Arch. Environ. Contam. Toxicol. 24, 16–20 (1993)

ARCHIVES OF
Environmental
Contamination
and Toxicology

The Growth Response of the Green Alga *Chlorella vulgaris* to Combined Divalent Cation Exposure

Joseph W. Rachlin and Albania Grosso

Department of Biological Sciences, Lehman College of the City University of New York, Bedford Park Boulevard West, Bronx, New York 10468-1589, USA

Abstract. Using the growth response of the green alga *Chlorella vulgaris* as a model system, the effects of combinations of the environmentally active cations Cd, Co, and Cu were evaluated. The 96-h static EC_{50} for these cations to *C. vulgaris* were, respectively, 0.89 μM, 9.0 μM, and 2.8 μM, yielding a toxicity series such that Cd > Cu > Co. The cation combinations of Cd + Cu, and Cu + Co acted synergistically, while Cd + Co, and the tri-metallic combination Cd + Cu + Co resulted in antagonistic interactions. Examination of these toxic combinations at 24, 48, 72, and 96 h indicate that the cellular response is not a uniform one. Failure of energy dispersive X-ray spectrophotometric analysis to demonstrate any intracellular incorporation of these cations (except for a weak cytoplasmic Cu peak at the 8.0 KEV position) suggests that the toxic actions of these cations at EC_{50} concentrations are exerted at the level of the plasma membrane.

Recent reviews (Boudou and Ribeyre 1989a, 1989b), and studies (Taylor 1989; Taylor and Stadt 1990; Visviki and Rachlin 1991) have recognized that examining the effects of divalent cations in various combinations is more representative, of the actual environmental problems faced by organisms, than are single metal studies. This recognition results from the realization that environmental loadings of cations from anthropogenic sources rarely involve single cation contributions, and if they do, the introduced cation will interact with a host of chemicals native to the receiving system. Thus, organisms potentially impacted by these toxicants face a multiple rather than a single toxicant insult. Working with algal models representative of the base of the aquatic food chain, our laboratory team has evaluated, under a variety of conditions, several key cations for their influence on growth (Rachlin *et al.* 1983; Rai *et al.* 1990; Rachlin and Grosso 1991; Visviki and Rachlin 1991). Using population growth as an end-point, we now report the interactions of the cations cadmium, cobalt, and copper, on the green alga *Chlorella vulgaris*, in terms of their actions being either additive, antagonistic, or synergistic.

Materials and Methods

The alga, *Chlorella vulgaris* (UTEX 30) was obtained as pure isolates from the Starr Culture Collection of Algae, University of Texas at Austin, and was grown and maintained in chelator-free modified Bristol's medium (Bold 1949) at a pH of 6.5 in 125 ml Erlenmeyer flasks. These flask cultures, containing 50 ml of the Bristol's medium, were incubated in a Sherer-Gillett RI-24 LTP growth chamber illuminated with Sylvania cool white fluorescent lamps supplemented with a 25-W incandescent light bulb to provide red light. Stock and experimental cultures were maintained in log phase of growth by the removal of 20 ml of medium and cells every seven days and replacing this with an equal volume of fresh sterile medium. Illumination was maintained at 280 foot candles (3.08 Klux, 7.84 W M^{-2}). The day/night program within the chamber was constant at 16 : 8 h and the incubation temperature was maintained at 19 ± 1°C.

Test solutions consisted of the modified Bristol's medium (pH 6.5) as control, or solutions of this medium containing the 96-h EC_{50} concentrations of the cations Cd, Co, and Cu, in the following combinations: Cd + Cu, Cd + Co, Cu + Co, and Cd + Cu + Co. Test solutions were made in modified Bristol's medium using the chloride salts of the cations in appropriate concentrations so that results are reported as concentrations of the cations alone (Rosko and Rachlin 1977; Rachlin *et al.* 1983). The EC_{50} concentrations used for Cu and Cd were, respectively, 0.18 ppm (2.8 μM) and 0.10 ppm (0.89 μM) and had been previously determined by Rosko and Rachlin (1977). The 96-h EC_{50} value for Co was determined in the current study and found to be 0.56 ppm (9.5 μM). All control and test concentration trials were run in triplicate, following the procedures described in Rachlin and Grosso 1991, for a maximum 96-h exposure period, with enough replicates (24 per test concentration) so that triplicate cell counts could be made every 24 h. Cell counts were made with a brightline hemocytometer, on well-mixed cultures, and since the cell counts for each flask of a triplicate run were within 10% of each other, the data for each triplicate were pooled for the determination of percent growth and 95% confidence intervals (Rachlin and Farran 1974; Rachlin and Grosso 1991).

Cation interactions were evaluated using modifications of Colby's formula as reviewed by Rai *et al.* (1981), and applied by Visviki and Rachlin (1991). Colby's original formula is, E = XY/100 (where E is the expected population growth of the alga as a percent of control growth. X and Y are the percent of control growth of the alga after exposure to cations X and Y, respectively. Values of E greater or less than the percent of control growth, respectively, indicate synergistic and antagonistic cation interactions, and values of E equal to the percent of control growth indicate an additive interaction). This for-

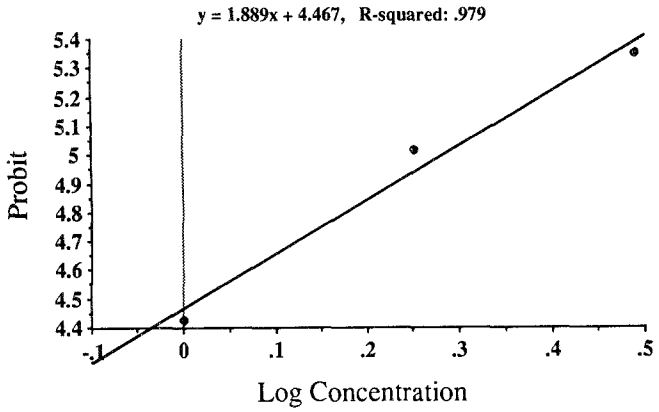

Fig. 1. Regression line of the probit response of *Chlorella vulgaris* to selected concentrations of cobalt

Table 1. Estimation of the EC_{50} values of cobalt from the percent response of *Chlorella vulgaris* after 96 h exposure, with respective regression equation

Conc (μM)	Conc (ppm)	Log conc	% Control growth	Empirical probit
5.4	0.32	−0.4949	63.8	5.3531
9.5	0.56	−0.2518	50.7	5.0175
17.0	1.00	0.0000	28.4	4.4290

$Y = 1.889X + 4.467$, $r^2 = 0.979$, Log $EC_{50} = -0.2821$, $EC_{50} = 0.52 \pm 0.038$ ppm (9.0 μM), probability >95%. Confirmation run of 0.56 ppm (9.5 μM) Co yielded 50.7% reduction in growth

mula was modified in the following manner; if three variables are required, $E = (ABC)/100^2$, and if four variables are required, $E = (ABCD)/100^3$. Thus a general formula was developed, where $E = (V_1V_2...V_n)/100^{n-1}$ and n is the number of variables (V) being considered.

Cation incorporation into the exposed cells was evaluated for all test conditions by means of the X-ray energy dispersive approach, using a Hitachi H7000 EM equipped with a PGT System 4 Plus energy dispersive X-ray spectrometer in STEM mode at 75 Kv. This system was used on air dryed cation exposed and control cells following the procedures outlined in Baxter and Jensen (1980), Jensen *et al.* (1982), and Rai *et al.* (1990).

Results

The data for the regression line (Figure 1) and the regression equation of the probit response analysis from which the EC_{50} value for cobalt was determined is presented in Table 1. The estimated EC_{50} of 0.522 ± 0.38 ppm (9.0 μM) was confirmed in a series of runs in which test concentrations of cobalt at 0.56 ppm (9.5 μM) yielded a 50.7% reduction in growth of the test *Chlorella* cultures. Using this value, 9.5 μM for Co, and values of 2.8 μM and 0.89 μM, respectively representing the 96 h static trial EC_{50} concentrations of Cu and Cd for *Chlorella vulgaris*, a series of cation combination studies were performed. These results are presented in the "observed" column of Table 2. All metal combinations resulted in greater than 50% reduction in growth after 96-h of exposure when compared to control cultures. Assuming control growth to represent 100%, then combinations of Cd + Cu and Cu + Co resulted in respective growth reductions of 93.9% and 92.7%. The combinations of Cd + Co, and the three cation combinations (Cd + Cu + Co) resulted in essentially the same growth reduction (63.4% and 63.6%). Clearly, copper combined with either cadmium or cobalt is more toxic than the cadmium-cobalt combination. Surprisingly, the three cation combination is no more toxic than the cadmium-cobalt combination, and considerably less toxic than either the cadmium-copper or copper-cobalt combinations.

Table 2. Comparison between expected and observed percent of control growth of *Chlorella vulgaris* (± 95% confidence intervals) following 96 h exposure to metal combination

Metal combinations	Expected	Observed
Cd + Cu	25.0	6.1 ± 0.09
Cu + Co	25.0	7.3 ± 0.09
Cd + Co	25.0	36.6 ± 0.17
Cd + Cu + Co	12.5	36.4 ± 0.17

Table 3. Percent of control growth of *Chlorella vulgaris* (± 95% confidence intervals) at each 24 h interval of exposure to metal combinations

Time (h)	Cu + Co	Cd + Cu	Cd + Co	Cd + Cu + Co
24	12.8 ± 0.12	18.9 ± 0.14	44.0 ± 0.18	101.0 ± 0.04
48	31.5 ± 0.17	13.6 ± 0.12	58.7 ± 0.18	37.8 ± 0.17
72	14.2 ± 0.12	4.9 ± 0.08	39.9 ± 0.18	19.4 ± 0.14
96	7.3 ± 0.09	6.1 ± 0.09	36.6 ± 0.17	36.4 ± 0.17

To evaluate whether these cation combinations are interacting in an additive, synergistic, or antagonistic manner, a modification of Colby's formula was applied. Table 2 presents data in which the expected and observed growth responses are compared. It can be seen that the combinations of cadmium-copper, and copper-cobalt both produced greater reductions in growth than would be predicted by application of Colby's formula; and that both the cadmium-cobalt, and three cation combinations

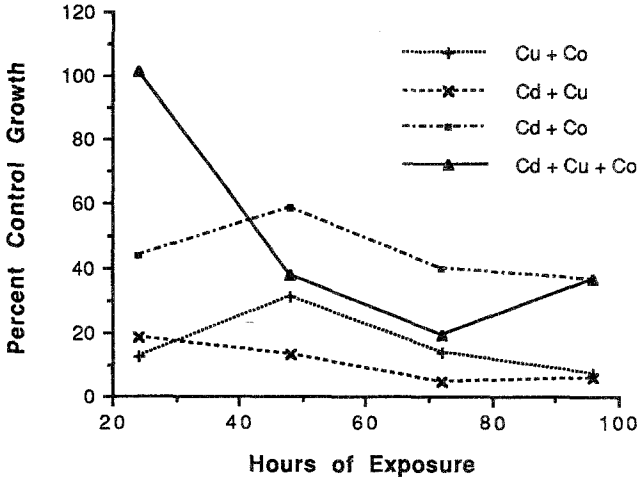

Fig. 2. Percent response of *Chlorella vulgaris* to exposure to various cation combinations

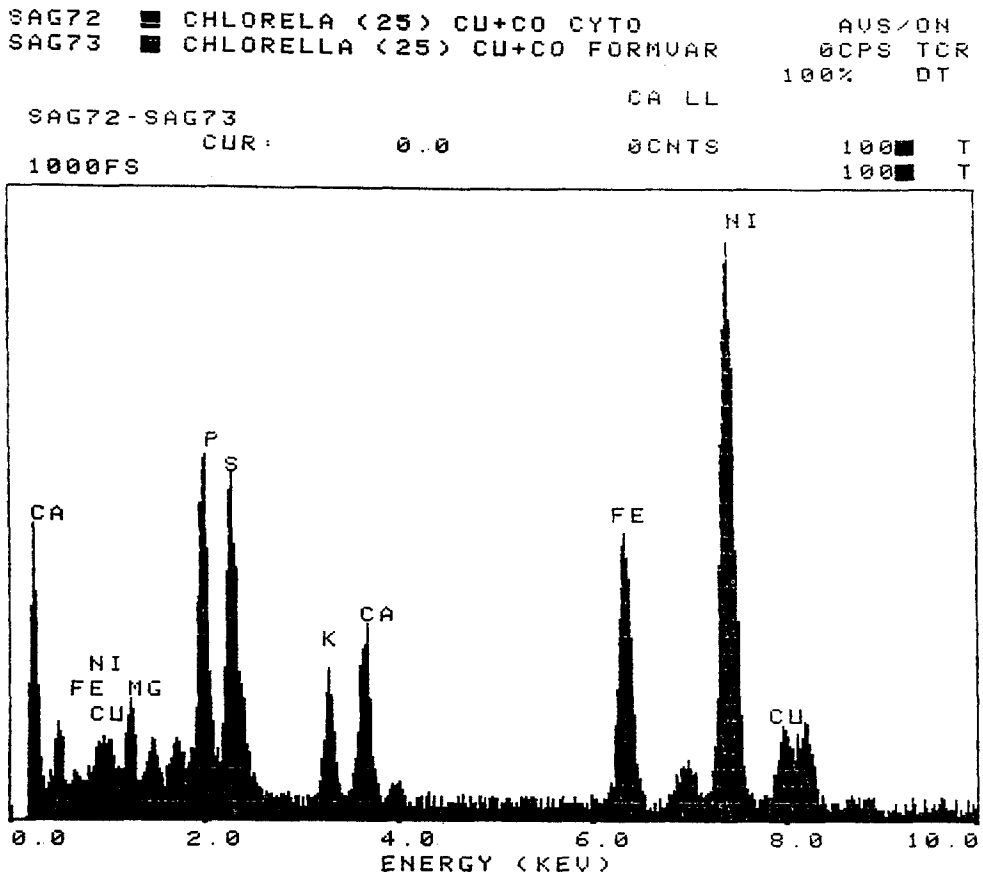

Fig. 3. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Cytoplasm with formvar platform background subtracted

resulted in growth responses greater (lower percent reduction) than predicted by application of the formula.

In order to evaluate the growth response of *Chlorella vulgaris* to these metal combinations in time frames shorter than the standard 96-h static trial procedure, replicate cultures were set up so that counts could be made at 24 h intervals over the entire 96 h test procedure. In all the bimetal combinations (Table 3, Figure 2) growth of the cells was less than 50% of control values at 24 h. The curves for Cu + Co and Cd + Co were approximately parallel throughout the 96 h exposure, with both showing a slight recovery at 48 h and then a continued decline in growth to the final 96 h low values. The Cd + Cu curve does not show this 48 h recovery, but instead shows a continued decline in growth through 72 h and then leveled off for the remaining 24 h of the study. The tri-metal combination showed a different response; at 24 h growth was same as in control cultures, and then there was a dramatic decline in growth at 48 h (38% of control), and at 72 h (19.4% of control). At 96 h, there was a slight recovery to 36.4% of control, which is the same level achieved by the Cd + Co bimetallic combination at the end of the 96 h test run.

Energy dispersive X-ray spectrometric studies for evaluation of cellular incorporation of metals yielded negative results for all metal combination trials. That is there was no evidence of any intracellular metal incorporation. Only the Cu + Co combination indicated any metal incorporation (Figures 3, 4, and 5); these figures, respectively, show the Cu + Co spectra for (1) the cytoplasm with the formvar platform background subtracted, (2) the spectra of the polyphosphate body, and (3) the spectra of the polyphosphate body with the background cytoplasm subtracted. A weak cytoplasmic copper peak, indicating slight cellular incorporation, is clearly evident (Figures 3, 4) at the 8.0 KEV position.

Discussion

The toxicity of cobalt to *Chlorella vulgaris* (9.0 μM) is less than either the toxicity of Cu (2.8 μM) or Cd (0.89 μM). The series for toxicity of these three cations to *Chlorella vulgaris* is Cd > Cu > Co. In confirmation runs a concentration of 9.5 μM cobalt yielded 50.7% reduction in the population growth of the alga after 96 h of exposure (Table 1). This was the cobalt dose selected for use in the combined metal studies, as it represents an actual tested EC_{50} concentration rather than the value of 9.0 μM estimated from the regression response curve (Figure 1).

All metal combinations resulted, after 96 h of exposure, in greater than the 50% growth reduction of the single cation treatments. Using our modification of Colby's formula as the response predictor, combinations of Cd + Cu and Cu + Co resulted in reductions in growth greater than that expected for these bimetallic combinations, and are, therefore, acting in a synergistic fashion (Table 2). This contrasts with the bimetallic combination of Cd + Co, and the tri-metal combination (Cd + Cu + Co), which gave growth reductions less than ex-

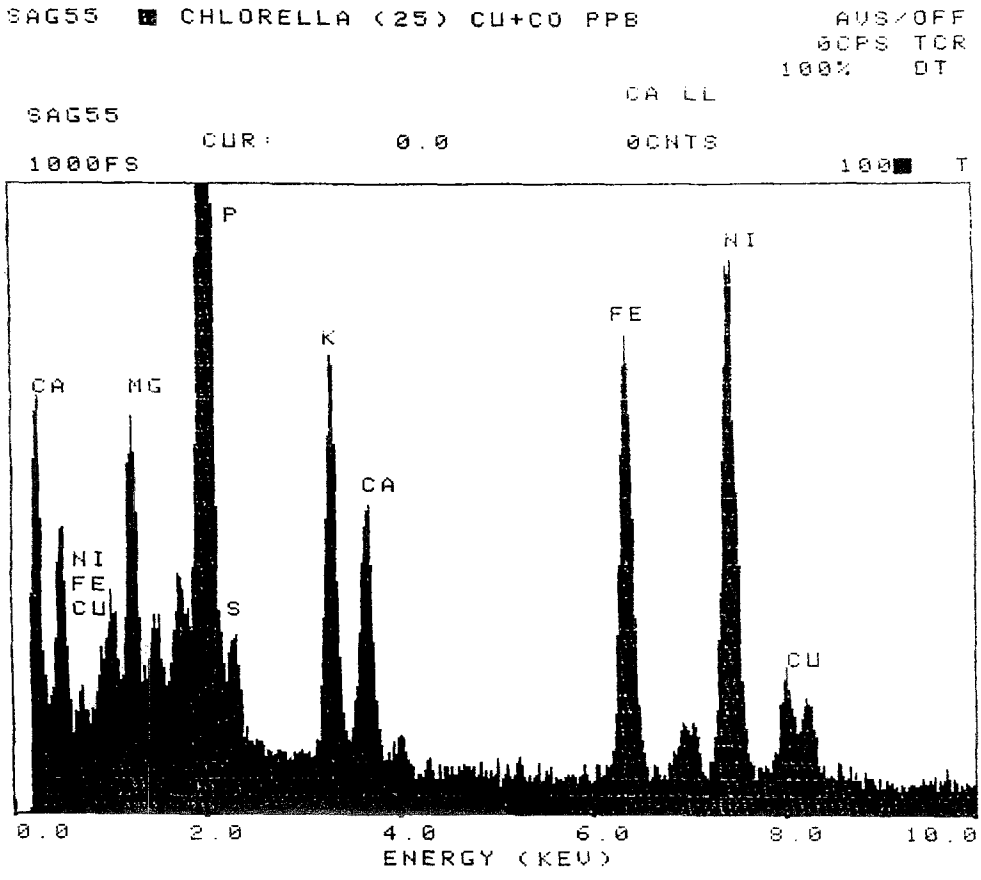

Fig. 4. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Polyphosphate body

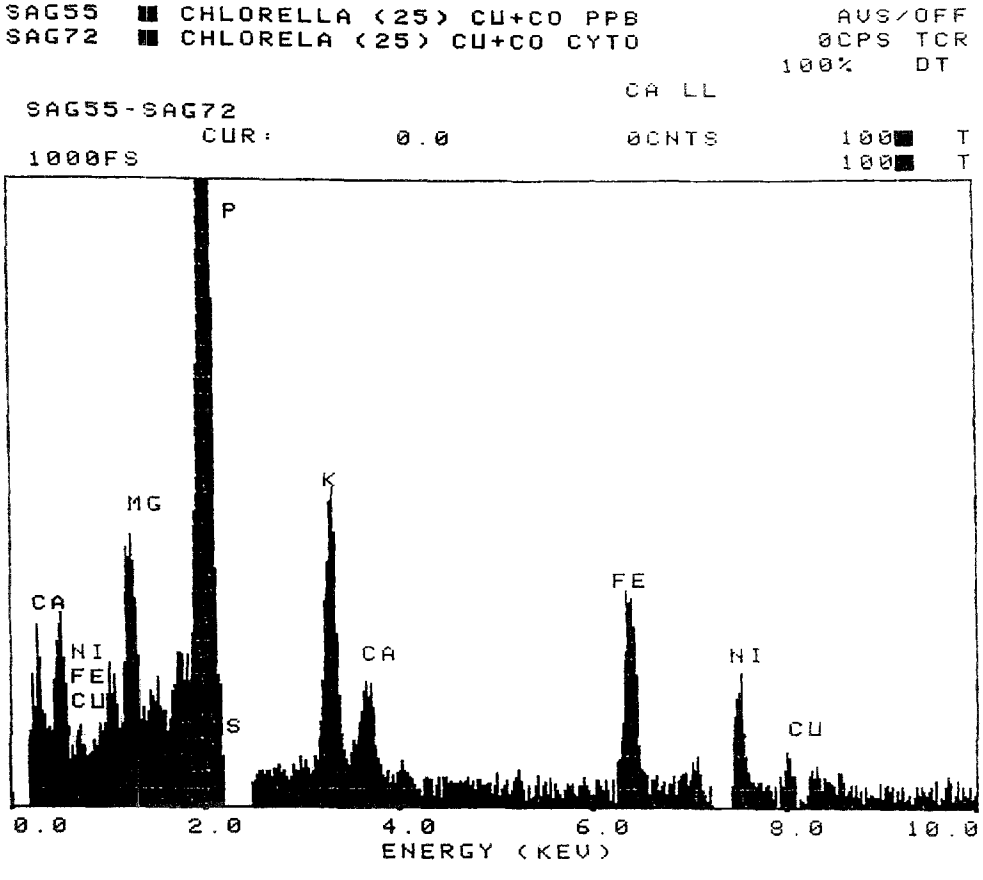

Fig. 5. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Polyphosphate body with background cytoplasm subtracted

pected for these combinations, indicating antagonistic interactions. These data demonstrate that Cu combined with either Cd or Co is more toxic than either Cd combined with Co or the tri-metal combination. The combination of the most toxic cation (Cd) with the least toxic action (Co) resulted in antagonistic interactions, even when Cu was added. The antagonistic interaction of Cd and Co seems to be great enough to overcome the demonstrated synergism between Cd and Cu. These results, coupled with the results of the energy dispersive X-ray spectrometric studies, in which no intracellular metal incorporation, other than a weak cytoplasmic copper peak at the 8.0 KEV position, was found (Figure 3, 4, and 5) strongly suggest that the effects of these metal interactions result from membrane phenomena rather than intracellular toxicity. That metals compete for adsorption sites on the plasma membrane and evoke a toxicity response by affecting what traverses the membrane is well known (Rosko and Rachlin 1975, 1977; Rai *et al.* 1981; Rachlin *et al.* 1982, 1984, 1985; Sunda 1988/89; Majidi *et al.* 1990; Xue and Sigg 1990; Ionouhe *et al.* 1991; Visviki and Rachlin 1991). The non-linear response to these cation combinations, when examined at each 24 h time interval (Table 3, Figure 2), indicates that the toxic response is not uniform over time. This is consistent with a response resulting from alterations in membrane permeability. That cations interact with sulfhydryl groups on proteinaceous membranes to produce -S-metal-S-bridges, which can then alter membrane permeability was demonstrated by Rothstein (1959), and Simkiss (1979) indicated the importance of proteins and lipoproteins in trapping metals, which provides a mechanism for removing cations from the cell as a detoxification mechanism. These actions might account for the slight recovery (Figure 2) observed at 48 h in the Cd + Co and Cu + Co trials and in the tri-metal (Cd + Cu + Co) combination at 96 h.

Acknowledgments. This research was supported in part by National Institute of General Medical Sciences Grant # 2SO6 GMO8 225/07, Subproject No. 443080 to J.W. Rachlin.

References

Baxter M, Jensen TE (1980) A study of methods for *in situ* X-ray energy dispersive analysis of polyphosphate bodies in *Plectonema boryanum*. Arch Microbiol 126:213–215

Bold HC (1949) Morphology of *Clamydomonas chlamydogama* sp. nov. Bull Torr Bot Club 76:101–108

Boudou A, Ribeyre F (1989a) Aquatic ecotoxicology: Fundamental concepts and methodologies. Vol. I CRC Press Inc, Boca Raton, FL

———, ———(1989b) Aquatic ecotoxicology: Fundamental concepts and methodologies. Vol. II. CRC Press Inc, Boca Raton, FL

Ionouhe M, Inagawa A, Morita M, Tohoyama H, Joho M, Murayama T (1991) Contributions of cell wall and metal-binding peptide to Cd and Cu tolerances in suspension-culture cells of tomato. Bot Mag Tokyo 104:217–482

Jensen TE, Rachlin JW, Jani V, Warkentine B (1982) An X-ray energy dispersive study of cellular compartmentalization of lead and zinc in *Chlorella saccharophila* (Chlorophyta), *Navicula incerta* and *Nitzschia closterium* (Bacillariophyta). Environ Exp Bot 22:319–328

Majidi V, Laude DA, Holcombe JA (1990) Investigations of the metal-algae binding site with ^{113}Cd nuclear magnetic resonance. Environ Science Technol 24:1309–1312

Rachlin JW, Farran M (1974) Growth response of the green algae *Chlorella vulgaris* to selected concentrations of zinc. Water Res 8:575–577

Rachlin JW, Grosso A (1991) The effects of pH on the growth of *Chlorella vulgaris* and its interactions with cadmium toxicity. Arch Environ Contam Toxicol 20:505–508

Rachlin JW, Jensen TE, Baxter M, Jani V (1982) Utilization of morphometric analysis in evaluating response of *Plectonema boryanum* (Cyanophyceae) to exposure to eight heavy metals. Arch Environ Contam Toxicol 11:323–333

Rachlin JW, Jensen TE, Warkentine B (1983) The growth response of the diatom *Navicula incerta* to selected concentrations of the metals: cadmium, copper, lead and zinc. Bull Torr Bot Club 110:217–223

———, ———, ———(1984) The toxicological response of the alga *Anabaena flos-aquae* (Cyanophyceae) to cadmium. Arch Environ Contam Toxicol 13:143–151

———, ———, ———(1985) Morphometric analysis of the response of *Anabaena flos-aquae* and *Anabaena variabilis* (Cyanophyceae) to selected concentrations of zinc. Arch Environ Contam Toxicol 14:395–402

Rai LC, Gaur JP, Kumar HD (1981) Phycology and heavy metal pollution. Biol Rev 56:99–151

Rai LC, Jensen TE, Rachlin JW (1990) A morphometric and X-ray energy dispersive approach to monitoring pH-altered cadmium toxicity in *Anabaena flos-aquae*. Arch Environ Contam Toxicol 19:479–487

Rosko JJ, Rachlin JW (1975) The effect of copper, zinc, cobalt and manganese on the growth of the marine diatom *Nitzschia closterium*. Bull Torr Bot Club 102:100–106

———, ———(1977) The effect of cadmium, copper, mercury, zinc and lead on cell division, growth, and chlorophyll a content of the chlorophyte *Chlorella vulgaris*. Bull Torr Bot Club 104:226–233

Rothstein A (1959) Cell membrane as site of action of heavy metals. Fed Proc Fed Amer Soc Exp Biol 18:1026–1028

Simkiss K (1979) Metal ions in cells. Endeavour 3:2–6

Sunda WG (1988/89) Trace metal interactions with marine phytoplankton. Biol Oceanog 6:411–442

Taylor GJ (1989) Multiple metal stress in *Triticum aestivum*. Differentiation between additive, multiplicative, antagonistic, and synergistic effects. Can J Bot 67:2272–2276

Taylor GJ, Stadt KJ (1990) Interactive effects of cadmium, copper, manganese, nickel, and zinc on root growth of wheat (*Triticum aestivum*) in solution. In: Van Beusichem ML (ed) Plant nutrition—physiology and applications. Proceedings of the 11th international plant nutrition colloquium, Wageningen, The Netherlands, July 30 to August 4, 1989. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 317–322

Visviki I, Rachlin JW (1991) The toxic action and interactions of copper and cadmium to the marine alga *Dunaliella minuta*, in both acute and chronic exposure. Arch Environ Contam Toxicol 20:271–275

Xue HB, Sigg L (1990) Binding of Cu(II) to algae in a metal buffer. Water Res 24:1129–1136

Manuscript received June 4, 1992 and in revised form July 3, 1992.