodactylum tricornutum* mises en présence de différentes concentrations en cuivre (mg l^{-1}).

	cell density	growth rate
Control	52.25±2.34	1.26
0.05	48.81±5.64	1.02
0.10	38.88±8.28	0.70
0.25	33.81±5.55	0.49
0.50	27.13±2.03	0.22
1.00	16.00±1.03	-

Previous assays have shown that the ^{3}H-thymidine incorporation rate in *Phaeodactylum tricornutum* cells was linear during the first hour after thymidine addition (non published data). The proportion of ^{3}H-thymidine assimilated decreased as copper concentration in the medium increased (Table 2). Minimum copper concentration assayed (0.05 mg l^{-1}) reduced the thymidine assimilation rate by 15% (12.07 fmol ml^{-1} h^{-1}) compared with the mean value obtained in control cultures (14.01 fmol ml^{-1} h^{-1}) (Table 2). Cultures with the highest copper concentrations, 0.5 and 1 mg l^{-1}, showed ^{3}H-assimilation rates near to 0 (Table 2). The EC_{50} of ^{3}H-thymidine assimilation after 12 hours of copper exposure is 0.079 mg l^{-1}.

Table 2. ^{3}H-thymidine (fmol ml^{-1} h^{-1}) and ^{14}C-bicarbonate (μg carbon l^{-1} h^{-1}) assimilation rates, and percentage of cellular viability of *Phaeodactylum tricornutum* cultures with different copper concentrations (expressed as mg l^{-1}).

Table 2. Taux d'assimilation de ^{3}H-thymidine (fmol ml^{-1} h^{-1}) et de ^{14}C-bicarbonate (μg carbone l^{-1} h^{-1}) et pourcentage de viabilité cellulaire de cultures de *Phaeodactylum tricornutum* mises en présence de différentes concentration en cuivre (mg l^{-1}).

	^{3}H-thymidine	^{14}C-bicarbonate	Viability
Control	21.01±1.78	617.81±5.03	99.45
0.05	12.07±1.17	578.60±5.36	98.98
0.10	9.23±0.68	477.31±3.98	98.74
0.25	7.58±0.89	310.21±4.02	98.86
0.50	2.67±0.39	300.21±0.69	98.29
1.00	1.98±0.25	94.28±0.19	97.41

Growth rates of *P. tricornutum* cultures after 24 hours of copper exposure correlated well (r = 0.928) with ^{3}H-assimilation rates after 12 hours copper exposure (Fig. 1).

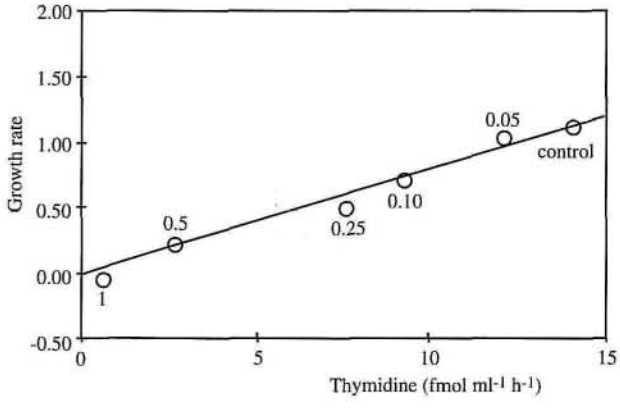

Figure 1. Correlation between ^{3}H-thymidine assimilation rate (expressed as fmol ml^{-1} h^{-1}) and growth rate (expressed as day^{-1}) of *Phaeodactylum tricornutum* cultures with different copper concentrations in mg l^{-1}.

Figure 1. Corrélation entre le taux d'assimilation de la ^{3}H-thymidine (fmol ml^{-1} h^{-1}) et le taux de croissance ($jour^{-1}$) dans des cultures de *Phaeodactylum tricornutum* mises en présence de différentes concentrations en cuivre (mg l^{-1}).

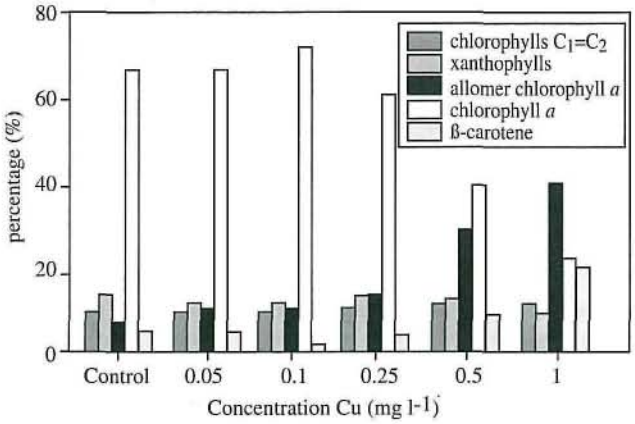

Figure 2. Photosynthetic pigments profile, expressed as percentage of total pigments, of *Phaeodactylum tricornutum* cultures with different copper concentrations in mg l^{-1}.

Figure 2. Profils des pigments photosynthétiques, exprimés en pourcentage de pigments totaux de cultures de *Phaeodactylum tricornutum* en présence de différentes concentrations en cuivre (mg l^{-1}).

The incorporation of ^{14}C-bicarbonate was assayed as a photosynthetic rate index. Minimum copper concentration assayed (0.05 mg l^{-1}) had no effect on carbon assimilation rate (Table 2), with values near to those obtained in control cultures. A copper concentration of 0.10 mg l^{-1} reduced the photosynthetic rate by 23% compared with control cultures, with values of 477.31 and 617.81 µg C l^{-1} h^{-1}, respectively (Table 2). The highest copper concentration assayed, 1 mg l^{-1}, provoked a strong decrease in the photosynthetic rate: 94.28 µg C l^{-1} h^{-1} (Table 2). After 12 hr of the light period and copper exposure, the EC_{50} for the photosynthesis was 0.405 mg l^{-1}.

The analysis of photosynthetic pigments by HPLC showed that copper affected the pigment pattern of *P. tricornutum* after 24 h. of copper exposure (Fig. 2). Chlorophyll *c* and xanthophylls proportions are highly constant in cells exposed to the different copper concentration assayed (Fig. 2). ß-carotene proportion with respect to the total of pigments is 4.51% in control cultures and 4.14% in cultures with 0.05 mg l^{-1} of copper; this proportion decreases in cultures with 0.10 and 0.25 mg l^{-1}, and increase in the remaining cultures, being maximum in cultures with 1 mg l^{-1}, with a 19.32% of ß-carotene. However, most important changes are observed in the proportions of chlorophyll *a* and its allomer (Fig. 2): the proportion of the chlorophyll *a* allomer increased when copper concentration increased in the medium, while the proportion of chlorophyll *a* decreased; the maximum percentage of chlorophyll *a* was observed in cultures with 0.1 mg l^{-1} of copper or less, with values near to 70%, whereas the proportion of the chlorophyll *a* allomer in these cultures was approximately 7%. Higher concentrations of copper provoked an inversion in the proportions of both pigments; chlorophyll *a* decreased while its allomer increased, being maximum at 1 mg l^{-1} of copper (39.31%).

The study of chlorophyll *a* fluorescence by flow cytometry showed a significant increase in this fluorescence in *Phaeodactylum tricornutum* cells exposed to 0.5 and 1 mg l^{-1} of copper, after 24 hr of metal exposure (Fig. 3). If the chlorophyll *a* fluorescence in control cultures is considered 100%, the fluorescence increase in affected cells was 19% and 38%, in cultures with 0.5 and 1 mg Cu l^{-1}, respectively.

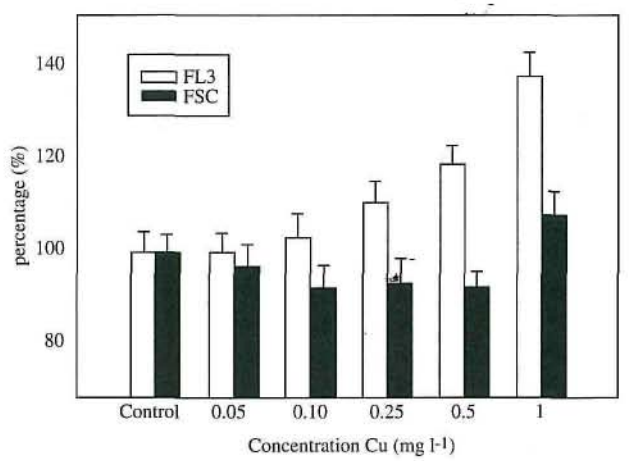

Figure 3. Chlorophyll *a* autofluorescence (FL3) and forward scatter signal (FSC) after 24 hours of copper exposure in *Phaeodactylum tricornutum* cells. Data are expressed as the percentage of the fluorescence of control cells, according to the equation of Reader *et al.* (1993). Results are the means of three replicates.

Figure 3. Autofluorescence de la chlorophylle *a* (FL3) et signal de diffusion aux petits angles (FSC) après 24 h d'exposition au cuivre dans des cellules de *Phaeodactylum tricornutum*. Les données sont exprimées en pourcentage de fluorescence des cellules témoins, selon l'équation de Reader *et al.* (1993). Les résultats sont les moyennes de trois mesures.

Copper exposure induced changes in cellular volume after 24 hr of culture (Fig. 3). Copper concentrations of 0.5 and 1 mg l^{-1} provokes an increase of cell volume of 13 and 20% respect to the control cells, respectively. Remaining cultures showed FSC signals lower than those obtained for the control cultures, but not significantly differents.

The percentage of non viable cells in control cultures is close to 0 after 24 hours of culture (Table 2). Remaining cultures, except cultures with 1 mg l^{-1} of copper, showed near 1% of cells stained with PI, being non viable cells. Cultures with 1 mg l^{-1} presented 2.6% of non viable cells after 24 hours of copper exposure.

Discussion

Heavy metals toxicity tests with marine microalgae have been used during the last decades. An exposure period of 72-96 hours is used in most toxicity tests and is also applicable to the algal tests (Walsh & Merrill, 1984). Although most papers about heavy metal toxicity on microalgae report results in a 96 hours period, the present work shows that 24 hours is enough to provoke measurable alteration in *Phaeodactylum tricornutum* cells and therefore can be considered as an interesting study period.

One of the most studied toxic effect of copper on microorganisms is the effect on growth. Copper concentration that affects growth in microalgae is largely variable and depends on the species assayed, cell density, composition of the medium, physical culture conditions, etc. (Stauber & Florence, 1985b). Results obtained showed important differences in growth with the different copper concentrations assayed (Table 1). The EC_{50} for growth after 24 hours of copper exposure was 0.667 mg l^{-1}. This value is higher than those found for other diatoms, like *Nitzschia closterium* or *Asterionella japonica* (Stauber & Florence, 1987), observed after 48 or 96 hours of metal exposure.

^{3}H-thymidine assimilation assay is generally used in environmental studies (Tubbing & Admiraal, 1991a,b). There are some studies about the ^{3}H-thymidine assimilation by eucaryotic algae, protozoa, yeasts and other fungi, reviewed by Karl (1982). This method allows the indirect study of the nucleic acid synthesis in microorganisms, as the basis of cellular growth. Following copper transport into the cytosol, copper may react with -SH enzyme groups and free thiols (e.g. glutathione), disrupting enzyme-active sites and cell division (Florence & Stauber, 1986; Stauber & Florence, 1985a). In the present work it has been observed that ^{3}H-thymidine assimilation rate and growth are proportionally related in *P. tricornutum* cultures (Fig. 1). This result shows that the decrease of the growth rate observed in this marine diatom could be provoked by a default in the DNA synthesis.

Copper may also exert its toxicity in subcellular organelles, as chloroplasts, altering photosynthesis and also ATP accumulation (Cid *et al.*, 1995). Photosynthetic rate has been used as a common toxicity test with microalgae. Photosynthesis inhibition by copper has been observed previously in other diatoms (Stauber & Florence, 1987; Wong & Chang, 1991). Copper concentration that affects both growth and photosynthesis depends on the species assayed. Wong & Chang (1991) have observed that 0.1 mg Cu l^{-1} produced a slight inhibition of photosynthesis in *Chlorella pyrenoidosa*, while 0.25 mg Cu l^{-1} produced total inhibition. In *Nitzschia closterium* photosynthesis was affected at copper concentrations above 0.1 mg Cu l^{-1} (Lumsden & Florence, 1983). Results obtained in the present work showed that the ^{14}C-bicarbonate assimilation rate of *P. tricornutum* cultures used as a photosynthesis rate index, decreased when copper concentration increased; 0.5 mg Cu l^{-1} reduced by 50% the photosynthetic rate (Table 2). Therefore, photosynthesis is more affected by copper than growth. This uncoupling between division rate and photosynthesis can be due to copper inhibiting the process of cell division independently of any effect of this metal on the production of the new cell material (Stauber & Florence, 1987).

Regarding the photosynthetic pigments analysis by HPLC, copper provoked important changes on chlorophyll *a*, increasing the proportion of its allomer (Fig. 2), this being the most important conclusion of this analysis; however the information obtained for the remaining pigments is not clear and then is not useful for this short time studies. An increase in the intracellular pH has been proposed to explain this increase of the chlorophyll *a* allomer (Cid *et al.*, 1995). Since the chlorophyll *a* allomer has a less photosynthetic effectiveness, the decrease in the photosynthetic rate observed in *P. tricornutum* cultures with the highest copper concentrations assayed can therefore be explained.

Researchers examining the effects of heavy metals on uni- or multicellular algae have reported that, at least for some species, cells respond to toxic levels of metals by increasing cell size (Fisher *et al.*, 1981; Stauber & Florence, 1987; Bolaños *et al.*, 1992; Abalde *et al.*, 1995). Heavy metals change cell membrane permeability to small cations (Overnell, 1975), probably leading to this increase in cell volume. Results obtained in the present study agree with these reports, since an increase in the FSC signal by flow cytometry (related with cell volume) has been observed in *P. tricornutum* cells exposed to the higher copper concentrations (Fig. 3).

Chlorophyll *a* fluorescence is particularly sensitive to the functioning of photosystem II (PSII). Chlorophyll *a* fluorescence results obtained by flow cytometry show that copper provokes an inhibitory effect on PSII activity in *P. tricornutum*, at concentrations of 0.25, 0.50 y 1 mg l^{-1} (Fig. 3).

Among the many methods providing an evaluation of toxic effects on cultured cells, viability assays are the oldest and most widespread technique. The major criteria employed in viability assays is the membrane integrity. Cells that have lost the integrity of their plasma membrane, and have become permeable to external compounds, such as dyes and enzymes, are considered to be non-viable and they are not metabolically active (Frankfurt, 1990). However, viability data of *P. tricornutum* after 24 hours of copper exposition (Table 2) reveal that this analysis is not adequate to this short-time assessments.

Regarding the sensitivity of the different parameters assayed, ^{3}H-thymidine assimilation was more sensitive than

growth or photosynthesis. Among the assays carried out by flow cytometry, FSC and chlorophyll *a* fluorescence variations were the most sensitive. Pigment analysis by HPLC also provided some useful information.

Acknowledgements

This work was supported by a grant from Xunta de Galicia. Santiago de Compostela, Spain (XUGA10301B93).

References

Abalde J., Cid A., Reiriz S., Torres E. & Herrero C. 1995. Response of the marine microalga *Dunaliella tertiolecta* (Chlorophyceae) to copper toxicity in short time experiments. *Bulletin of Environmental Contamination and Toxicology,* **54**: 317-324.

Bolaños L., García-González M., Mateo P. & Bonilla I. 1992. Differential toxicological response to cadmium in *Anabaena* strain PCC7119 grown with NO_3- and NH_4+ as nitrogen source. *Journal of Plant Physiology*, **140**: 345-349.

Cid A., Fidalgo P., Herrero C. & Abalde J. 1996. Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry. *Cytometry,* **25**: 32-36.

Cid A., Herrero C., Torres E. & Abalde J. 1995. Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. *Aquatic Toxicology,* **31**: 165-174.

Fisher N.S., Jones G.J. & Nelson D.M. 1981. Effects of copper and zinc on growth, morphology and metabolism of *Asterionella japonica* (Cleve). *Journal of experimental marine Biology and Ecology,* **51**: 37-56.

Florence T.M. & Stauber J.L. 1986. Toxicity of copper complexes to the marine diatom *Nitzschia closterium. Aquatic Toxicology,* **8**: 11-26.

Frankfurt O.S. 1990. Flow cytometric measurement of cell viability using DNase exclusion. In: Methods in Cell Biology. vol. 33. Flow Cytometry, (Z. Darzynkiewicz & H.A. Crissman eds), pp. 13-18. Academic Press: San Diego.

Fuhrman J.A. & Azam F. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in the Southern California Bight. *Marine Biology,* **66**: 109-120.

Hörnström E. 1990. Toxicity test with algae. A discussion on the batch method. *Ecotoxicology and Environmental Safety,* **20**: 343-353.

Karl D.M. 1982. Selected nucleic acid precursors in studies of aquatic microbial ecology. *Applied and Environmental Microbiology,* **44**: 891-902.

Lumsden B.R. & Florence T.M. 1983. A new algal assay procedure for the determination of the toxicity of copper species in seawater. *Environmental and Technological Letters,* **4**: 271-276.

Ormerod M.G. 1990. Analysis of DNA. General methods. In: *Flow Cytometry. A Practical Approach* (M.G. Ormerod ed), pp. 69-87. Oxford University Press: Oxford.

Overnell J. 1975. The effect of heavy metals on photosynthesis and loss of cell potassium in two species of marine alga *Dunaliella tertiolecta* and *Phaeodactylum tricornutum. Marine Biology,* **29**: 99-103.

Poole R.K. & Gadd G.M. (eds.) 1989. *Metal-microbe interactions*. IRL Press: Oxford.

Reddy G.N. & Prasad M.N.V. 1990. Heavy metal binding proteins/peptides: occurence, structure, synthesis and functions. A Review. *Environmental and Experimental Botany,* **30**: 251-264.

Stauber J.L. & Florence T.M. 1985a. The influence of iron on copper toxicity to the marine diatom *Nitzschia closterium (Ehrenberg) W. Smith. Aquatic Toxicology,* **6**: 297-305.

Stauber J.L. & Florence T.M. 1985b. Interactions of copper and manganese: a mechanism by which manganese alleviates copper toxicity to the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquatic Toxicology,* **7**: 241-254.

Stauber J.L. & Florence T.M. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology,* **94**: 511-519.

Tubbing D.M.J. & Admiraal W. 1991a. Inhibition of bacterial and phytoplanktonic metabolic activity in the lower River Rhine by ditallowdimethylammonium chloride. *Applied and Environmental Microbiology,* **57**: 3616-3622.

Tubbing D.M.J. & Admiraal W. 1991b. Sensitivity of bacterioplankton in the Rhine River to various toxicants measured by thymidine incorporation and activity of exoenzymes. *Environmental and Toxicological Chemistry,* **10**: 1161-1172.

Visviki, I. & Rachlin, J.W. 1991. The toxic action and interactions of copper and cadmium to the marine alga *Dunaliella minuta*, in both acute and chronic exposure. *Archives of Environmental Contamination and Toxicology,* **20**: 271-275.

Walsh G.E. & Merrill R.G. 1984. Algal bioassays of industrial and energy process effluents. In: *Algae as Ecological Indicators* (L.E. Shubert ed), pp. 329-360. Academic Press: London.

Wong P.K. & Chang L. 1991. Effects of copper, chromium and nickel on growth, photosynthesis and chlorophyll *a* synthesis of *Chlorella pyrenoidosa* 251. *Environmental Pollution,* **72**: 127-139.

Wright S.W., Jeffrey S.W., Mantoura R.F.C., Llewellyn C.A., Bjorland T., Repeta D. & Welschmeyer N. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series,* **77**: 183-196.