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Acute and chronic effects of copper and cadmium on the growth and ultrastructure of *Dunaliella minuta*, *Dunaliella salina* and *Chlamydomonas bullosa*

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ACUTE AND CHRONIC EFFECTS OF COPPER AND CADMIUM ON THE GROWTH AND ULTRASTRUCTURE OF *DUNALIELLA MINUTA*, *DUNALIELLA SALINA* AND *CHLAMYDOMONAS BULLOSA*.

by

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Abstract

ACUTE AND CHRONIC EFFECTS OF COPPER AND CADMIUM ON THE GROWTH AND ULTRASTRUCTURE OF *DUNALIELLA MINUTA*, *DUNALIELLA SALINA* AND *CHLAMYDOMONAS BULLOSA*.

by

Ioanna Visviki

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The effective copper and cadmium concentrations that limited the growth of three chlorophytes by 50%, EC(50), in 96 hour static experiments were determined. EC(50)s were 7.57 uM copper and 0.34 uM cadmium for *Dunaliella minuta*, 5.94 uM copper and 4.55 uM cadmium for *Dunaliella salina*, and 0.78 uM copper and 0.025 uM cadmium for *Chlamydomonas bullosa*. The relationship of the two cations was antagonistic towards the growth of the first species but synergistic for the latter two. At the ultrastructural level the relationship varied depending on the cellular component examined.

Exposure to 4.9×10^{-4} uM copper or 4.5×10^{-6} uM cadmium for eight months increased the tolerance of *D. minuta* towards its established Cu and Cd EC(50)s by 34% and 19% respectively, and the co-tolerance of Cu-treated cultures by 26%. Cd-treated *D. salina* was 50% more tolerant towards this cation, whereas Cu-treated cultures showed extreme sensitivity towards copper and "co-sensitivity" towards cadmium. Cu and Cd-treated *C. bullosa* cultures were 29% and

26% more sensitive towards copper and cadmium respectively. No changes in co-tolerance were observed.

At the ultrastructural level cadmium was shown to be more toxic than copper. Exposure to copper and/or cadmium affected a variety of cellular structures including chloroplast, pyrenoid, nucleus, starch granules, polyphosphate bodies, lipids, vacuoles, cell wall and periplasmalemmal space. In light of the observed ultra-structural changes several mechanisms of toxicity, as well as posssible detoxifying mechanisms are discussed.

Rachlin et al. (1982c) proposed that toxicological responses could reflect phylogenetic relationships and thus can be ordered within a taxonomic framework. The validity of the phylogenetic hypothesis was tested using the growth responses of the three chlorophytes to metal stress. Analysis of covariance of the cadmium generated regression lines indicated that the responses of all three chlorophytes were significantly different. Analysis of the growth data under copper stress showed, contrary to expectations, that the two non-related species, C. bullosa and D. minuta, responded to the cation in similar fashion, whereas the responses of D. salina and D. minuta were significantly different. It is concluded that the phylogenetic hypothesis does not hold true for the lower levels of taxonomic organization.

To my parents Dora and Athanassios
and my sisters Julie and Dimitra
with all my love

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Introduction

Heavy metals differ from other aquatic pollutants in that, unlike herbicides and pesticides, they do not biodegrade. Thus they can persist and accumulate in sedimentary sinks from where they may be re-released into the water column. Their persistance in water bodies coupled with continuous influx from anthropogenic sources threatens the productivity of aquatic ecosystems, and may alter the density, diversity, structure and species composition of aquatic communities (Moore and Ramamoorthy, 1984). Movement of these cations into the aquatic food web and their attendant bioaccumulation can even pose a threat to human health as was demonstrated for both mercury and cadmium in Japan (Forstner and Whittman, 1981; Mance, 1987).

Metals are elements which are good conductors of electricity, with electrical resistance directly proportional to the absolute temperature. Additionally, they exhibit high thermal conductivity, malleability and ductility (Forstner and Whittman, 1981). These physical properties make metals ideal for a wide variety of applications. Heavy metals have been usually distinguished from other metals by their high densities in excess of 5.0 g/cm^3. A precise and informative classification of metals based on metal chemical properties, and specifically on the kinetics of ligand formation, has been proposed by Nieboer and Richardson (1980). According to their classification

Class A Metals include those which preferentially bind to oxygen donor atoms in ligands, such as alkaline metals and alkaline-earth metals. Class B Metals include those which are attracted to sulphur groups in ligands (lead, mercury, precious and semi-precious metals). Borderline metals exhibit preferences intermediate between the other two classes in the process of ligand formation and include cadmium, copper, manganese, nickel and zinc. It is of interest that the elements which possess toxic properties, and have been traditionally called heavy metals, belong to the latter two classes.

Trace amounts of some heavy metals such as iron, cobalt, copper, zinc, manganese are necessary for the metabolic processes of organisms. Higher concentrations, however, are toxic. Heavy metals in general compete with essential metals for active enzyme or membrane sites, and react with biologically active groups such as carboxyl, phosphoryl, sulphhydryl, amino and thiol groups. Thus, they modify the active conformation of biomolecules, block their functional groups and displace essential metal ions, thereby interfering with metabolic pathways essential for normal cell functioning (Ochiai cited by Borovik, 1990).

Heavy metals are being added to waterbodies from industrial effluents, sewage, atmospheric fallout, geological weathering, mining and drilling. Once in the water the metal concentration is lowered, after the initial dilution, by precipitation, adsorption and absorption by

aquatic organisms. Precipitation occurs if the concentration of a metal is higher than the solubility of the least soluble compound that can be formed between the metal and ions in the water (Bryan, 1971).

Newly deposited precipitates are susceptible to physical and chemical remobilization. As organic material is degraded, metals are released in the order Cd > Zn > Cu > Pb. Subsequently their fate depends on the redox potential of the sediments and the water column (Livett, 1988).

Adsorption of metals from solution occurs on clay minerals, iron/manganese oxides and sulphides, carbonates, phosphates, on organic matter and phytoplanktonic organisms (Bryan, 1971; Livett, 1988). Thus heavy metals are partitioned among the water, the sediment and the biota. Their toxicity and availability to aquatic organisms depend on the concentration of free metal ions, the pH, and the extent of metal complexation by organic and inorganic ligands (Breteler et al., 1984; Sunda, 1991). It can also be influenced by temperature, salinity, water hardness, the presence of other pollutants, the sediment type and the microbial mass present (Rai et al., 1981; Berk and Colwell, 1981). Metal uptake may be affected by the age and physiological state of the organisms, as well as by aerobic and anaerobic conditions (Silverberg et al., 1976).

As has already been noted, metals, due to their unique toxic characteristics, pose a serious threat to aquatic ecosystems. An additional reason for the sensitivity of

these ecosystems to metal pollution, results from the structure of aquatic food webs. Compared to their terrestrial counterparts, aquatic trophic webs include a greater variety of trophic levels, whereby accumulation of metals can be enhanced (Forstner and Whittman, 1981). Therefore it is imperative to examine the effects of heavy metals on the various components of these trophic levels, and particularly the algae. Algae constitute the base of aquatic food webs and are almost exclusively responsible for marine net primary production which is estimated at 55.0×10^9 tons/year (Whittaker, 1975). Consequently, metal accumulation by phytoplankton and/or decrease of its productivity could have serious repercussions on other trophic levels. Additionally algae can be used as biological indicators to monitor spatial and temporal pollution trends (Phillips, 1977; Forstner and Whittman, 1981). Specifically, they can be used to identify contaminated areas, as well as to determine improvement, deterioration or stability of affected communities (Mance, 1987).

In spite of the importance of algae in photosynthesis and marine food webs, relatively little attention has been focused on them in terms of pollution research. Algae are usually chronically exposed to several metals, however, most studies offer little information on the effects of chronic metal exposure, or on metal interactions. In addition, few studies (Sicko-Goad and Stoermer, 1979; Silverberg et al., 1976; 1977; Rachlin et al., 1984; 1985) examine

simultaneously more than one level of organization correlating, for example, changes in physiological parameters with changes in cellular ultrastructure.

The present study attempts to contribute to the literature focusing on the effects of cadmium and copper on the growth and ultrastructure of the marine chlorophytes Chlamydomonas bullosa and Dunaliella minuta and the halophilic Dunaliella salina. Cadmium and copper have been selected for study, because the former poses the greatest threat for human health, while the latter is detrimental for natural ecosystems (Breteler et al., 1984). The effective concentrations, EC(50)s, of these cations that limit population growth by 50% in 96 hour static exposures are first determined. This information will contribute to an increasing data base which will allow the establishment of realistic, yet relatively safe pollutant levels by environmental agencies. The growth responses of the three chlorophytes to increasing metal concentrations are used to test the phylogenetic hypothesis advanced by Rachlin et al. (1982c). These researchers proposed that toxicological responses, like other physiological traits, are taxonomic characters. Thus algal responses to metal stress could reflect phylogenetic relationships, and therefore can be ordered within a taxonomic framework. An organizational framework of this kind could be a very valuable predictive tool. If it holds true, knowledge of the toxicological response of one taxon, could provide information about the

potential responses of other members of the taxon, and of other closely related taxa. The phylogenetic hypothesis has previously been tested only at the level of the division (Warkentine and Rachlin, 1986). In the present study its validity at the familial level is examined.

The acquisition of heavy metal tolerance in algae from polluted areas, like the acquisition of pesticide resistance in insects, or herbicide resistance in weeds is an example of evolution in action (Shaw, 1990). It may be due to genetic adaptation or due to physiological changes resulting from metal exposure. Here the possibility that low level, chronic metal exposure leads to metal adaptation is examined. The acquisition of co-tolerance is also investigated. Additionally, the nature of copper and cadmium interactions and their effects on algal growth are examined. Furthermore, morphometric analysis is carried out to determine the effects of these cations on chlorophyte ultrastructure after short term sublethal monometal exposure, short term dimetal exposure, and chronic low level monometal exposure. Morphometric data can provide much needed information on metal toxicity mechanisms and on algal defenses to metal stress. However, before these issues are explored, some background information on copper, cadmium and the target organisms selected is appropriate.

Copper is widely distributed in nature in free state, in sulfides, arsenides, chlorides and carbonates. It is universally used in the electrical, construction, plumbing

and automotive industries (Moore ana Ramamoorthy, 1984) and therefore, poses a potential environmental hazard through anthropogenic input. The total flux of copper is approximately 75,000 metric tons per year, of which 5,000-13,000 tons are deposited into the ocean through both wet and dry deposition (Nriagu, 1979a). Anthropogenic sources are responsible for 75% of the atmospheric emissions (Nriagu, 1979b). Additionally approximately 17,000 metric tons/year of solid copper waste are deposited into the ocean through mine tailings, flyash, fertilizer production, municipal and industrial sewage (Nriagu, 1979a). Copper residence time in the ocean is estimated to vary from 1,500 to 78,000 years (Nriagu, 1979c).

Copper is an essential component of the photosynthetic electron transport system and a component or cofactor of several enzymes (Kaplan et al., 1984). Trace amounts, therefore, are required for the metabolic processes of algae (Rai et al., 1981). Higher concentrations, however, are toxic, and copper in the form of copper sulphate has been widely used as an algicide to control undesirable algal growth. Copper's dual role as micronutrient and toxicant has been demonstrated by Sandman and Boger (1980). They reported that Skenedesmus acutus required 0.1-1.0 uM of copper for optimum growth. Chlorophyll became bleached in Cu-depleted cultures and growth decreased by 50%. Copper concentrations exceeding 10 uM on the other hand, were temporarily toxic leading to chlorophyll degradation and

oxidation of lipids. Other algal studies report that copper inhibits growth and photosynthesis, and like other metals, affects the permeability of the plasma membrane, causing loss of organic matter (Steeman-Nielsen and Wium Andersen, 1971), loss of potassium (Rai et al., 1981) and reduction in the uptake of essential elements and compounds, such as manganese (Sunda and Huntsman, 1983) and silicic acid (Rueter et al., 1981). Additionally, it has been shown to interfere with cell volume regulation in Dunaliella marina tranferred in anisotonic media (Riisgard, 1979; Riisgard et al., 1980), inhibit autospore liberation in Chlorella pyrenoidosa (Steeman-Nielsen et al., 1969), cause loss of motility in Gonyaulax tamarensis (Anderson and Morel, 1978), and to inhibit normal cell division leading to uncoupling of photosynthetic and division rates (Rosko and Rachlin, 1977; Fisher and Jones, 1981).

Cadmium is most commonly found associated with zinc in carbonate and sulfide ores. It is obtained as a by-product in the refining of copper, lead and zinc. In industry cadmium is mainly used for electroplating and for the production of pigments, plastic stabilizers and batteries (Moore and Ramamoorthy, 1984). Worldwide annual emissions of cadmium from natural sources, such as airborne soil particles, volcanogenic aerosols and forest fires, are approximately 8.43×10^5 kg. Industrial activities contribute 7.19×10^6 kg/year (Nriagu, 1979c). Precipitation effectively removes cadmium from the atmosphere. Its

residence time in the oceans, in turn, varies from 10^4 to 10^5 years (Balistrieri et al., 1981 cit. by Livett, 1988).

Cadmium, in contrast to copper, is believed to have no nutritive value for algae or other organisms. The findings of a recent study (Price and Morel, 1990), however, which showed that Cd can replace zinc in Zn-limited cultures of Thalassiosira weissflogii, suggest that the nutritional role of this cation should be reconsidered. Cadmium has been reported to inhibit photosythetic $^{14}CO_2$ uptake (Stratton and Corke, 1979) and calcium uptake (Pick et al., 1986a; 1986b), decrease algal growth rates (Rachlin et al., 1982b), cause loss of motility in Euglena gracilis (Fennikoh et al.,1978), and induce filament elongation, and loss of cellular contents in the apical cells of the filaments in Anabaena inaequalis (Stratton and Corke, 1979).

In humans the most serious case of cadmium poisoning, called Itai-itai disease, was diagnosed in residents of the Toyama Prefecture in Japan from 1940-1960. Untreated mine wastes discharged in local rivers heavily contaminated both drinking water and rice fields. Patients suffering from the disease exhibited skeletal deformation and renal dysfunction. Additionally epidemiological studies have established a causal link between cadmium exposure and cancer incidence (Moore and Ramamoorthy, 1984).

The selective criterion for the organisms used in this study was their taxonomic relationship, rather than their

ecological importance. Indeed, these species constitute a minor component of the algal flora of natural waters, with the exception of hypersaline waters, where Dunaliella dominates.

All three species belong to the division Chlorophyta, class Chlorophyceae (Mattox and Stewart, 1984), order Volvocales (Bold and Wynne, 1978). Ettl (1981) proposed the new class Chlamydophyceae to include chlamydomonad flagellates and nonmotile genera such as Chlorococcum. His assumption that the latter represent evolutionarily arrested stages of chlamydomonad development has been questioned. Additionally, his classification scheme has been criticized for placing closely related genera in different classes based on small differences (Mattox and Stewart, 1984). Thus, he assigns Dunaliella, which is very similar to Chlamydomonas, to the Chlorophyceae because it lacks a cell wall. The loss of the cell wall is secondary, and it is an adaptation to high salinity habitats. It is not significant enough to warrant recognition at such high taxonomic levels (Mattox and Stewart, 1984). Mattox and Stewart, on the other hand, have divided the traditional order Volvocales into Chlamydomonadales (unicellular flagellates) and Volvocales (motile colonies), because they consider the latter true unicellular organisms. However, according to Pickett-Heaps (1975) the intercellular connections present in Volvox are not equivalent to plasmodesmata of higher plants which are considered true indicators of

multicellularity. They are larger than those of higher plants, and usually contain a strand of endoplasmic reticulum and sometimes other small organelles. Therefore Volvox and its relatives are colonial organisms and since they are very similar to Chlamydomonas they should remain in the same order. For the reasons outlined above the traditional higher classification is followed here (Fritsch, 1935; Round, 1966).

The genus Chlamydomonas (family Chlamydomonadaceae) has worldwide distribution and is found in a variety of habitats. Species have been collected from tropical and temperate areas, arctic and alpine habitats, and they are found in soil, freshwater, marine and brackish waters (Harris, 1989). The most recent work on the genus (Ettl, 1976) lists 459 species divided into nine groups. Most species are ellipsoid or ovoid in shape and have clearly polar structure, with two anterior flagella and a single basal chloroplast. The cell wall is mainly composed of hydroxyproline-rich glycoproteins. Asexual reproduction takes place by longitudinal division of the cell. Usually two successive divisions occur to form four daughter cells, which are subsequently released from the mother cell wall (Bold and Wynne, 1978). Chlamydomonas has been considered the prototypical motile green alga and extensive research has been done on its physiology, biochemistry, genetics and ultrastructure (reviewed by Harris, 1989). Unfortunately, investigators have focused on a few favorite species such as

C. reinhardtii and C. moewisii. Very little information has been published on Chlamydomonas bullosa. Its ultrastructure has been described by Cann and Pennick (1982), and it occasionally appears in surveys of aquatic habitats.

The genus Dunaliella (family Polyblepharidaceae) contains 29 species (Massyuk, 1973 reviewed by Borowitzka and Borowitzka, 1988) which are widely distributed. They are found in fresh water, in the sea, on saline soil and hypersaline waters. They are unicellular, biflagellate organisms, morphologically similar to Chlamydomonas, but lacking a cell wall. They exhibit wide temperature and salinity tolerance. Dunaliella salina is the most halotolerant eukaryote known, but D. minuta has lower salinity optima (Borowitzka and Borowitzka, 1988). Due to their osmoregulatory capacity members of the genus have been studied intensively and extensive information about their physiology (Enhuber and Gimmler, 1980; Loeblich, 1982; Pick et al., 1986a; 1986b) and biochemistry (Tornabene et al., 1980; Sheffer et al., 1986) is known. In hypertonic media Dunaliella spp. produce and accumulate glycerol as an osmoregulatory solute (Borowitzka and Brown, 1974; Ben-Amotz et al., 1982b). In addition, D. salina produces and accumulates large amounts of β-carotene, which function to protect photosynthesis at high light intensities (Loeblich, 1982; Ben-Amotz and Avron, 1983). High glycerol and β-carotene production along with extreme halotolerance have made D. salina an ideal organism for mass culture

(Ritchmond, 1984; Ben-Amotz and Avron, 1982).

Materials and Methods

A-- Culture Maintenance

Axenic cultures of *Chlamydomonas bullosa* (CCAP 11/83) and *Dunaliella minuta* (CCAP 19/5) were obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Research Laboratory, Scotland. A pure isolate of *Dunaliella salina* (UTEX 1644) was obtained from the Starr Culture Collection of Algae, University of Texas. *Chlamydomonas bullosa* was maintained in Bristol's solution (Bold, 1949) to which proteose had been added (1g proteose/liter Bristol's). *D. salina* was maintained in AS100 (artificial water with 100g NaCl) (Starr, 1978). *D. minuta* grew successfully in modified LDM medium (Starr, 1978). AS100 and LDM were based on artificial water instead of natural seawater, in order to avoid metal complexation by natural chelators. Bristol's solution is chelator free, while the other two media contain very small amounts of B12, biotin and Tris buffer that could act as chelators (Sunda and Guillard, 1976). Therefore, the effective concentrations determined for cultures maintained in these media, can be slightly overestimated. All glassware was prewashed and aged in acid to prevent metal contamination. Subsequently, glassware and media were sterilized by standard autoclaving procedure.

Stock cultures were maintained in log phase in 50 ml of the appropriate medium in stoppered 125 ml glass Erlenmeyer flasks. Stock and test cultures were incubated in a

Sherer-Gillett RI-24 LTP growth chamber, illuminated with Arc-Ray cool white fluorescent lamps. Mean light intensity around the flasks was determined to be 14 Watts M^{-2} (60 uE M^{-2} sec^{-1}, photosynthetically active radiation 400-700 nm). The day/night program was 16:8 hours, and the incubation temperature was maintained at 15±1° C. D. salina was incubated in a Sherer CEL 25-7HL growth chamber at 25±1° C, and subjected to the same day/night regime. Mean light intensity in the chamber was maintained at 56 Watts M^{-2}.

B-- Acute Sublethal Monometal Exposure

Stock solutions of copper and cadmium five times the desired concentration were prepared by dilution of copper chloride and cadmium chloride respectively in deionized water. For the test runs 1 ml of the appropriate metal stock was added to 4 ml of sterile medium. Preliminary toxicity runs indicated the range within which the 96 hour EC(50) for each metal was expected to fall.

The cell count of the stock cultures was determined and dilution with either control or test medium was made to yield a final count of 2.5×10^5 cells/ml. Five ml of test or control medium, containing that number of cells/ml, were inoculated into sterile Falcon 30 ml plastic tissue culture flasks, and the initial pH was determined. The flasks were placed into the growth chamber for the 96 hour exposure period, and were agitated once every 24 hours with a Vortex-Genie table top mixer, to ensure homogeneous exposure

to the metal, and to prevent the establishment of anaerobic conditions, which have been shown to affect metal uptake (Silverberg et al., 1976) and toxicity (Hassal, 1962; Mc Brien and Hassal, 1967). Each test was run in triplicate. At the end of the 96 hour test period the pH was again determined and the cultures were agitated for homogeneity. Two replicate cell counts from each flask were made using a brightline hemocytometer. The EC(50) for each metal was calculated from the pooled data, by the method of probit analysis (Finney, 1964a; 1964b). Subsequently, the calculated EC(50) values were tested in triplicate to confirm accuracy.

The probit regression lines generated during the determination of the metal EC(50)s were compared by analysis of covariance (Sokal and Rohlf, 1981), in order to determine whether closely related taxa respond to metal stress in similar fashion.

C-- Acute Sublethal Dimetal Exposure

The effects of the simultaneous presence of copper and cadmium on growth were determined by exposing the three species to their established EC(50) concentrations for these metals for 96 hours, under the previously described conditions. Colby's formula (Colby, 1967) was used with the growth data to calculate the expected toxicity of the metals if the dimetallic action were additive (Appendix

I). Observed toxicities greater or less than the expected, respectively indicate synergistic or antagonistic interactions.

D-- Chronic Low Level Exposure

For chronic low level experiments, cultures of all three chlorophytes were centrifuged, the medium was removed, and the cells were overlaid with 50 ml of fresh medium containing the appropriate exposure concentrations of the test metals. Exposure concentrations were 4.9×10^{-4} uM Cu^{++} and 4.5×10^{-6} uM Cd^{++}. These values represent the median soluble water column metal concentrations found in the Lower New York Bight (Breteler et al., 1984). The values were chosen so that long term exposure concentrations would approximate metal levels found in natural environments.

Triplicate cultures kept in logarithmic growth phase and agitated daily were maintained in the growth chamber, under the temperature and light conditions previously described, for eight months. At the end of that period the cultures were centrifuged and covered with fresh metal-free medium. Four ml of culture were inoculated into sterile 30 ml plastic tissue culture flasks. To this was added either 1 ml of control (distilled wated) or 1 ml of test medium containing a metal concentration equal to five times the determined EC(50) concentration for that metal. The cell concentration was approximately 2.5×10^5/ml. The culture flasks were incubated in the growth chamber under the previously stipulated conditions for 96 hours. In addition,

the metal exposed cultures were challenged in parallel studies with the EC(50) concentration of the other test metal of this study. Thus, it was possible to determine whether chronic low level exposure to metal A conferred co-tolerance towards metal B. Based on the results, Indices of Tolerance were calculated using Hall's modified formula (Appendix I) (Hall, 1980).

E-- Morphometric Analysis

Algal specimens, following their exposure to copper and cadmium under the conditions described in sections B, C, D, were prepared for morphometric analysis. After the exposure period the cells were harvested via centrifugation, fixed in 3.5% gluteraldehyde for one hour at room temperature and postfixed in 1% osmium tetroxide for one hour at room temperature. Subsequently, they were dehydrated in 50%, 70%, 95% and 100% ethanol series and were embedded in Spurr's low viscocity medium under vacuum following standard procedures. The fixation technique used was a modification of that proposed by Haas and Saghy (reviewed by Hyat, 1972). The samples were polymerized overnight in a 65° C oven. Blocks were sectioned using a LKB (type 8801) ultra-microtome with a diamond knife. Ultrathin (90 mu) sections were picked up on 400-mesh copper grids and post stained with uranyl acetate for 10 minutes at room temperature. The sections were rinsed in absolute methanol, 100%, 80%, and 50% ethanol and finally in distilled water. In addition they were post stained in lead citrate for 30 seconds and were rinsed with

distilled water. The post staining methods used were modifications of the method proposed by Stampak and Ward (1964) and Reynolds (1963) for uranyl acetate and lead citrate respectively.

The sections were examined by transmission electron microscopy using an HITACHI HS-9. Chlamydomonas bullosa and Dunaliella salina cells were examined at a magnification of 5000x, whereas Dunaliella minuta cells were examined at a magnification of 7000x. A minimum of 30 photomicrographs, each representing a random section through a cell, were obtained for every treatment. Sections containing only cell wall or cell wall with no identifiable features were not included. Estimates of volume densities of various cellular components were obtained by grid point-counting (Fig. 1). Using the procedures for morphometric analysis (Sicko-Goad et al., 1977; Sicko-Goad and Stoermer, 1979; Rachlin et al., 1982a; 1985) the cells were analyzed for relative volume of nucleus, nucleolus, pyrenoid and chloroplast, the number and relative volume of Golgi, lipids, polyphosphate bodies and vacuoles, and the relative volume of mitochondria. For Chlamydomonas bullosa the relative volume of the cell wall, the relative volume of the periplasmalemmal space under the cell wall, the number and relative volume of membranous organelles and intranuclear vacuoles were also calculated. Additionally, the relative numbers (no./cell volume) of lipids, polyphosphate bodies, vacuoles, membranous organelles and intranuclear vacuoles were estimated, to

ensure that changes in the numbers of these cellular components were genuine, and not due to increases or decreases in cell volume. The mean and standard error were obtained for each measurement and the statistical significance of difference was determined by analysis of variance.

Results

A-- Acute Monometal Exposure

The regression equations of the probit response lines for copper and cadmium concentrations from which the respective EC(50) values were estimated are shown in Tables 1 to 3. The metal concentrations, EC(50s), which reduced the growth of Dunaliella minuta, D. salina and Chlamydomonas bullosa by 50% after 96 hours of static exposure are given in Table 4. From this table it is apparent that cadmium has a more toxic effect than copper on the growth of all three chlorophytes. 0.34 uM Cd^{++} reduced the growth of D. minuta by 50% compared to 7.57 uM Cu^{++}, indicating that this alga is 22.3 times more sensitive to the former metal. Cadmium sensitivity is even more pronounced in Chlamydomonas bullosa where the EC(50) values are 0.78 uM copper and 0.025 uM cadmium. Thus, C. bullosa is 31.2 times more tolerant toward copper. 5.94 uM Cu^{++} and 4.55 uM Cd^{++} are required to limit the growth of D. salina to 50% of the control. This species is only 1.3 times more sensitive towards cadmium than copper. Overall there is great variation in the sensitivity exhibited by the chlorophytes examined. Chlamydomonas bullosa is the most sensitive species being 9.7 times more susceptible to copper than the most copper resistant species, namely Dunaliella minuta. The difference in cadmium sensitivity is even more apparent with D. salina being 182.0 times more tolerant than Chlamydomonas. Evidently tolerance is not a function of cell size, since D. minuta which has

the greatest surface area/volume ratio, is the most tolerant species towards copper and intermediate in cadmium resistance.

Confirmation runs showed that the estimated EC(50) values produced a decrease in cell growth within 8.5% of the expected 50% reduction. Specifically, 7.57 uM Cu^{++} led to 54% of control growth or 8% of the expected response for Dunaliella minuta. In the same species cadmium concentration of 0.34 uM yielded 46.29% of control growth or 7.42% of the expected response. 5.94 uM Cu^{++} and 4.55 uM Cd^{++} respectively limited the growth of D. salina to 52.67% and 53.58% of the control or to 5.34% and 7.16% of the expected growth. Finally, Chlamydomonas bullosa challenged with 0.78 uM Cu^{++} or 0.025 uM Cd^{++} responded within 7.56% and 8.46% of the expected reduction, giving 53.78% and 54.23% of control growth for copper and cadmium respectively. It is concluded, therefore, that the estimated values for copper and cadmium are fair representations of the actual concentrations which will reduce the growth of these chlorophytes by 50%. Additionally, since the initial and final pH values for all test and control cultures remained at a constant 7.4 for D. minuta, 7.0 for C. bullosa and 7.5 for D. salina, it is evident that the reductions in growth are indeed due to metal toxicity and not pH related effects.

The regression lines generated from the determination of the metal EC(50)s were subjected to analysis of covariance

(Table 5) to determine whether the toxicological responses of D. minuta and D. salina were more parallel to each other, than either of them to the response of C. bullosa. ANCOVA of the copper generated regression lines showed that their slopes differ significantly (Table 5), and thus they cannot be represented by a single common regression line. Subsequent pairwise analysis of covariance revealed that the slopes of the growth responses of Dunaliella minuta and D. salina were significantly different ($P < 0.05$). In contrast, the slopes of the growth responses of D. minuta and C. bullosa did not differ significantly, indicating that the two not closely related species responded to copper in a more similar fashion than closely related ones. Analysis of the cadmium generated regression lines also showed significant differences in the responses of all three species (Table 5). Further pairwise analysis of covariance indicated that the toxicological responses of the three chlorophytes were unique and their slopes were not parallel.

B-- Acute Dimetal Exposure

The growth responses of the three species to combined metal exposure are presented in Table 6. The results clearly show that the relationship of the two metals varies depending on the target organism. D. minuta challenged with 7.57 uM Cu^{++} and 0.34 uM Cd^{++} exhibited 55.1% of control growth. D. salina exposed to 5.94 uM Cu^{++} and 4.55 uM Cd^{++} yielded only 7.60% of control growth. The toxic effect of both cations was even more pronounced in C. bullosa.

Simultaneous exposure to 0.78 uM Cu^{++} and 0.025uM Cd^{++} resulted in population growth 3.46% of the control. Had the dimetallic action been additive, a final population growth representing 25% of control growth would have been expected for all three chlorophytes. It is apparent, however, that copper and cadmium synergistically limit the growth of C. bullosa and D. salina, but act antagonistically towards D. minuta.

C-- Chronic Low level Exposure

The results of the long term copper exposure studies are presented in Table 7. D. minuta cultures exposed to $4.9x10^{-4}$ uM Cu for eight months increased their tolerance toward their established EC(50) copper concentration by 34%. In contrast, C. bullosa and D. salina, similarly treated, exhibited increased sensitivity. C. bullosa was 29% less tolerant to 0.78 uM Cu^{++}, while D. salina showed even more dramatic results decreasing below the initial population. Cu-treated cultures did not develop co-tolerance towards cadmium. D. minuta and C. bullosa challenged with their established EC(50) Cd^{++} concentrations responded within 14% and 6% of their expected tolerance respectively. D. salina, on the other hand, decreased below the initial population, thus indicating that chronic low level copper exposure resulted in heightened sensitivity towards both metals.

Indices of tolerance of cultures exposed to $4.5x10^{-6}$ uM Cd^{++} for eight months are shown on Table 8. D. minuta and D. salina increased their tolerance towards their established

cadmium EC(50)s by 19% and 50% respectively. Chlamydomonas bullosa became 26% more sensitive towards this cation, but showed no change in co-tolerance towards copper. D. minuta increased its co-tolerance towards 7.57 uM Cu^{++} by 26%, thus showing that chronic low level exposure to cadmium enhanced both the tolerance and co-tolerance of this alga. Cd-treated D. salina became extremely sensitive towards copper decreasing below the initial population level, while similarly treated C. bullosa maintained its co-tolerance towards copper responding within 8% of the expected.

D-- Morphometric analysis

The ultrastructural profiles of Dunaliella minuta control cells (Fig. 6), cells challenged with acute, sublethal copper and/or cadmium concentrations and cells chronically exposed to low levels of copper or cadmium are presented in Table 9 and 10. Comparison of the two tables clearly shows that short-term sublethal metal exposure produced greater effects on the cellular level than low level chronic exposure. Furthermore, cadmium or a combination of cadmium and copper were more toxic to D. minuta than copper. In fact the latter caused no significant changes in the cellular structures examined when compared with control. Exposure to 0.34 uM Cd^{++} caused a 23.27% decrease in relative chloroplast volume (Fig. 2), a 44.58% decrease in lipid number (Fig. 3). It also led to a 74.6% increase in the relative volume of starch granules (Fig. 4). As shown in Fig. 5-7, cells simultaneously exposed to 7.55 uM copper and

0.34 uM cadmium had 33.49% larger total cell volumes compared to controls. They exhibited a 41.46% decrease in relative pyrenoid volume (Fig. 8), but showed no significant difference in relative total chloroplast volume. Additionally their relative lipid volume increased by 130% (Fig. 9). The toxic power of the two cations was reversed in the chronically exposed cultures, with copper being toxic while cadmium produced no discernible effects. As shown in Fig. 8 cells grown in 4.9×10^{-4} had a 28% decrease in relative pyrenoid volume. Analysis of variance of the morphometric data, presented in Table 11, revealed that the changes discussed above are significant at the 5% probability level.

Comparison of the morphometric data with the toxicological results indicates that no simple correlation exists between the effects of the two metals on growth and their effects on algal ultrastructure. Their action is antagonisic towards the growth of *D. minuta* but varies at the cellular level depending on the cellular componed examined. For example, there is a significant decrease in relative chloroplast volume when the cells are exposed to cadmium, however, no such change is apparent in cells exposed to both metals. Thus, it seems that copper mitigates this Cd-induced effect, and in this case the relationship of the two metals is antagonistic. On the other hand, exposure to both cations produces a significant reduction of the pyrenoid, which is not present after short term monometal

exposure, but is evident after long term low level exposure to copper. Therefore, cadmium appears to contribute to the diminution of this organelle, accentuating the toxic effects of copper.

The morphometric data of Dunaliella salina control cells, cells exposed to acute sublethal copper and/or cadmium concentrations and cells chronically exposed to low levels of copper or cadmium are presented in Table 12 and 13. Comparison of control and metal treated cells clearly shows that acute exposure to cadmium has greater effects on cellular ultrastructure than copper exposure. Additionally, sublethal cadmium concentration affects a greater number of cellular components than low level concentration or combined sublethal metal concentrations. Fig. 10-12 show that 4.55 uM Cd^{++} causes a 70% increase in total cell volume, and a 24.35% decrease in the relative volume of the nucleus (Fig. 13). It does not affect the number of lipids, but as shown in Fig. 14, it causes a 91.72% increase in relative lipid volume. Simultaneous exposure to copper and cadmium has a negative effect on both relative lipid volume and lipid number (Fig. 15), leading to decreases of 68.28% and 57.05% respectively. Chronic treatment with 4.9×10^{-4} uM copper on the other hand, increases lipid numbers by 71.02% (Fig. 15) and the relative lipid volume by 211.72% (Fig. 14), while long term exposure to 4.5×10^{-6} uM Cd increases the lipid number by 28.75% and the relative lipid volume by 143%. ANOVA (Table 14) has shown that these

ultrastructural changes are significant at the 5% probability level.

The growth data of D. salina cultures exposed to both cations are not congruent with the morphometric results. Copper and cadmium act synergistically to severely limit the growth of this chlorophyte, however, at the cellular level their action is both synergistic and antagonistic. Acute exposure to cadmium leads to significant increase in total cell volume, decrease in relative nuclear volume and increase in relative lipid volume. In joint exposure copper appears to counteract these Cd-induced effects and no changes in total cell volume or relative nuclear volume are apparent, whereas relative lipid volume decreases by approximately 160% when compared with Cd-challenged cells, apparently the result of synergistic copper and cadmium action.

The ultrastructural profiles of Chlamydomonas bullosa control cells (Fig. 16), cells exposed to acute, sublethal concentrations of copper and/or cadmium, and cells grown in the presence of either 4.9×10^{-4} Cu^{++} or 4.5×10^{-6} Cd^{++} for eight months are shown in Table 15 and 16. Comparison of the morphometric data indicates that 96 hour exposure to sublethal copper and cadmium concentrations is more toxic to this chlorophyte than acute exposure to copper or cadmium alone. Acute sublethal exposure to the former leads to 30.95% decrease in the number of polyphosphate bodies

(Fig. 17), but does not alter their relative volume. Cells exposed to 0.025 uM Cd^{++} have total volumes 49.73% larger than controls (Fig. 18). Their relative pyrenoid volume, however, is reduced by 50.19% (Fig. 19). 0.78 uM Cu^{++} and 0.025 uM Cd^{++} cause a 72.56% increase in total cell volume (Fig. 18, 20a, 20b) and a 64.9% decrease in relative pyrenoid volume. At the same time they cause a tremendous increase in relative starch volume by 96.52% (Fig. 21), which effectively reduces the effective chloroplast volume (total chloroplast volume minus pyrenoid and starch volume) by 26.99% as shown in Fig. 22. Exposure to 4.9×10^{-4} uM Cu^{++} for eight months leads to a 24.24% increase in total cell volume and a 28.44% decrease in relative cell wall volume (Fig. 23). Fig. 24 shows that the space between the cell wall and the cell membrane is also reduced by 24.73%. Cells chronically exposed to low levels of cadmium have total volumes 51.89% larger than controls. It is obvious from Fig. 18 that their mean total cell volume is in fact very similar to that of cells challenged with 0.025 uM Cd^{++} for 96 hours, thus indicating that acute sublethal exposure and chronic low level exposure have the same effect on the cell volume of this chlorophyte. As is shown in Fig. 23 chronic exposure to this cation also leads to the reduction of the relative cell wall volume by 18.84%, and a 72.7% increase in the relative volume of vacuoles (Fig. 25). Analysis of variance has shown that all the ultrastructural changes discussed above are significantly different (Table 17).

An additional difference between control and experimental cells concerns the presence of membranous organelles (Fig. 26). Even though the relative number and relative volume of these structures is not significantly different in challenged and control cells, the frequency of cells carrying these organelles increases with metal exposure. 21.05% of control cells have membranous organelles compared to 45.45% and 43.33% of cells exposed to 0.025 uM Cd^{++} and $4.5x10^{-6}$ uM Cd^{++} respectively. 63.33% of cells challenged with both cations, 55.88% of cells chronically exposed to $4.9x10^{-4}$ uM Cu^{++} and 60.6% of cells exposed to 0.78 uM Cu^{++} also exhibit such organelles. Furthermore, 39.39% of C. bullosa cells exposed to this latter treatment have intranuclear vacuoles compared to only 5.26% of controls. It should be noted that there is no significant difference in the relative number or relative volume of intranuclear vacuoles of control or copper-treated cells.

Overall Chlamydomonas bullosa is the most sensitive of the species examined in this study. All the experimental treatments, but one, caused a significant increase in total cell volume. It is believed that in most cases this is due to metal inhibition of normal cell division, rather than due to changes in cell wall and cell membrane permeability. We are led to that conclusion because the periplasmalemmal space is not significantly different in treated cells (except after long term Cu-treatment) when compared to controls.

Comparison of the morphometric data with the toxicological results indicates that there is no simple correlation between the effects of copper and cadmium on growth, and their effects on algal ultrastructure. These cations act synergistically to limit the growth of C. bullosa, but their relationship at the cellular level varies depending on the cellular component under consideration. Exposure to 0.78 uM Cu^{++} causes a significant reduction in the relative number of polyphosphate bodies, whereas dimetal exposure has no effect, thus showing that the action of the two cations is antagonistic towards the number of polyphosphate bodies. Exposure to 0.025 uM Cd^{++} gives rise to cells with larger volumes than controls. Exposure to both metals enhances that trend, indicating that copper and cadmium act additively or synergistically to inhibit normal cell division.

Cu or Cd-treated cultures subsequently challenged with CuEC(50) or CdEC(50) respectively were shown to be more sensitive to these metals (Table 6 and 7). Morphometric analysis revealed that chronically exposed cells have significantly smaller relative cell wall volumes than controls. Their increased sensitivity, therefore could be at least partly attributed to this change, since a thiner cell wall would be a less effective barrier to heavy metal uptake.

Table 1. Estimation of the EC(50) values of copper and cadmium from the percent response of Dunaliella minuta after 96-hour exposure with respective regression equations.

	Conc. (uM)	Log Conc.	Percent Response	Empirical Probit
	4.910	0.6911	63.46	5.3425
Copper	7.397	0.8690	51.76	5.0426
	8.924	0.9505	44.00	4.8490

$Y = -1.867x + 6.641$ $r^2 = 0.992$

Log EC(50) = 0.8790

sLog EC(50) = 0.0004 S.E = 0.007

EC(50) = 7.57 $\pm$ 0.007 uM Cu^{++}

	0.107	-0.9706	85.62	6.0625
Cadmium	0.320	-0.4948	50.18	5.0050
	0.543	-0.2652	35.14	4.6174

$Y = -2.075x + 4.031$ $r^2 = 0.996$

Log EC(50) = -0.4670

sLog EC(50) = 0.0004 S.E = 0.0003

EC(50) = 0.34 $\pm$ 0.0003 uM Cd^{++}

Table 2. Estimation of the EC(50) values of copper and cadmium from the percent response of Dunaliella salina after 96-hour exposure with respective regression equations

	Conc. (uM)	Log. Conc.	Percent Response	Empirical Probit
	5.2085	0.7167	100.00	8.7190
Copper	5.9481	0.7744	48.79	4.9699
	$Y = -65.017x + 55.317$		$r^2 = 1.00$	
	Log EC(50) = 0.7744			
	EC(50) = 5.94 uM Cu^{++} *			
	3.8167	0.5817	100.00	8.7190
Cadmium	4.3683	0.6403	80.65	5.8669
	$Y = -48.651x + 37.019$		$r^2 = 1.00$	
	Log EC(50) = 0.6577			
	EC(50) = 4.55 uM Cd^{++} *			

* Due to the steep slope of the regression line, a third metal concentration could not be tested, and therefore the standard error could not be estimated.

Table 3. Estimation of the EC(50) values of copper and cadmium from the percent response of Chlamydomonas bullosa after 96-hour exposure with respective equations

	Conc. (uM)	Log. Conc.	Percent Response	Empirical Probit
	0.0441	-1.3560	100.00	8.7190
Copper	1.1172	0.0481	35.71	4.6335
	2.2345	0.3492	7.14	3.5316

$Y = -3.001x + 4.669$ $r^2 = 0.998$

Log EC(50) = -0.1103

sLog EC(50) = 0.142 S.E = 0.25

EC(50) = 0.78 $\pm$ 0.25

	0.0178	-1.7497	62.24	5.3107
Cadmium	0.0356	-1.4487	38.55	4.7102
	0.0534	-1.2726	23.65	4.2840

$Y = -2.135x + 1.586$ $r^2 = 0.997$

Log EC(50) = -1.5737

sLog EC(50) = 0.0382 S.E = 0.0023

EC(50) = 0.025 $\pm$ 0.0022 uM Cd^{++}

Table 4. The 96-hour EC(50) concentration of copper and cadmium to *Dunaliella minuta*, *D. salina* and *Chlamydomonas bullosa*

Species	Cu EC(50) (uM)	Cd EC(50) (uM)
Dunaliella minuta	7.57 ± 0.007	0.34 ± 0.0003
Dunaliella salina	5.94	4.55
Chlamydomonas bullosa	0.78 ± 0.25	0.025 ± 0.0022

Table 5. Analysis of covariance of regression lines generated from the determination of metal EC(50)s.

Metal	Species	MS	S^2yx	F-statistic
Copper	D. minuta			
	D. salina	3.1561	0.0661	F(2,3)=47.75 *
	C. bullosa			
	D. minuta			
	D. salina	6.205	0.0894	F(1,2)= 69.446 *
	D. minuta			
	C. bullosa	0.0447	0.0105	F(1,2)= 4.257
	D. salina			
	C. bullosa	6.262	0.0986	F(1,2)= 63.511 *
Cadmium	D. minuta			
	D. salina	2.7616	0.0027	F(2,3)= 1022.8 *
	C. bullosa			
	D. minuta			
	D. salina	3.8708	0.0033	F(1,2)= 1172.97*
	D. minuta			
	C. bullosa	1.4231	0.0029	F(1,2)= 490.724*

Table 5. Analysis of covariance of regression lines generated from the determination of metal EC(50)s.

Metal	Species	MS	S^2yx	F-statistic
Cadmium	*D. salina*			
	C. bullosa	4.565	0.0019	F(1,2)= 2402.63*

MS = mean square among slopes

S^2yx = average unexplained MS for groups

* significant difference at $P < 0.05$

Table 6. Effects of simultaneous exposure to copper and cadmium on population density

Species	Cu EC(50)+Cd EC(50) (uM)	Expected	Observed	Type of Interaction
D. minuta	7.57 + 0.34	25.00	55.10	Antagonistic
D. salina	5.94 + 4.55	25.00	7.60	Synergistic
C. bullosa	0.78 + 0.025	25.00	3.46	Synergistic

Table 7. Indices of tolerance of cultures exposed to 4.9×10^{-4} uM copper for eight months, followed by cation challenge.

Species	Challenge Cu EC(50)	Cation Cd EC(50)
Dunaliella minuta	0.84	0.43
Dunaliella salina	Below initial population	Below initial population
Chlamydomonas bullosa	0.21	0.53

Table 8. Indices of tolerance of cultures exposed to 4.5×10^{-6} uM cadmium for eight months, followed by cation challenge.

Species	Challenge Cu EC(50)	Cation Cd EC(50)
Dunaliella minuta	0.76	0.69
Dunaliella salina	Below initial population	1.00
Chlamydomonas bullosa	0.46	0.24

Table 9. Summary of morphometric data (mean value ± S.E) of Dunaliella minuta control cells and cells exposed to acute sublethal copper and/or cadmium concentrations (uM).

	Control	7.55 Cu^{++}	0.34 Cd^{++}	7.55 Cu + 0.34 Cd
Cell volume	61.54±3.63	72.51±3.85	69.97±2.81	82.15±3.74
Chloroplast rel. vol.	46.65±3.91	46.00±3.38	35.80±2.72	37.95±3.04
Golgi no.	1.15±0.10	1.25±0.13	1.32±0.15	1.57±0.16
Golgi r.v.	1.85±0.40	1.90±0.36	1.35±0.19	1.70±0.23
Lipid no.	5.81±0.65	8.06±1.17	3.22±0.36	9.92±1.07
Lipid r.v.	0.20±0.10	0.50±0.18	0.00	0.54±0.12
Polyphosphate body no.	2.27±0.21	1.86±0.18	1.38±0.18	1.56±0.16
Polyphosphate body r.v.	1.01±0.36	1.05±0.27	0.60±0.20	1.64±0.35
Vacuole no.	3.15±0.26	2.86±0.26	3.23±0.29	4.40±0.29
Vacuole r.v.	13.67±1.43	17.39±1.81	13.88±1.59	20.66±2.29
Mitochondria r.v.	3.41±0.40	2.96±0.33	3.06±0.31	2.50±0.31
Nucleus r.v.	15.10±1.32	14.90±1.02	15.70±1.12	13.90±1.00
Nucleolus r.v.	2.70±0.36	2.20±0.23	2.90±0.79	2.60±0.42
Pyrenoid r.v.	7.13±0.59	5.50±0.80	5.18±0.74	4.21±0.51
Starch r.v.	6.33±0.88	7.56±0.95	11.14±1.33	6.00±0.71

Table 10. Summary of morphometric data (mean value ± S.E) of *Dunaliella minuta* cells exposed to low levels of copper or cadmium for eight months.

	4.9×10^{-4} uM Cu^{++}	4.5×10^{-6} uM Cd^{++}
Cell volume	70.00±3.92	69.68±3.11
Chloroplast r.v.	44.90±2.98	38.60±2.98
Golgi no.	1.13±0.13	1.08±0.11
Golgi r.v.	1.70±0.40	2.27±
Lipid no.	3.8±0.80	6.61±0.95
Lipid r.v.	0.21±0.12	0.39±0.15
Polyphosphate body no.	2.08±0.22	2.76±0.31
Polyphosphate body r.v.	2.80±0.45	1.84±0.30
Vacuole no.	3.97±0.32	4.14±0.28
Vacuole r.v.	17.39±1.92	16.10±1.47
Mitochondria r.v.	4.79±0.93	2.42±0.29
Nucleus r.v.	13.20±1.03	16.90±0.94
Nucleolus r.v.	2.80±0.76	2.50±0.22
Pyrenoid r.v.	4.71±0.63	5.40±0.61
Starch r.v.	3.99±0.65	6.20±0.95

Table 11. Analysis of variance of cellular parameters of Dunaliella minuta control cells, cells exposed to acute sublethal copper and/or cadmium concentrations, and cells chronically exposed to low level copper or cadmium concentrations (uM).

Cellular Parameter	Treatment	Mean	SA^2	SW^2	F-statistic
Chloroplast					
r.v.	Control	46.65			
	0.34 Cd^{++}	35.80	21.50	3.9905	F(1,67)=5.388
Lipids r.no.	Control	9.40			
	0.34 Cd^{++}	4.60	3.410	0.2281	F(1,26)=14.95
Starch r.v.	Control	6.33			
	0.34 Cd^{++}	11.14	3.686	0.440	F(1,62)=8.377
Total Cell	Control	61.54			
	7.57 Cu^{++} + 0.34 Cd^{++}	82.15	7926.88	513.50	F(1,73)=15.44
Lipid r.v.	Control	0.20			
	7.57 Cu^{++} + 0.34 Cd^{++}	0.54	0.0194	0.00375	F(1,63)=5.173

Table 11 (continued)

Cellular Parameter	Treatment	Mean	SA^2	SW^2	F-statistic
Pyrenoid r.v.	Control	7.13			
	7.57 Cu^{++} + 0.34 Cd^{++}	4.21	0.698	0.04988	F(1,16)=13.994
Pyrenoid r.v.	Control	7.13			
	4.9×10^{-4} Cu^{++}	4.71	0.503	0.0676	F(1,33)=7.44

SA^2 = variation among treatments

SW^2 = variation within treatments

Table 12. Summary of the morphometric data (mean value ± S.E) of Dunaliella salina control cells and cells exposed to acute sublethal copper and/or cadmium concentrations.

	Control	5.94 Cu^{++}	4.55 Cd^{++}	5.94 Cu^{++} + 4.55 Cd^{++}
Cell volume	73.06±4.06	84.74±4.37	124.27±8.00	69.45±3.99
Chloroplast r.v.	56.04±4.51	55.00±3.41	69.60±15.00	47.60±3.64
Golgi no.	2.09±0.17	1.75±0.16	2.05±0.24	1.89±0.17
Golgi r.v.	1.79±0.23	1.70±0.22	1.35±0.19	1.60±0.35
Lipid no.	35.34±3.13	32.14±2.80	52.30±4.66	15.19±2.02
Lipid r.v.	1.45±0.31	1.40±0.22	2.78±0.42	0.46±0.17
Polyphosphate body no.	1.83±0.48	1.25	1.20	3.33
Polyphosphate body r.v.	-	-	-	-
Vacuole no.	6.12±0.60	5.53±0.49	4.18±0.70	4.76±0.32
Vacuole r.v.	1.94±0.43	1.75±0.22	1.96±0.37	2.03±0.26
Mitochondria r.v	-	-	-	-
Nucleus r.v.	16.47±1.11	16.95±1.42	12.46±1.32	16.70±1.11
Nucleolus r.v.	1.66±0.26	1.56±	1.00±0.29	1.19±0.24
Pyrenoid r.v.	6.23±0.72	7.60±0.72	10.60±0.62	5.60±0.78
Starch r.v.	14.90±1.71	13.70±1.40	10.70±1.22	16.69±1.87

Table 13. Summary of morphometric data (mean value ± S.E) of *Dunaliella salina* cells exposed to low levels of copper or cadmium for eight months.

	4.9×10^{-4} uM Cu^{++}	4.5×10^{-6} uM Cd^{++}
Cell volume	67.00±3.78	67.87±4.40
Chloroplast r.v.	57.34±5.48	53.80±4.99
Golgi no.	2.00±0.23	2.00±0.23
Golgi r.v.	2.06±0.32	1.36±0.39
Lipid no.	60.44±5.02	45.50±3.97
Lipid r.v.	4.52±0.55	3.53±0.43
Mitochondria r.v.	-	-
Nucleus r.v.	15.19±1.00	16.70±1.11
Nucleolus r.v.	-	1.00±0.32
Pyrenoid r.v.	5.5±0.56	6.40±0.77
Starch r.v.	16.49±1.83	16.95±2.75

Mitochondrial relative volumes were omitted because some mitochondrial cross sections under both control and experimental conditions appeared to be swollen.

Table 14. Analysis of variance of cellular parameters of Dunaliella salina control cells, cells exposed to acute sublethal metal concentrations and low level chronic metal concentrations (uM).

Cellular Parameter	Treatment	Mean	SA^2	SW^2	F-statistic
Total cell	Control	73.06			
	4.55 Cd^{++}	124.27	4516.64	1318.78	F(1,67)=34.25
Lipid r.v.	Control	1.45			
	4.55 Cd^{++}	2.78	0.3037	0.04589	F(1,31)=6.618
Nucleus r.v.	Control	16.47			
	4.55 Cd^{++}	12.46	1.9603	0.3397	F(1,48)=5.771
Lipid no.	Control	35.34			
	5.94 Cu^{++} + 4.55 Cd^{++}	15.18	115.537	4.971	F(1,65)=23.242
Lipid r.v.	Control	1.45			
	5.94 Cu^{++} + 4.55 Cd^{++}	0.46	0.1612	0.02204	F(1,64)=7.314
Lipid no	Control	35.34			
	4.9×10^{-4} Cu^{++}	60.44	324.24	12.992	F(1,67)=24.95

Table 14 (continued).

Cellular Parameter	Treatment	Mean	SA^2	SW^2	F-statistic
Lipid r.v.	Control	1.45			
	4.9×10^{-4}				
	Cu^{++}	4.52	1.6183	0.0677	F(1,67)=23.90
Lipid no	Control	35.34			
	4.5×10^{-6}				
	Cd^{++}	45.50	65.71	8.537	F(1,63)=7.697
Lipid r.v.	Control	1.45			
	4.5×10^{-6}				
	Cd^{++}	3.53	0.696	0.04316	F(1,63)=16.126

SA^2 = Variation among treatments

SW^2 = Variation within treatments

Table 15. Summary of the morphometric data (mean value ± S.E) of Chlamydomonas bullosa control cells and cells exposed to acute sublethal copper or cadmium concentrations (uM).

	Control	0.78 Cu^{++}	0.025 Cd^{++}
Cell volume (A)	109.16±6.92	125.55±7.13	163.45±8.33
Cell without cell wall (B) r.v.	85.80±5.64	84.90±5.10	82.00±4.43
(A-B) r.v.	14.15±1.02	15.00±1.12	16.60±1.16
Cell wall r.v.	8.65±0.42	10.42±0.95	9.30±0.43
Chloroplast r.v.	49.22±4.33	47.90±3.87	48.50±3.16
Golgi no.	1.62±0.14	1.58±0.19	1.67±0.13
Golgi r.v.	0.63±0.16	0.60±0.17	0.46±0.08
Lipid no.	19.42±2.03	12.25±1.08	32.27±3.08
Lipid r.v.	0.45±0.10	0.20±0.06	0.44±0.13
Polyphosphate body no.	22.45±2.33	15.50±1.74	28.39±3.02
Polyphosphate body r.v.	1.70±0.23	2.10±0.33	2.20±0.28
Vacuole no.	7.92±0.74	10.12±0.80	13.82±1.13
Vacuole r.v.	9.68±0.01	11.00±1.18	10.70±0.84

Table 15 (continued)

	Control	0.78 Cu^{++}	0.025 Cd^{++}
Membranous organelle no.	1.75±0.37	2.00±0.25	1.87±0.31
Membranous organelle r.v.	0.80±0.20	0.79±0.20	0.60±0.16
Mitochondria r.v	2.82±0.40	2.83±0.29	2.18±0.22
Nucleus r.v.	9.96±1.02	9.00±0.95	9.10±1.04
Nucleolus r.v.	1.56±0.30	1.36±0.20	1.40±0.30
Pyrenoid r.v.	7.75±1.06	4.98±0.01	3.86±0.70
Starch r.v.	4.60±0.77	4.80±0.91	4.10±0.55

Table 16. Summary of the morphometric data (mean value ± S.E) of Chlamydomonas bullosa cells exposed to acute sublethal copper and cadmium concentrations, and cells exposed to low levels of copper or cadmium (uM) for eight months.

	0.78 Cu^{++} + 0.025 Cd^{++}	$4.9x10^{-4}$ Cu^{++}	$4.5x10^{-6}$ Cd^{++}
Cell volume (A)	188.37±13.49	135.62±5.65	165.80±9.40
Cell without cell wall (B) r.v.	84.40±5.47	89.30±3.88	88.00±5.17
(A-B) r.v	15.60±2.17	10.65±0.83	11.45±1.26
Cell wall r.v.	9.65±0.90	6.19±0.43	7.02±0.57
Chloroplast r.v.	43.28±3.23	46.90±2.86	45.00±3.00
Golgi no.	2.33±0.23	1.50±0.14	1.60±0.13
Golgi r.v.	0.50±0.10	0.56±0.08	0.60±0.09
Lipid no.	41.13±3.50	28.26±2.41	22.07±3.44
Lipid r.v.	0.46±0.10	0.17±0.07	0.12±0.06
Polyphosphate body no.	20.14±3.41	23.59±2.13	24.79±3.27
Polyphosphate body r.v.	1.40±0.19	2.40±0.42	3.30±0.39
Vacuole no.	17.17±1.57	10.50±0.49	10.60±0.84
Vacuole r.v.	13.35±1.33	12.28±0.70	17.69±1.50

Table 16 (continued)

	0.78 Cu^{++} + 0.025 Cd^{++}	4.9×10^{-4} Cu^{++}	4.5×10^{-6} Cd^{++}
Membranous organelle no.	2.42±0.22	1.79±0.17	1.69±0.26
Membranous organelle r.v.	0.47±0.12	0.50±0.17	0.50±0.17
Mitochondria r.v.	2.50±0.30	2.20±0.21	2.20±0.22
Nucleus r.v.	7.56±0.79	9.30±0.95	9.90±0.77
Nucleolus r.v.	1.10±0.23	1.40±0.14	1.70±0.002
Pyrenoid r.v.	2.72±0.40	5.40±0.54	5.00±0.71
Starch r.v.	13.64±1.44	4.40±0.74	4.60±0.97

Table 17. Analysis of variance of cellular parameters of Chlamydomonas bullosa control cells, cells exposed to acute sublethal copper and/or cadmium concentrations and cells chronically exposed to low levels of copper or cadmium (uM).

Cellular	Treatment	Mean	SA^2	SW^2	F-statistic
Treatment					
Polyphosphate body no.	Control	20.57			
	0.78 Cu^{++}	12.35	0.1166	0.0222	F(1,68)=9.5409
Total Cell	Control	109.16			
	0.025 Cd^{++}	163.45	52069.72	2041.96	F(1,69)=25.50
Pyrenoid r.v	Control	7.75			
	0.057 Cd^{++}	3.86	0.01476	0.0015	F(1,38)=10.04
Total Cell	Control	188.36			
	0.78 Cu^{++} $\pm$ 0.025 Cd^{++}	188.36	105182.3	3420.88	F(1,66)=30.75
Functional Chloroplast r.v	0.78 Cu^{++}	36.87			
	$\pm$ 0.025 Cd^{++}	26.92	0.2972	0.0307	F(1,66)=9.672

Table 17 (continued)

Cellular Treatment	Treatment	Mean	SA^2	SW^2	F-statistic
Pyrenoid r.v	Control	7.75			
	0.78 Cu^{+1} + 0.025 Cd^{++}	2.72	0.02084	0.001	F(1,31)=18.434
Starch r.v	Control	4.60			
	0.78 Cu^{++} + 0.025 Cd^{++}	13.64	0.1320	0.004	F(1,63)=34.137
Total Cell	Control	109.16			
	$4.9x10^{-4}$ Cu^{++}	135.62	12563.24	1473.22	F(1,70)=8.53
Cell Wall	Control	8.65			
	$4.9x10^{-4}$ Cu^{++}	6.19	0.01084	0.0007	F(1,70)=16.42

Table 17 (continued)

Cellular Treatment	Treatment	Mean	SA^2	SW^2	F-statistic
Treatment					
(A-B)* r.v	Control	14.15			
	4.9×10^{-4}				
	Cu^{++}	10.65	0.02194	0.003	F(1,70)=6.805
Total Cell	Control	109.16			
	4.5×10^{-6}				
	Cd^{++}	165.80	53786.68	2185.88	F(1,66)=24.61
Cell Wall	Control	8.65			
	4.5×10^{-6}				
	Cd^{++}	7.02	0.005	0.0008	F(1,68)=5.546
Vacuole r.v	Control	9.68			
	4.5×10^{-6}				
	Cd^{++}	17.69	0.1075	0.006	F(1,66)=16.86

SA^2 = Variation among treatments

SW^2 = Variation within treatments

(A-B) = Periplasmalemmal space

Figure 1: Electron micrograph of a Chlamydomonas bullosa control cell (magnification 14,750) overlayed with an one centimeter (magnification 1.15) counting grid.

Figure 2: Histogram showing the mean values ± S.E of the relative chloroplast volumes of *Dunaliella minuta* control cells (Cnt), and cells exposed to 0.34 uM cadmium (Cd) for 96 hours. Bold lines indicate the positions of mean values. The parallel hatched areas represent one standard error.

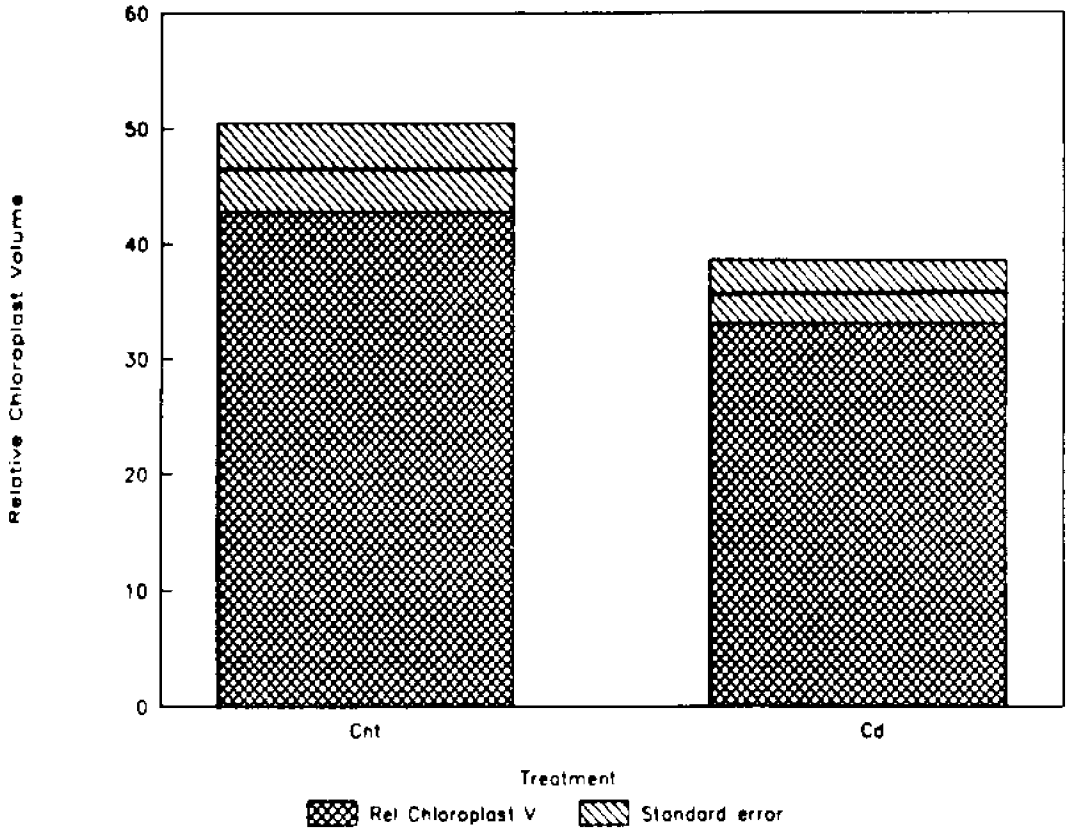

60
50
40
30
20
10
0
Relative Chloroplast Volume
Cnt
Cd
Treatment
Rel Chloroplast V
Standard error

Figure 3: Histogram showing the mean values ± S.E of lipid numbers of Dunaliella minuta control cells (Cnt) and cells exposed to 0.34 uM cadmium (Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

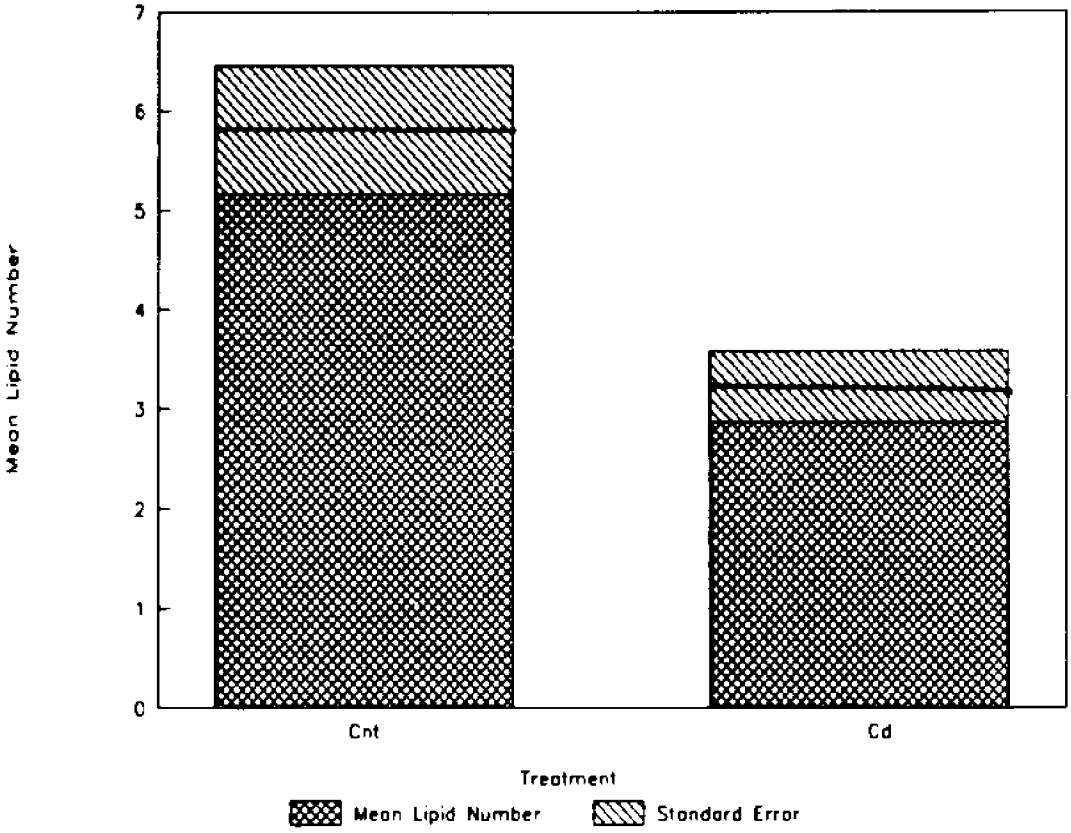

Mean Lipid Number
7
6
5
4
3
2
1
0
Cnt
Cd
Treatment
Mean Lipid Number
Standard Error

Figure 4: Histogram showing the mean values ± S.E of relative starch granule volumes of *Dunaliella minuta* control cells (Cnt), and cells exposed to 0.34 uM cadmium for 96 hours (Cd). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

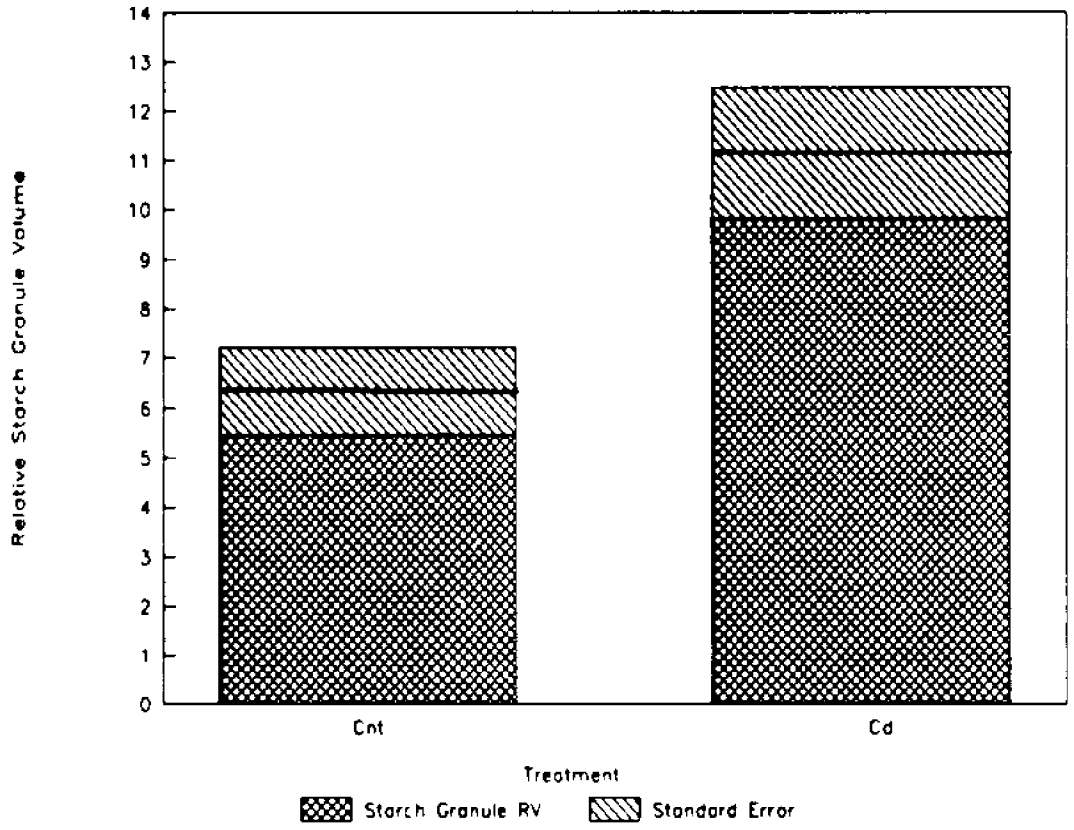

Relative Starch Granule Volume
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cd
Treatment
Starch Granule RV
Standard Error

Figure 5: Histogram showing the mean values ± S.E of total cell volumes of *Dunaliella minuta* control cells (Cnt), and cells exposed to 7.57 uM copper and 0.34 uM cadmium (Cu+Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

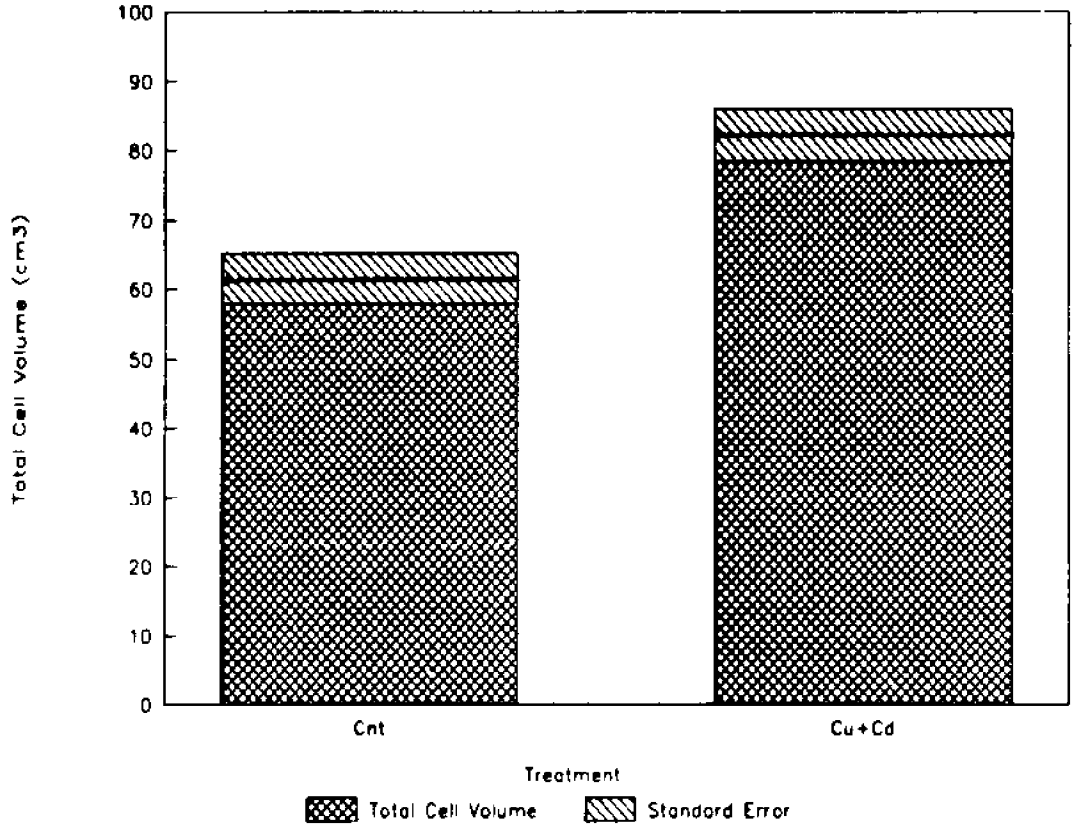

100
90
80
70
60
50
40
30
20
10
0
Total Cell Volume (cm3)
Cnt
Cu+Cd
Treatment
Total Cell Volume
Standard Error

Figure 6: Electron micrograph of a Dunaliella minuta control cell (magnification 12,880). c = chloroplast, f = flagellum, g = Golgi, m = mitochondrion, n = nucleus, p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids.

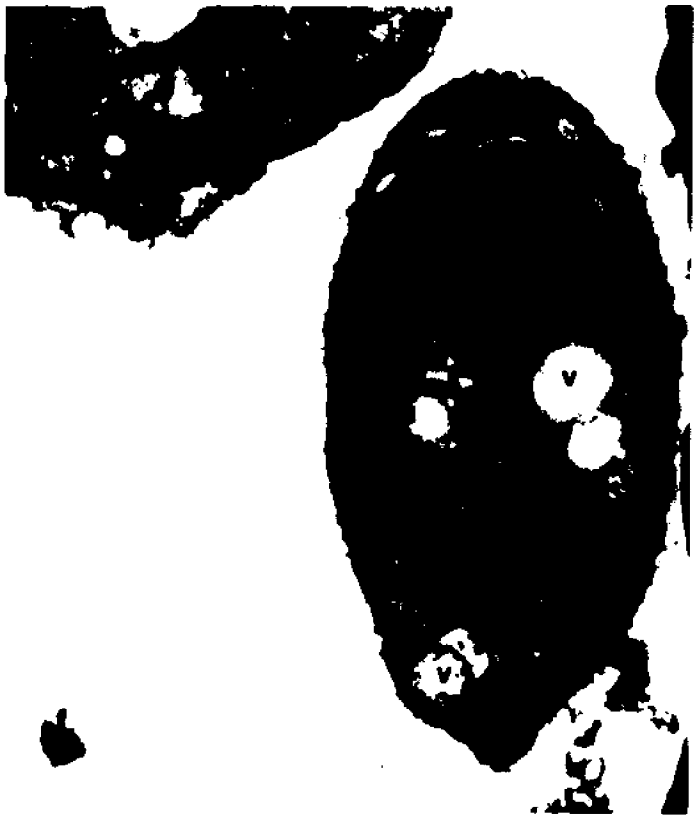


Figure 7: Electron micrograph of a Dunaliella minuta cell (magnification 12,880) exposed to 7.57 uM copper and 0.34 uM cadmium for 96 hours. c = chloroplast, g = Golgi, n = nucleus, nu = nucleolus, p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids.

Figure 8: Histogram showing the mean values ± S.E of the relative pyrenoid volume of Dunaliella minuta control cells (Cnt), cells exposed to 7.57 uM copper and 0.34 uM cadmium (Cu+Cd), and cells chronically exposed to 4.9×10^{-4} uM copper (Cu-LT). Bold lines indicate the position of mean values. Parallel hatched areas represent one standard error.

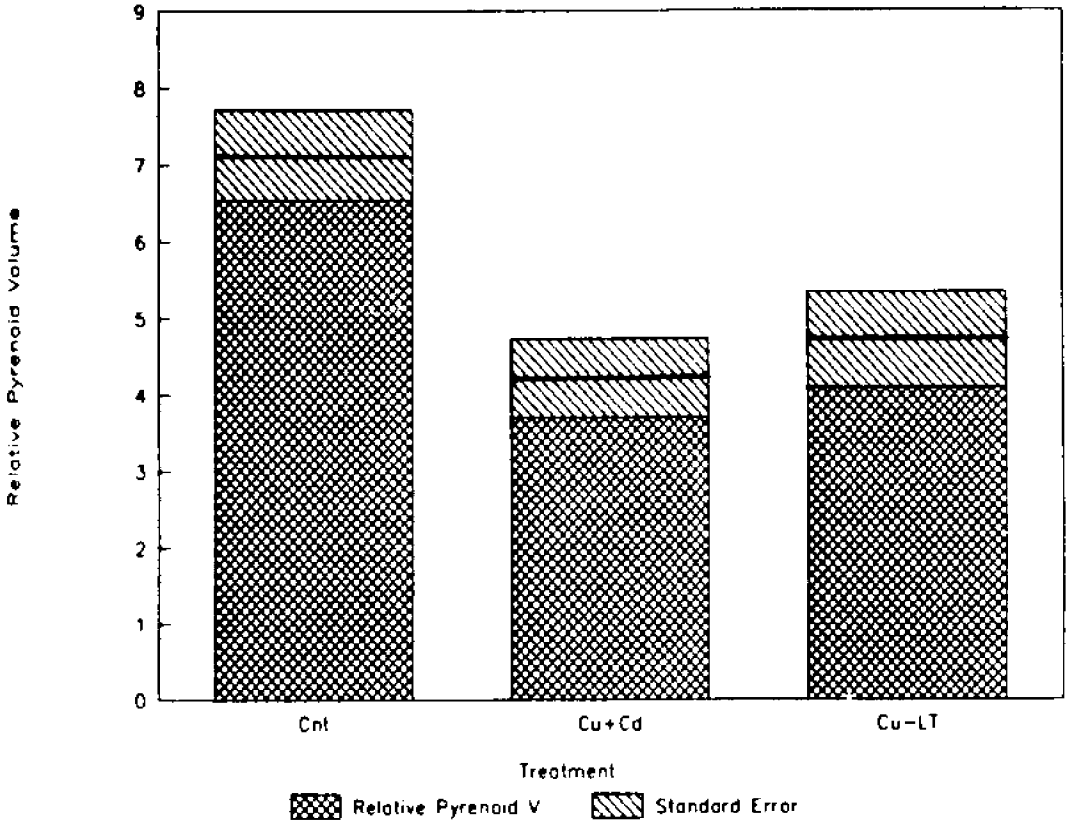

Relative Pyrenoid Volume
9
8
7
6
5
4
3
2
1
0
Cnt
Cu+Cd
Cu-LT
Treatment
Relative Pyrenoid V
Standard Error

Figure 9: Histogram showing the mean values ± S.E of relative lipid volumes of Dunaliella minuta control cells (Cnt) and cells exposed to 7.57 uM copper and 0.34 uM cadmium (Cu+Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

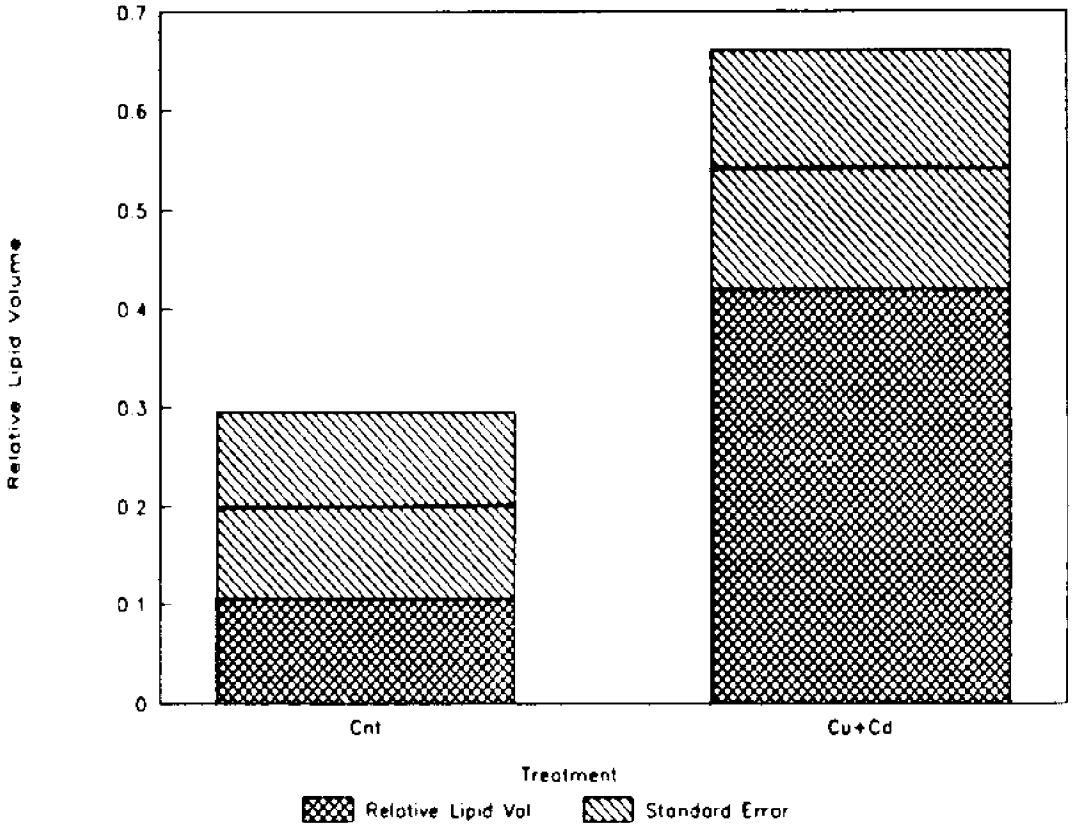

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative Lipid Volume
Cnt
Cu+Cd
Treatment
Relative Lipid Vol
Standard Error

Figure 10: Histogram showing the mean values ± S.E of total cell volumes of *Dunaliella salina* control cells (Cnt) and cells exposed to 4.55 uM cadmium (Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

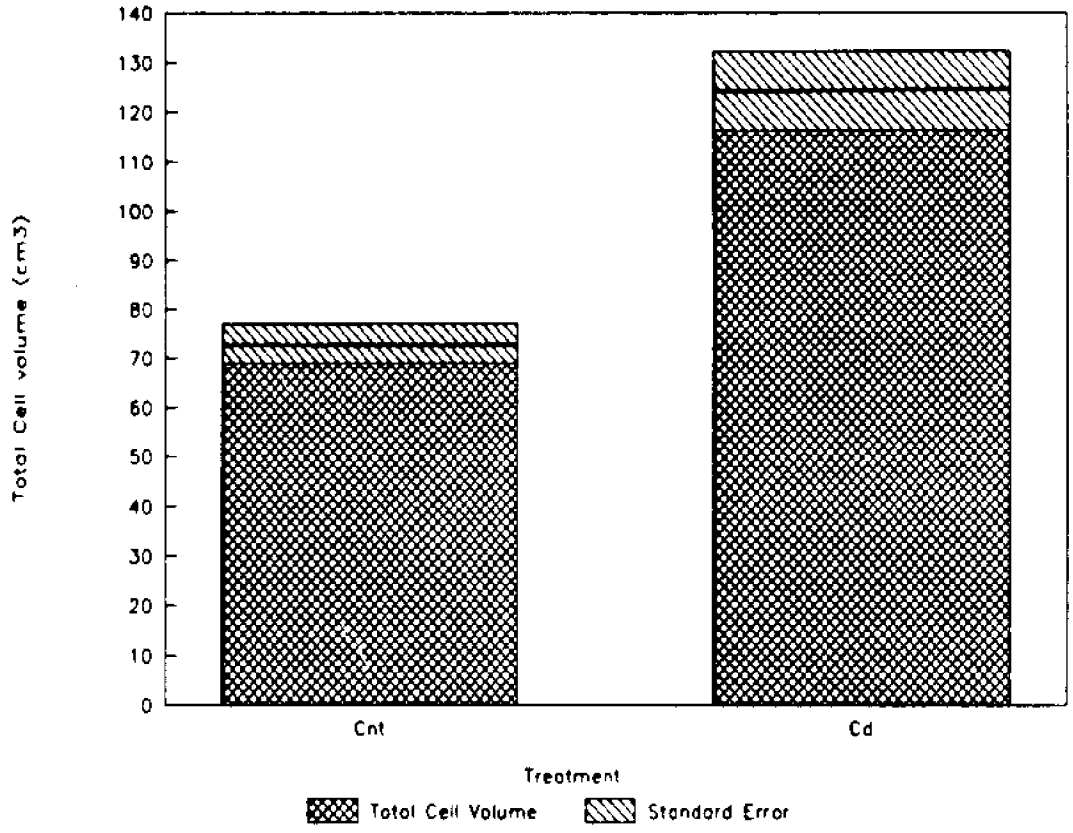

Total Cell volume (cm3)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Cnt
Cd
Treatment
Total Cell Volume
Standard Error

Figure 11: Electron micrograph of a Dunaliella salina control cell (magnification 9,200). c = chloroplast, g = Golgi, m = mitochondrion, n = nucleus, p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids.

Figure 12: Electron micrograph of a Dunaliella salina cell (magnification 9,200) cell exposed to 4.55 uM cadmium for 96 hours. c = chloroplast, g = Golgi, m = mitochondrion, n = nucleus, p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids.

Figure 13: Histogram showing the mean values ± S.E of relative nuclear volumes of *Dunaliella salina* control cells (Cnt) and cells exposed to 4.55 uM cadmium (Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

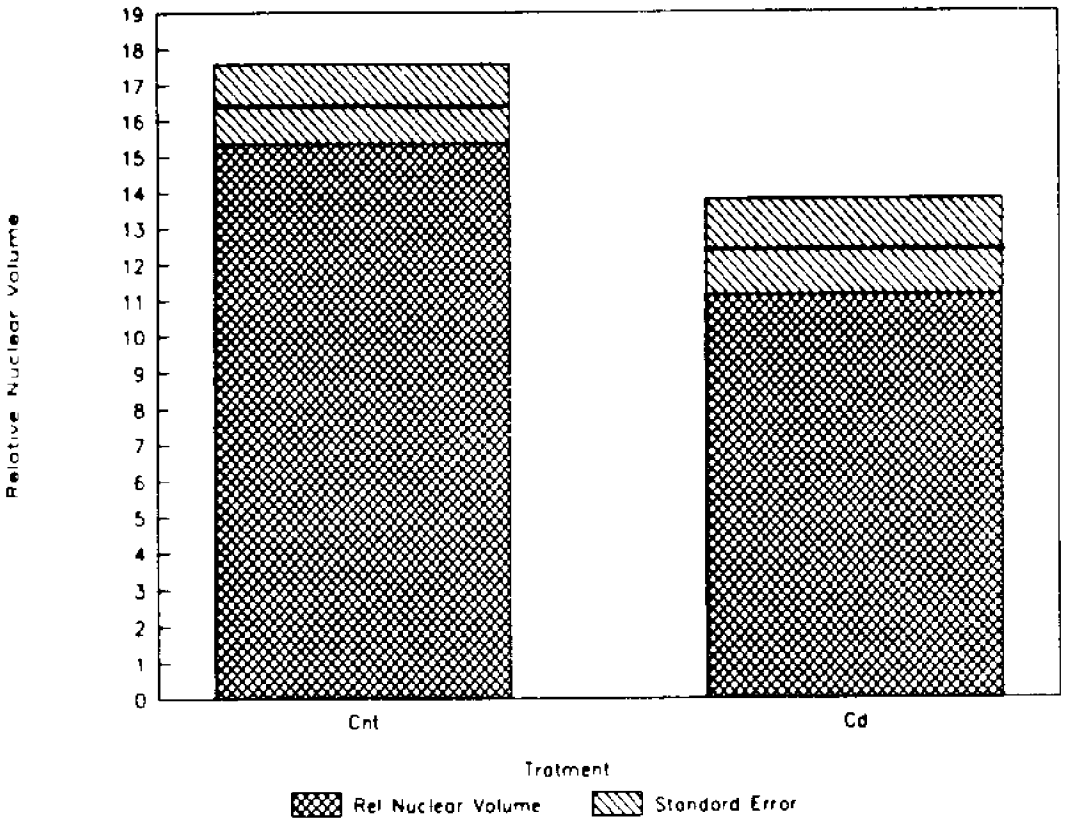

Relative Nuclear Volume
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cd
Tratment
Rel Nuclear Volume
Standard Error

Figure 14: Histogram showing the mean values + S.E of relative lipid volumes of *Dunaliella salina* control cells (Cnt), cells exposed to 4.55 uM cadmium (Cd) for 96 hours, cells exposed to 5.95 uM copper and 4.55 uM cadmium (Cu+Cd) for 96 hours, cells chronically exposed to 4.9×10^{-4} copper (Cu-LT), and cells chronically exposed to 4.5×10^{-6} cadmium (Cd-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

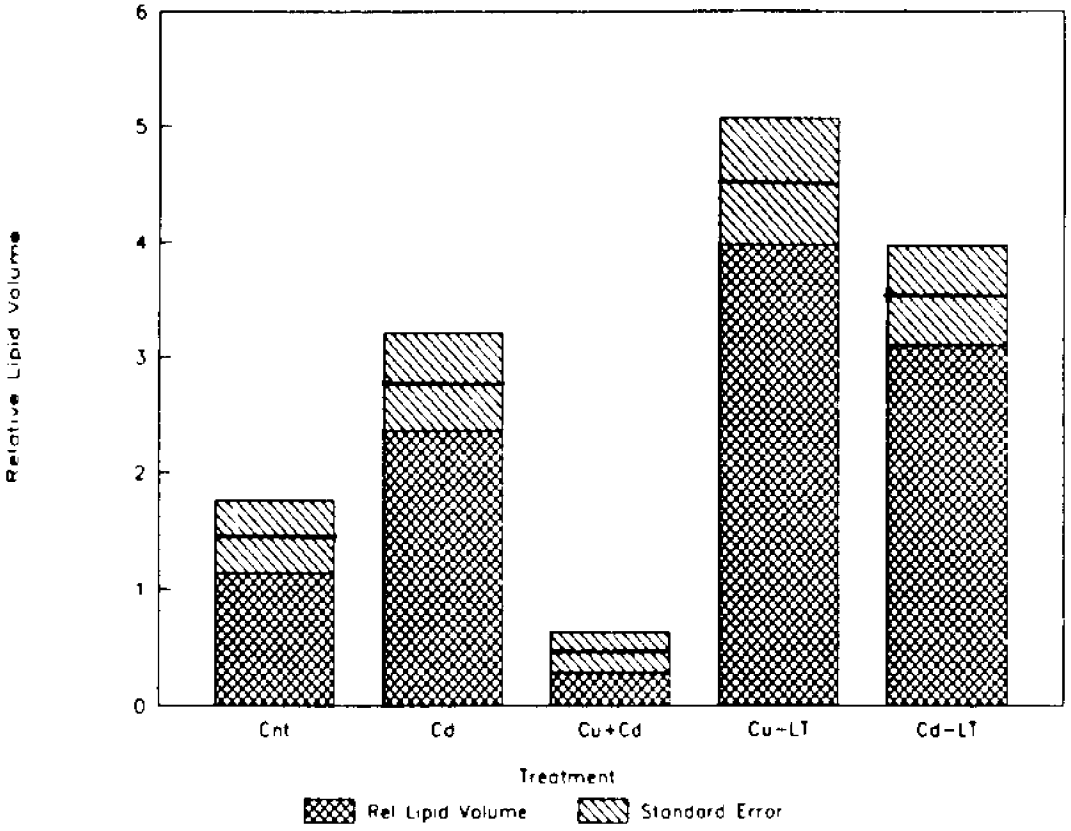

Relative Lipid Volume
6
5
4
3
2
1
0
Cnt
Cd
Cu+Cd
Cu-LT
Cd-LT
Treatment
Rel Lipid Volume
Standard Error

Figure 15: Histogram showing the mean values ± S.E of lipid numbers of Dunaliella minuta control cells (Cnt), cells exposed to 7.57 uM Cu and 0.34 uM Cd (Cu+Cd), cells chronically exposed to 4.9×10^{-4} copper (Cu-LT) and cells chronically exposed to 4.5×10^{-6} cadmium (Cd-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

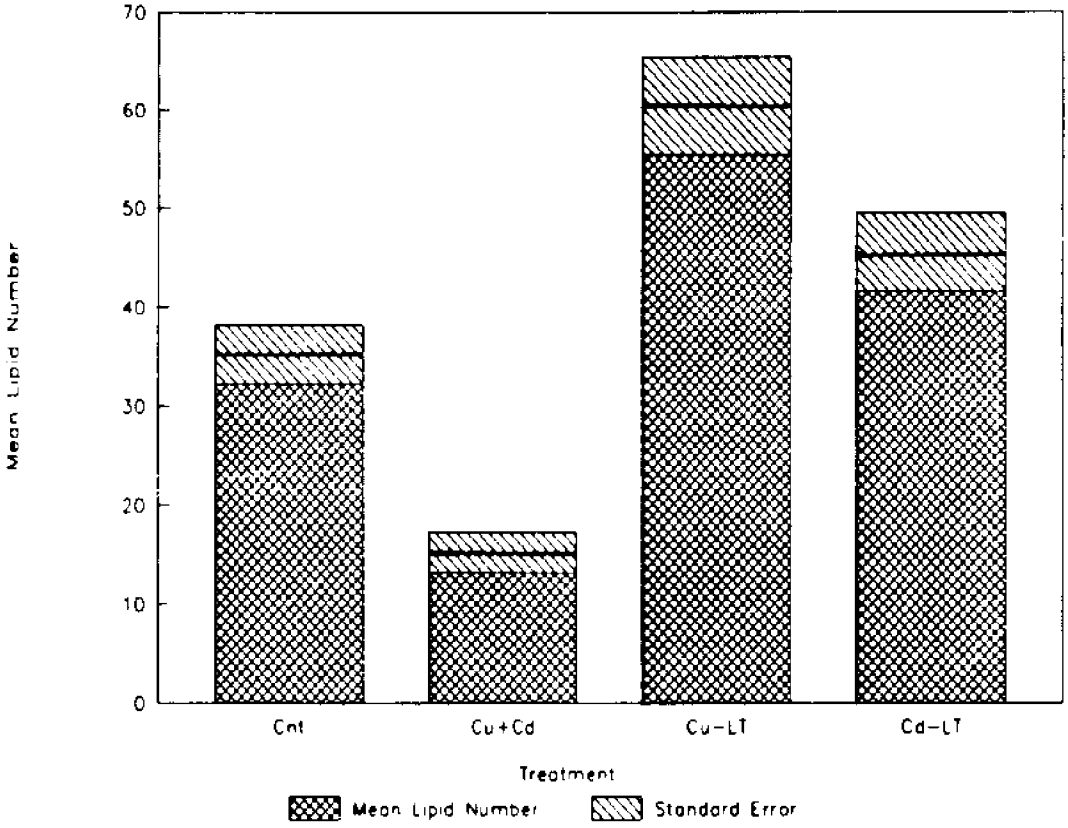

70
60
50
40
30
20
10
0
Mean Lipid Number
Cnt
Cu+Cd
Cu-LT
Cd-LT
Treatment
Mean Lipid Number
Standard Error

Figure 16: Electron micrograph of a Clamydomonas bullosa control cell (magnification 9,200). c = chloroplast, cm = cell membrane, cw = cell wall, f = flagellum, g = Golgi, m = mitochondrion, n = nucleus p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids. Large arrowheads indicate the position of polyphosphate bodies.

Figure 17: Histogram showing the mean values ± S.E of the numbers of polyphosphate bodies of *Chlamydomonas bullosa* control cells (Cnt) and cells exposed to 0.78 uM copper (Cu) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

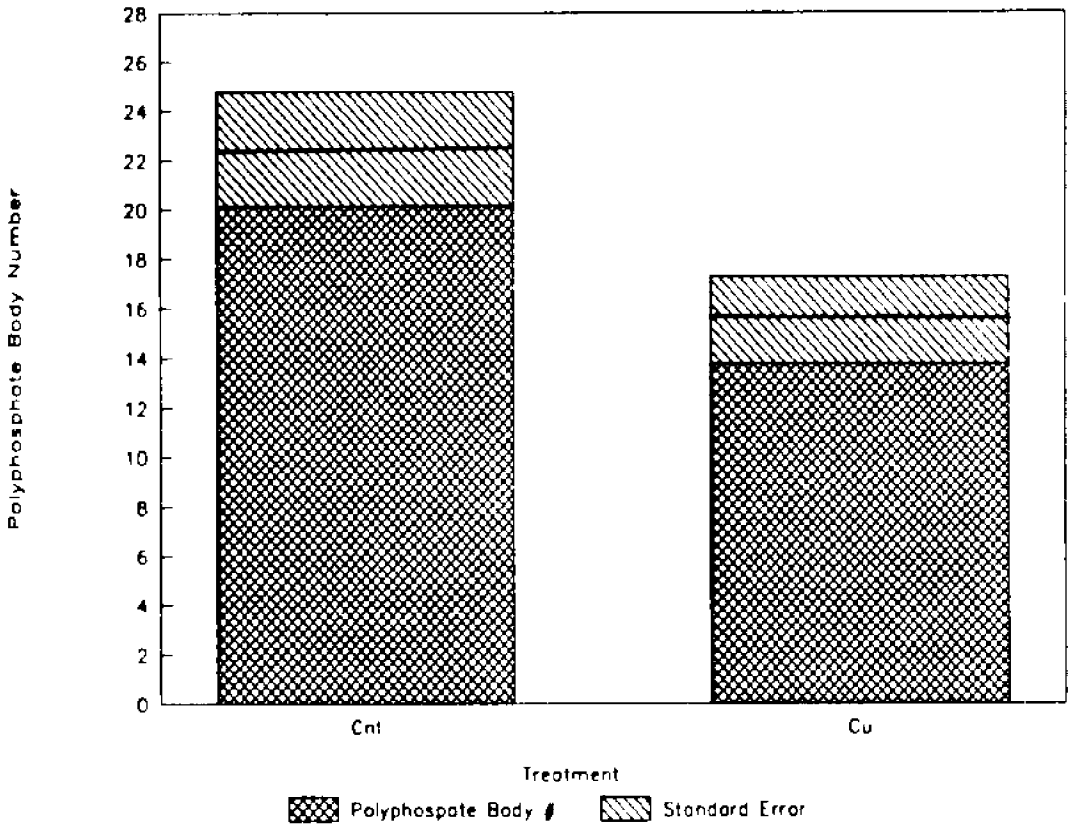

Polyphosphate Body Number
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Cnt
Cu
Treatment
Polyphospate Body #
Standard Error

Figure 18: Histogram showing the mean values ± S.E of total cell volumes of Chlamydomonas bullosa control cells (Cnt), cells exposed to 0.78 uM copper (Cu) for 96 hours, cells exposed to 0.025 uM cadmium (Cd) for 96 hours, cells exposed to 0.78 uM copper and 0.025 uM cadmium (Cu+Cd) for 96 hours, cells exposed to 4.9×10^{-4} uM copper for eight months (Cu-LT), and cells exposed to 4.5×10^{-6} uM cadmium for eight months (Cd-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

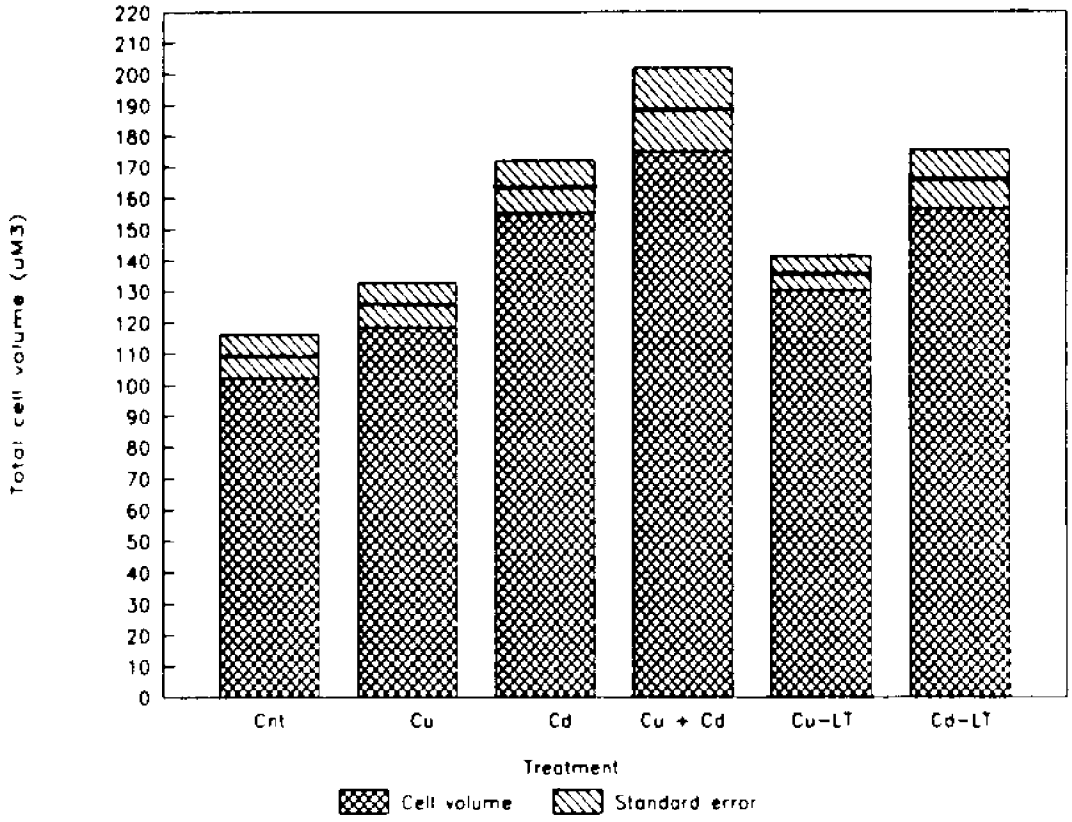

Total cell volume (uM3)
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Cnt
Cu
Cd
Cu + Cd
Cu-LT
Cd-LT
Treatment
Cell volume
Standard error

Figure 19: Histogram showing the mean values ± S.E of relative pyrenoid volumes of Chlamydomonas bullosa control cells (Cnt), cells exposed to 0.025 uM cadmium (Cd) for 96 hours, cells exposed to 0.78 uM copper and 0.025 uM cadmium (Cu+Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

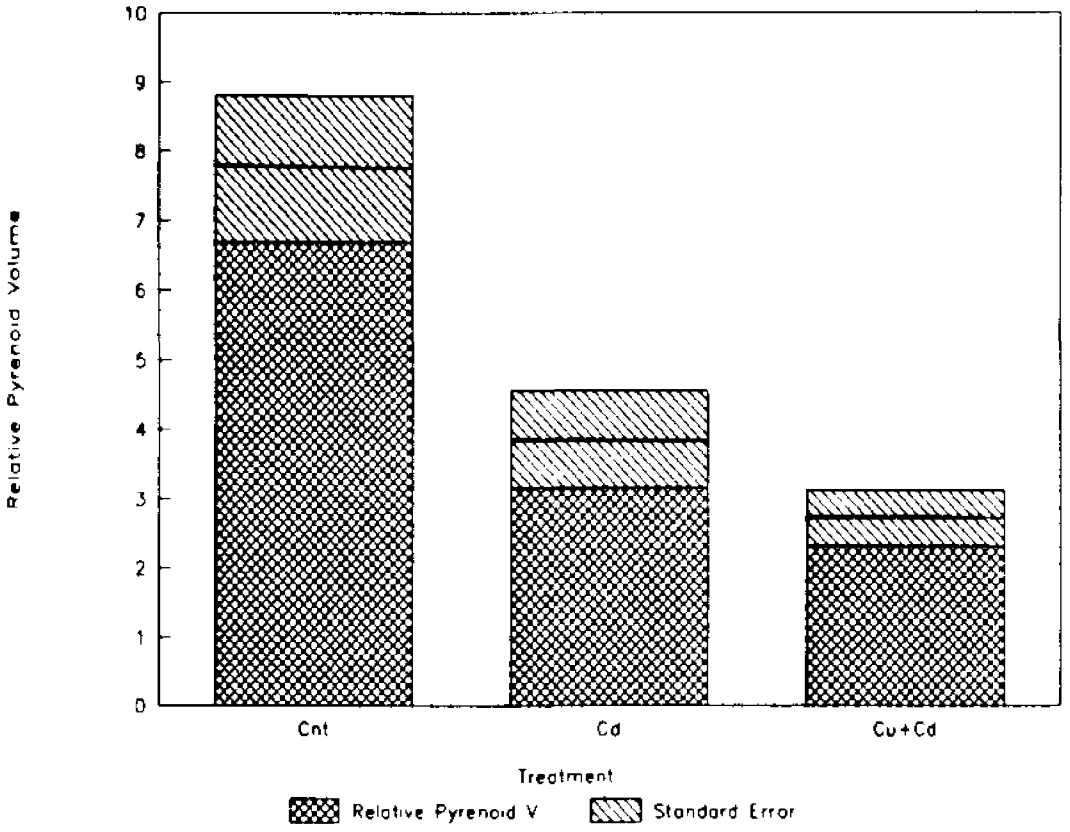

Relative Pyrenoid Volume
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cd
Cu+Cd
Treatment
Relative Pyrenoid V
Standard Error

Figure 20a: Electron micrograph of a Chlamydomonas bullosa control cell (magnification 9,200). c = chloroplast, m = mitochondrion, p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids. Large arrowheads indicate the position of polyphosphate bodies.

Figure 20b: Electron micrograph of a Chlamydomonas bullosa cell (magnification 9,200) exposed to 0.78 uM copper and 0.025 uM cadmium for 96 hours. cm = cell membrane, cw = cell wall, g = Golgi, m = mitochondrion, n = nucleus p = pyrenoid, s = starch, v = vacuole. Small arrowheads indicate the position of lipids. Large arrowheads indicate the position of polyphosphate bodies.

Figure 21: Histogram showing the mean values ± S.E of relative starch volumes of *Chlamydomonas bullosa* control cells (Cnt) and cells exposed to 0.78 uM copper and 0.025 uM cadmium (Cu+Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

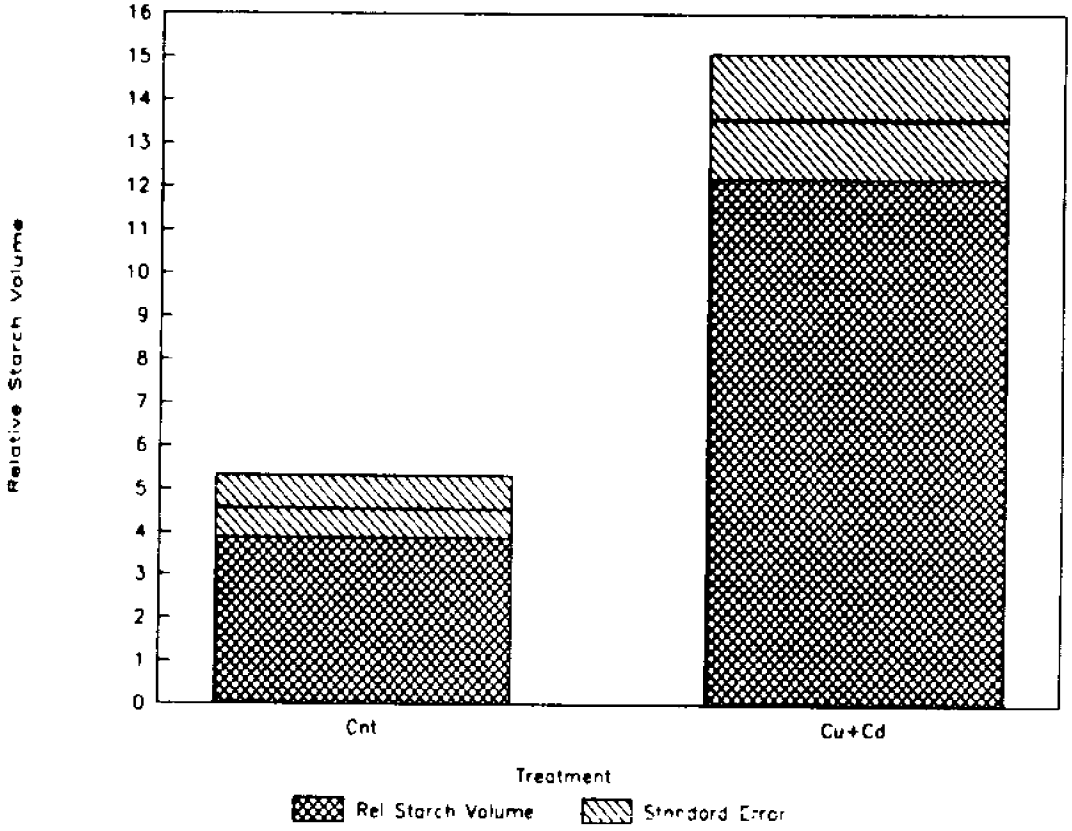

Relative Starch Volume
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cu+Cd
Treatment
Rel Starch Volume
Standard Error

Figure 22: Histogram showing the mean values ± S.E of relative effective chloroplast volumes of Chlamydomonas bullosa control cells (Cnt) and cells exposed to 0.78 uM copper and 0.025 uM cadmium (Cu+Cd) for 96 hours. Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

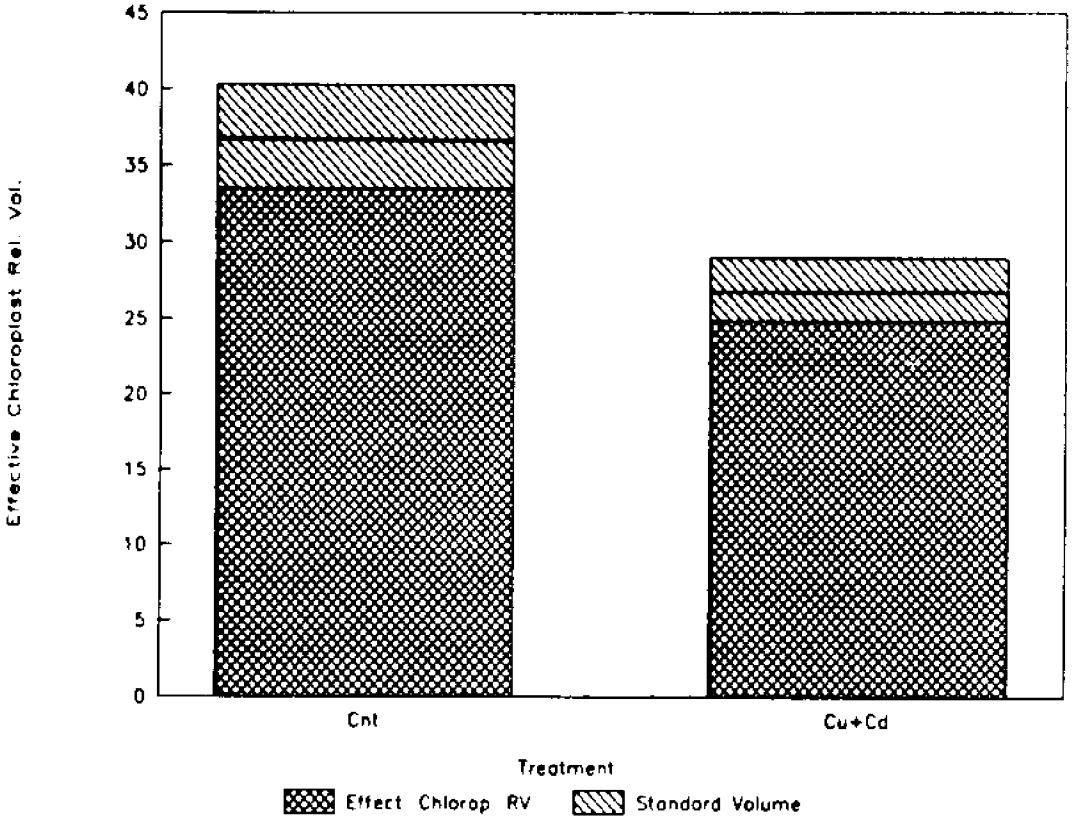

45
40
35
30
25
20
15
10
5
0
Effective Chloroplast Rel. Vol.
Cnt
Cu+Cd
Treatment
Effect Chlorop RV
Standard Volume

Figure 23: Histogram showing the mean values ± S.E of relative cell wall volumes of *Chlamydomonas bullosa* control cells (Cnt), cells chronically exposed to 4.9×10^{-4} uM copper (Cu-LT) and cells chronically exposed to 4.5×10^{-6} uM cadmium (Cd-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

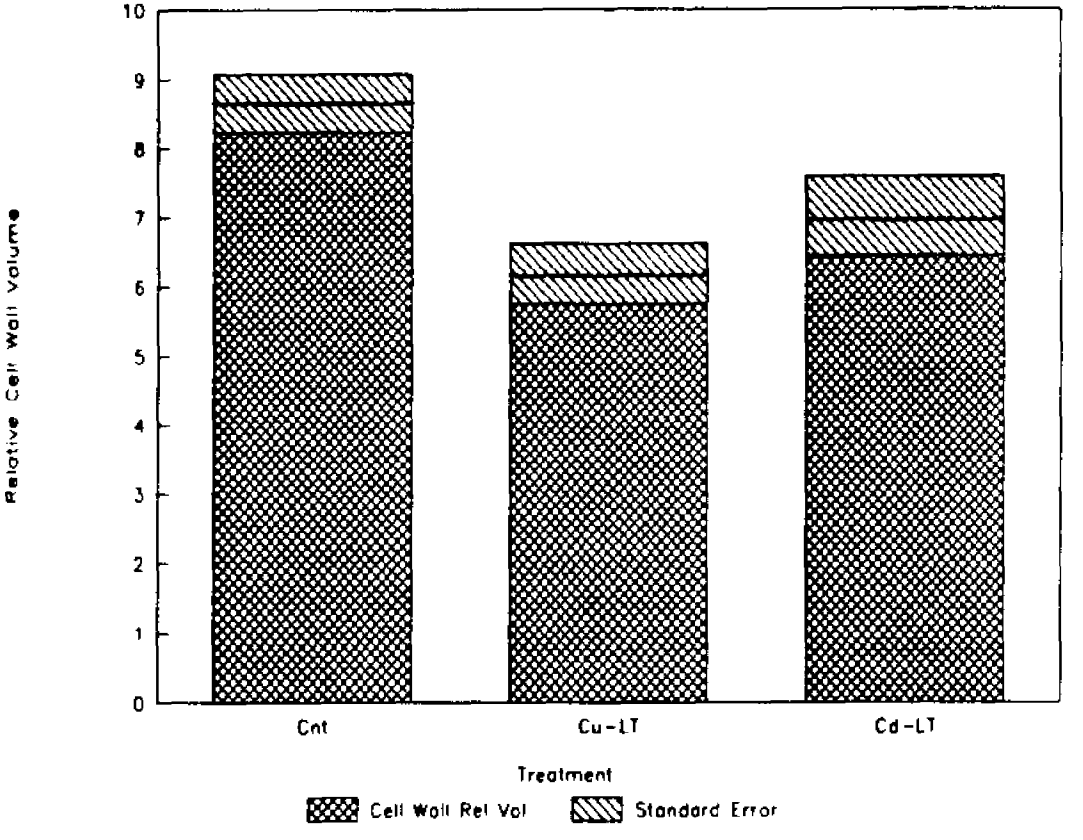

Relative Cell Wall Volume
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cu-LT
Cd-LT
Treatment
Cell Wall Rel Vol
Standard Error

Figure 24: Histogram showing the mean values + S.E of the periplasmalemmal relative volume of *Chlamydomonas bullosa* control cells (Cnt) and cells chronically exposed to 4.9×10^{-4} uM copper (Cu-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

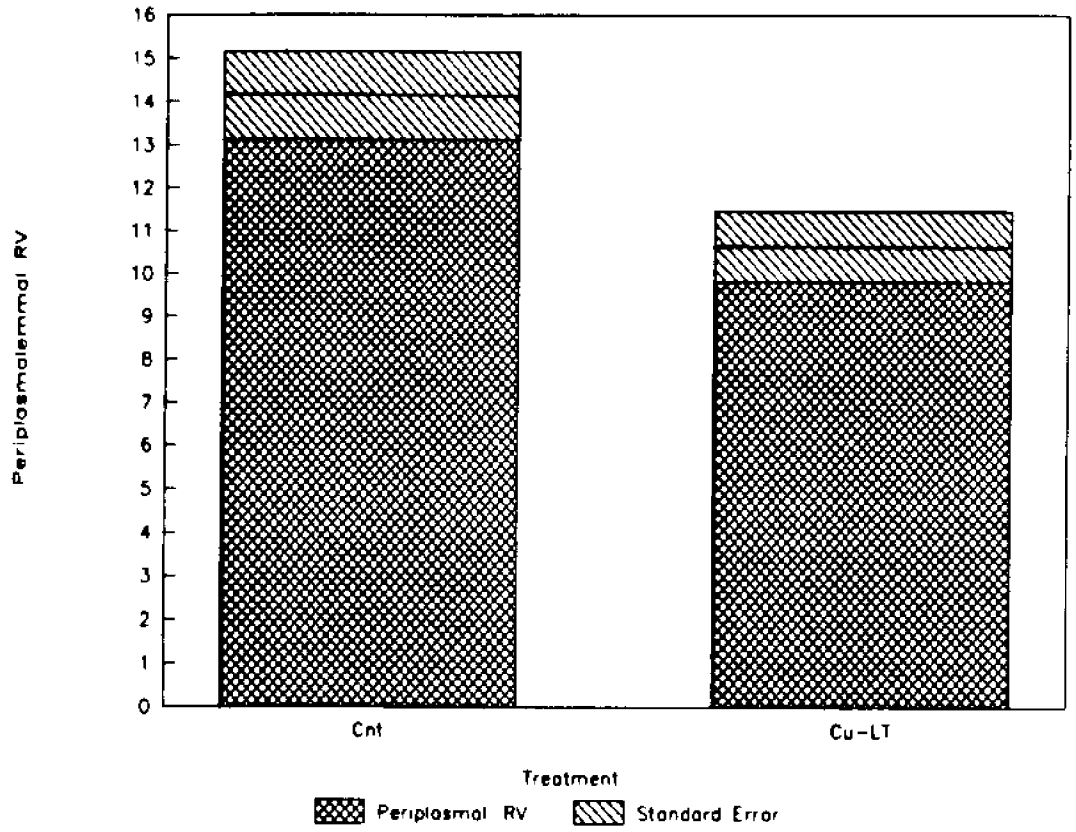

Periplasmal/mmal RV
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cu-LT
Treatment
Periplasmal RV
Standard Error

Figure 25: Histogram showing the mean values ± S.E of the relative vacuolar volumes of *Chlamydomonas bullosa* control cells (Cnt) and cells chronically exposed to 4.5×10^{-6} uM cadmium (Cd-LT). Bold lines indicate the positions of mean values. Parallel hatched areas represent one standard error.

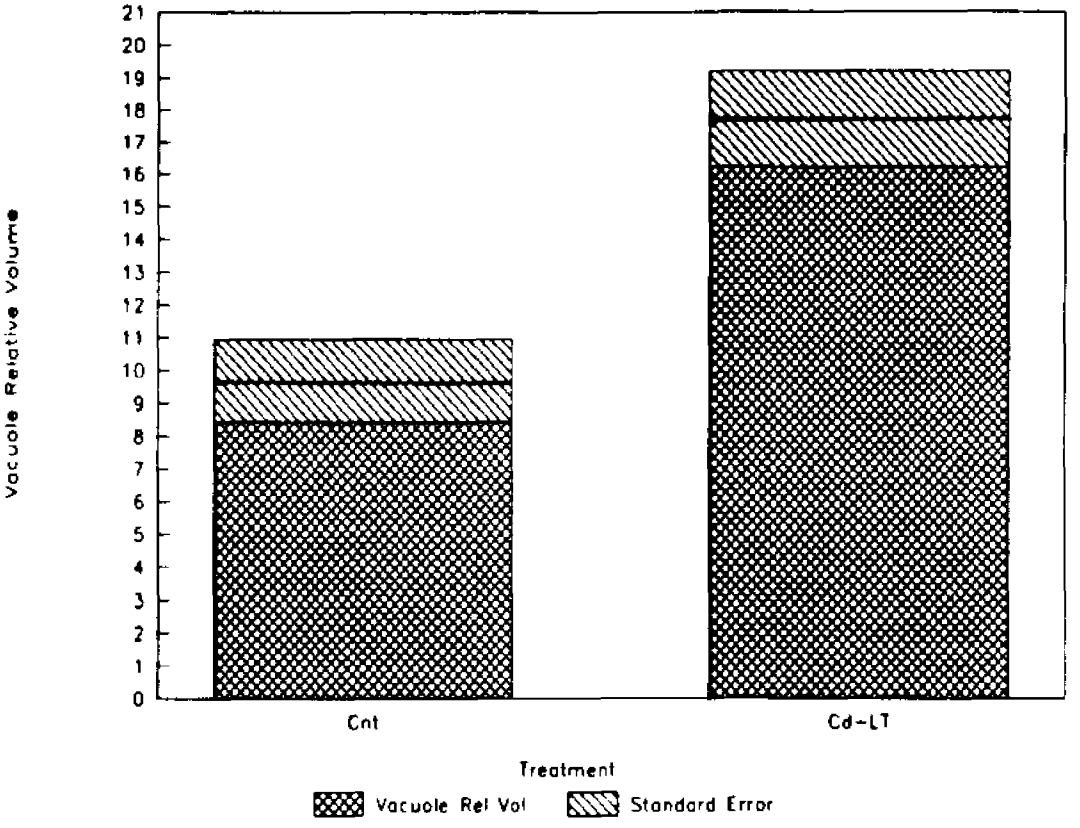
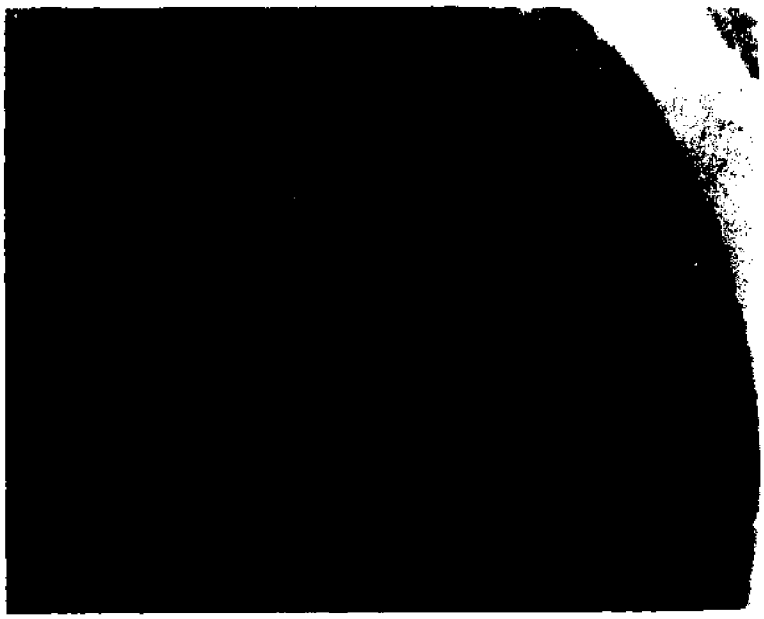

Vacuole Relative Volume
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Cnt
Cd-LT
Treatment
Vacuole Rel Vol
Standard Error

Figure 26: Electron micrograph of a Chlamydomonas bullosa control cell (magnification 20,125) with membranous organelles. c = cloroplast, g = Golgi. Small arrowheads indicate the position of lipids. Large arrowheads indicate the position of membranous organelles.



Discussion

The effective concentrations of copper and cadmium which reduced the population growth of the marine algae Dunaliella minuta and Chlamydomonas bullosa and the halotolerant alga Dunaliella salina by 50% of control values, EC(50)s, were determined in 96 hour static exposure tests. This particular parameter of toxicity was chosen because it has been reported to be a sensitive indicator of heavy metal toxicity (Rosko and Rachlin, 1977; Berland et al., 1977; Rivkin, 1979; Stratton and Corke, 1979). This was shown to be true in the present study, where comparison of growth and ultrastructural data showed that decreases in growth can occur in the absence of any discernible ultrastructural changes, i.e. D. minuta exposed to 7.57 uM copper. In addition to its sensitivity, this parameter can contribute to the information base required in order to evaluate the relative health, or pollution stress exerted on aquatic ecosystems (Rachlin et al., 1982c). However, before this information is applied to field conditions, the chemistry and chelating capacity of the natural water body should be estimated.

Dunaliella salina and Chlamydomonas bullosa cells exposed to their established cadmium EC(50)s had significantly larger volumes than controls. Growth reduction under these conditions is probably due to cation inhibition of normal cell division. Evidence favoring that conclusion is provided by the fact that: a) Cd-exposed D. salina cells had

significantly higher lipid volumes than controls. Increased lipid numbers and volumes usually occur in cells of the stationary rather than the logarithmic growth phase. b) Cd-exposed C. bullosa cells showed no change in periplasmalemmal volume indicating that the increase in cell volume is not due to osmotic effects. The decoupling of division rates from photosynthesis and growth when algal cells are exposed to metal stress have been reported for Isochrysis galbana(Davies, 1974) and Dunaliella tertiolecta (Davies, 1976) exposed to mercury, Chlorella vulgaris exposed to Cd, Cu and Hg (Rosko and Rachlin, 1977), Asterionella japonica exposed to copper and zinc (Fisher et al., 1981) D. tertiolecta exposed to copper (Lustigman, 1986), and Chlorella pyrenoidosa exposed to copper (Steeman Nielsen and Wium Andersen, 1970).

The decrease in cell division rates has been principally attributed to the binding of metals to sulphhydryl groups, which are important in regulating plant cell divisions (Fisher et al., 1981). Indeed, Fisher and Jones (1981) based on their own studies with A. japonica and previous findings on other organisms, have proposed that there could be a common basis of metal action in all biological systems, and that differences in metal toxicities are due to differential affinities of the metals for sulfur complexation. Thus, metals with high affinity for sulfur such as mercury and copper are expected to be more toxic

than metals which exhibit lower affinity for sulfur, such as cadmium. The assertion that copper is more toxic to algae than cadmium is repeatedly found in the literature (Rai et al., 1981; Moore and Ramamoorthy, 1984). However, our findings with D. minuta and C. bullosa show that cadmium is considerably more toxic than copper, or in the case of D. salina approximately equally toxic. These results are congruent with those reported for Chlorella vulgaris (Rosko and Rachlin, 1977), Chlorella saccharophila (Rachlin et al., 1982) Navicula incerta(Rachlin et al., 1983), Skeletonema costatum (Berland et al., 1977), Hymenomonas carterae, Thoracosphaera heimii, Emiliana huxleyi (Brand et al., 1986), and findings of other algal studies (Bentley-Mowat and Reid, 1977). These data, all showing a greater toxicity for cadmium, tend to contradict the "sulfur-affinity" hypothesis, and indicate that other toxicity mechanisms may play a more important role in these algae.

Decrease in growth rates can also be attributed to interference of toxic cations with the uptake and assimilation of essential nutrients. Indeed, it is the most plausible explanation in cases where growth reduction is not accompanied by increases in cell volume and accumulation of photosynthetic products. Foster and Morel (1982) demonstrated that cadmium inhibits the growth of the diatom Thalassiosira weissflogii, by inducing iron starvation. Cd competes with iron for binding and transport sites and

interferes with its intracellular assimilation or utilization. Cellular Mn content is the critical factor controlling the growth rate of T. oceanica and T. pseudonana (Sunda and Huntsman, 1983). Copper depresses the growth rates of these organisms by competitively binding on manganese uptake sites, and thus limiting their cellular Mn content.

Rachlin et al. (1982c) have attempted to provide an organizational framework for the ordering of algal responses to pollutants. They proposed that closely related taxa should respond to environmental stresses in a more similar fashion than non-closely related ones. Furthermore they suggested that knowledge about the toxicological response of one member of a taxon could be used to predict the potential responses of other members of the taxon, as well as the responses of members of closely related taxa. This hypothesis was formulated based on the responses of Chlorella saccharophila, Navicula incerta and Nitzschia closterium exposed to cadmium. Subsequently, it was successfully tested on the same organisms exposed to cadmium under suboptimal conditions.

Evidence from a variety of laboratory and field studies suggests that some broad phylogenetic differences are reflected in algal responses to heavy metals. A prokaryote-eukaryote trend in copper and nickel toxicity between freshwater cyanobacteria and green algae has been reported by Wood and Wang (1983; Wang and Wood, 1984). A similar

trend was observed by Brand et al. (1986), who examined differences in species sensitivity to copper and cadmium, based on the reproduction rates of 38 clones of marine phytoplankton. They found cyanobacteria to be the most sensitive to both cations followed by dinoflagellates and coccolithophores. Diatoms were the least sensitive. Discordant conclusions were reached by Saward et al. (1975). These researchers reviewed the literature on algal sensitivity to copper and reported that diatoms were the most sensitive organisms, green algae the least sensitive, with dinoflagellates and cyanobacteria being intermediate. In controlled ecosystem pollution experiments microflagellates and pennate diatoms were found to be more tolerant to copper stress than centric diatoms (Thomas and Seibert, 1977).

The present study examined the validity of the phylogenetic hypothesis in lower taxonomic groups using three species from the same order (Volvocales). The toxicological responses of the two congeneric species D. minuta and D. salina were compared to the response of C. bullosa. Based on the hypothesis, it was expected that D. minuta and D. salina subjected to heavy metal stress would exhibit similar responses, unlike C. bullosa. Analysis of covariance of the cadmium generated regression lines indicated that the responses of all three chlorophytes were significantly different. Analysis of the growth data under copper stress showed, contrary to expectations, that the two

non-related species, C. bullosa and D. minuta, responded to the cation in similar fashion, whereas the responses of D. salina and D. minuta were significantly different. Therefore, it is concluded that the phylogenetic hypothesis does not hold true for the lower levels of taxonomic organization. Its predictive value is probably limited to the higher taxonomic ranks, such as divisions.

One of the important factors affecting the toxicity of a particular metal in aquatic environments is the presence of other metals (Silverberg et al., 1976). Other metals present can act antagonistically, synergistically or additively on the uptake of the particular metal or on its effects on algal physiological and morphological parameters. The effects of metal combinations on algae depend on the algal species, the metal combinations used, as well as the order of metal introduction. Stratton and Corke (1979) reported that Cd and Hg acted synergistically, reducing the growth rate of Anabaena variabilis, when they were introduced simultaneously at sublethal concentrations. They exhibited the same relationship when Hg was introduced first, but showed antagonism when the order of introduction was reversed. We found that copper and cadmium acted antagonistically leading to greater growth of D. minuta than expected if their action were simply additive. Similar results were observed with Selenastrum capricornutum exposed to the same metals (Bartlett et al., 1974), Phaeodactylym tricornutum exposed to Cu and Zn, Skeletonema costatum and

P. tricornutum exposed to Zn and Cd (Braek et al., 1980), and Ascophyllum nodosum exposed to Cu and Zn (Stromgren, 1980).

It has been proposed that divalent metals share a common mode of uptake in some algae, and that protection offered by the addition of one metal against the toxicity of a second, may be due to competition for common adsorption sites on the cell surface as well as for transport and toxicity sites (Braek et al., 1976; Stratton and Corke, 1979; Harrison and Morel, 1983). This hypothesis is suported by uptake studies which show that Zn reduces the uptake of Cd in Phaeodactylum tricornutum and Skeletonema costatum (Braek et al., 1980), Cd inhibits Fe-uptake in Thalassiosira weissflogii, even when the latter is present at high concentrations (Foster and Morel, 1982), Cu reduces Cd uptake in Cricosphaera elongata, but not vice versa, (Gnassia-Barelli and Hardsted-Romeo, 1982), Cu inhibits Mn uptake in Thalassiosira pseudonana, T. oceanica (Sunda and Huntsman, 1983) and other species of phytoplankton (Sunda et al., 1981).

The action of copper and cadmium was synergistic towards the growth of D. salina and C. bullosa, indicating that in these chlorophytes the cations did not share common uptake, transport or toxicity sites. On the contrary, the presence of one probably facilitated the uptake and toxic effect of the other, as was the case with Scenedesmus which increased its nickel uptake in the presence of copper (Stokes,

1975). Synergistic effects on growth were also observrd with the dinoflagellate A. carteri, and the diatoms T. pseudonana and S. costatum (Clone Skel 5) exposed to Zn and Cu (Braek et al., 1976).

The toxic action of two or more heavy metal cations is not uniform on all the morphological and /or physiological parameters of the organism, but varies depending on the parameter under consideration. Combinations of Hg and Cd have been reported (Stratton and Corke, 1979) to act antagonistically on the growth yield of Anabaena inaequalis, but exhibited synergistic action on photosynthesis and nitrogenase activity. Hg and Ni acted in both synergistic and antagonistic manner towards growth and nitrogenase activity, depending on the metal combinations used, but showed a straight additive response towards photosynthesis. An equally complex picture emerged in the present study, upon examination of growth and ultrastructural data. Copper and cadmium acted antagonistically towards the growth and chloroplast volume of D. minuta. In the latter case Cu counteracted the toxic effects of Cd on the chloroplast, preventing significant reduction in the volume of this organelle. In contrast, the two metals acted additively or synergistically to limit pyrenoid volume. This effect was not evident after short term monometal exposure. Copper and cadmium, acting synergistically, severely limited the growth of Dunaliella salina, however, at the cellular level their relationship was both antagonistic and synergistic. Copper

counteracted Cd-induced effects, inhibiting changes in total cell volume and relative nuclear volume. Relative lipid volume, on the other hand, was greatly diminished due to their synergistic action. Similar results were obtained from C. bullosa. Copper and cadmium acted synergistically to limit the growth of this alga. Both metals acted additively or synergistically to greatly increase total cell volume. Copper, additively or synergistically, contributed to the Cd-induced decrease in relative pyrenoid volume, while cadmium inhibited the reduction in the number of polyphosphate bodies, which was evident after short term exposure to copper.

The study of ultrastructural changes due to metal stress, especially quantified by morphometric analysis, and correlated with physiological data can provide invaluable insight in the pathways of metal toxicity, and on algal detoxification mechanisms. Comparison of the morphometric data obtained after short term, sublethal, monometal exposure, clearly shows that cadmium has by far greater impact on the ultrastructure of the three species examined than does copper. This is in agreement with the growth data of the chlorophytes, and further indicates that toxicity mechanisms other than binding to sulphhydryl groups are more important in these organisms. The cellular targets of copper and cadmium are many and include chloroplast, pyrenoid, lipids, nucleus, polyphosphate bodies, vacuoles and cell wall.

The chloroplasts of D. minuta cells exposed to cadmium were significantly smaller than controls, indicating that cadmium stress caused a decrease in the photosynthetic potential of this alga. Reduction in chloroplast size and chloroplast organization has been reported for Chaetomorpha brachygona growing in the presence of several heavy metals including cadmium (Chan and Wong, 1987). Additionally, significant reduction in thylacoid surface area was observed in Anabaena flos-aquae exposed to various cadmium concentrations (Rachlin et al., 1984; Rai et al., 1990), and Anacystis cyanea exposed to environmental concentrations of copper and cadmium (Sicko-Goad, 1982). It has been suggested that changes in the chloroplast could be due to loss of chlorophyll and carotenoid (Rai et al., 1990). A significant decrease in the number of lipids (carotenoid globules) was detected in D. minuta cells after Cd-exposure, however, it was not accompanied by a decrease in lipid volume. Thus, it is concluded that lipid globules coalesced and loss of carotenoid did not take place. Cadmium is also known to affect the electron transport system by decreasing the cytochrome f:chlorophyll a ratio (Foster and Morel, 1982). However, in the absence of biochemical data, it is not known whether changes in chlorophyll or cytochrome f content took place in cadmium treated cells. Therefore, the role of these mechanisms in the observed chloroplast reduction is unclear.

C. bullosa cells exposed to sublethal concentrations of copper and cadmium showed no changes in overall chloroplast volume, but a significant decrease in their effective chloroplast volume. This reduction is an indirect effect, brought about by an inhibition of normal cell division, and the subsequent accumulation of starch granules. Nevertheless, it compromises the ability of the cell to photosynthesize.

The pyrenoid was affected under a variety of cation treatments. Its volume decreased significantly in C. bullosa cells exposed to sublethal concentration of cadmium, and in D. minuta cells chronically treated with low concentration of copper. Cells of both chlorophytes exhibited smaller pyrenoids after sublethal dimetal exposure. Reduction in the number of pyrenoids has been observed in C. brachygona subjected to high multimetal concentrations (Chan and Wong, 1987). The pyrenoid is the site of production of starch synthetase, which polymerizes glucose molecules into starch (Bold and Wynne, 1978). Reduction in the size of this organelle might signal the growing inability of the cells to produce energy storage molecules.

The relative lipid volume of Dunaliella minuta cells exposed to sublethal concentrations of copper and cadmium increased by 130% compared with controls. Similar increases were observed in Dunaliella salina cells exposed to acute sublethal concentration of cadmium. Such increases are known to occur in cells of the stationary growth phase (Eyden,

1975; Hoshaw and Maluf, 1981). In the present study they are correlated with significantly larger cell volumes. Therefore, they are attributed to metal inhibition of cell division which effectively uncouples growth rates from photosynthetic rates. Lipid accumulation has also been reported in D. salina exposed to high, sublethal concentrations of Cu and Pb (Pace et al., 1977), D. tertiolecta exposed to copper (Lustigman, 1986), and C. brachygona growing in the presence of several metals (Chan and Wong, 1987). D. salina cultures chronically exposed to low levels of copper or cadmium also exhibited significant increases in lipid volume, but not cell volume. Carotenogenesis in D. salina is known to increase under stressful conditions such as high salinity, high temperature, high light intensity, and nutrient limitation (Loeblich, 1982; Ben-Amotz et al., 1982a; Ben-Amotz and Avron 1983). Taking into consideration the tolerance spectra of this organism, the salinity, temperature and light intensity employed in this study were by no means extreme. It is suggested that the observed accumulation of β-carotene globules could be due to nutrient limitation induced by copper and cadmium. Evidence supporting that conclusion is provided by another algal study which showed that short term Cu-exposure (10^{-5}-10^{-4} M) limits the potassium content of D. tertiolecta cells (Overnell, 1975).

In contrast to the results discussed above, dimetallic exposure to copper and cadmium reduced the lipid volume of D. salina cells by 68.28%. Similar results have been reported for Anabaena flos-aquae exposed to high cadmium doses (Rachlin et al., 1984; Rai et al., 1990). The toxicity mechanism responsible for lipid reduction is not presently known, but Rai et al. (1990) have suggested that extensive Cd binding on lipid bodies could lead to their dissolution.

A 30.95% reduction in the number of polyphosphate bodies was observed in C. bullosa cells after sublethal 96 hour exposure to copper. Polyphosphate bodies are supposed to serve as phosphate storage areas, that can be degraded when phosphate availability is limited. X-ray dispersive studies have shown that heavy metals can be incorporated in these structures, causing a shift from their normal constituents (Mg, P, K, Ca) to the heavy metals the algae are exposed to (Sicko-Goad and Stoermer, 1979; Baxter and Jensen, 1980; Jensen et al., 1982a; 1982b; 1984; Rachlin et al., 1984). Decreases in the number of polyphosphate bodies have also been reported for Diatoma tenue exposed to copper (Sicko-Goad and Stoermer, 1979), and Anabaena flos-aquae exposed to high cadmium concentrations (Rachlin et al., 1984; Rai et al., 1990). The observed reductions in C. bullosa are not accompanied by reduction in polyphosphate volume, indicating that these structures coalesced. Thus copper

probably did not interfere with the phosphate uptake mechanism.

The nucleus was not immune to toxic cation action. Cd reduced the relative nuclear volume of D. salina cells by 24.35% of controls. Autoradiographs have demonstrated that cadmium can be incorporated into the nucleus (McLean and Williamson, 1977), and can alter the DNA content of affected cells by blocking the G1, S or G2 stage of the cell cycle. Cd-resistant cells, on the other hand, have the same DNA content as controls (Bonaly et al., 1980). Our results are consistent with those reported for lymphocytes exposed to zinc (Berger and Skinner, 1974), and can be attributed to cadmium induced inhibition of DNA replication.

A 72.70% increase in the relative volume of vacuoles was seen in C. bullosa cells chronically exposed to low levels of cadmium. Cytoplasmic vacuolation was reported for Skeletonema costatum exposed to sublethal concentrations of Hg, Cd and Zn (Smith, 1983). It was attributed to osmotic disorganization due to membrane damage. This explanation does not seem to apply to C. bullosa. Even though the total cell volume of Cd-treated cells increased, and their cell wall volume decreased, the space between the cell wall and the cell membrane did not change, indicating the absence of increased osmotic pressure within the cells. Sicko-Goad and Stoermer (1979) examined the effects of phosphate treatments and heavy metals on the diatom Diatoma tenue. They found that the relative volume of vacuoles increased

after phosphate starvation. It is unclear whether phosphate limitation played any role in C. bullosa; however, no decrease in the number of polyphosphate bodies was seen. Abundant vacuoles have also been observed in stationary phase cells (Hoshaw and Maluf, 1981), so the increase in vacuolar volume can be an indirect effect of Cd-induced inhibition of cellular division.

Chronic exposure of C. bullosa cells to copper or cadmium leads to decreases in the relative cell wall volume by 24.24% and 18.84% respectively. The cell wall of C. bullosa is thick and is normally composed of three not easily distinguishable layers (Cann and Pennick, 1982), which contain glycoproteins with hydroxyproline (Pickett-Heaps, 1975). Reduction in the relative volume of the cell wall indicates that the cells could be more susceptible to mechanical stress. Gradual degradation of the peptidoglycan layer has been observed in Anabaena flos-aquae exposed to high Cd concentrations (Rachlin et al., 1984; Rai et al., 1990). It is of interest that the present results have been obtained after treatment with very low concentrations of copper and cadmium.

The observed changes in cellular morphology are the direct or indirect effects of cation activity. Reductions in chloroplast, cell wall and nuclear volumes belong to the former, whereas accumulation of photosynthetic products and lipid globules belong to the latter. Examination of both direct and indirect effects reveals the pathways of heavy

metal action, or suggests future lines of research to elucidate the mechanisms of metal ion activity.

No correlation exists between Cu and Cd sensitivity and the habitat of an alga. Coastal or estuarine phytoplankton face greater fluctuations in salinity and temperature and are usually subjected to stress due to the presence of several pollutants. Algal cells from these areas, therefore, would be expected to develop special adaptations to counteract the toxic action of pollutants (Fisher and Frood, 1980). Examination of the reproduction rates of 38 clones of marine phytoplankton in the presence of copper or cadmium, however, failed to reveal consistent neritic-oceanic differences in tolerance. The results led Brand et al. (1986) to conclude that, unlike iron, zinc and manganese, copper and cadmium have not been important selective forces in phytoplankton evolution.

Several studies show that algae from metal polluted areas are more tolerant to heavy metals than algae from non-polluted areas (Russel and Morris, 1970; Bryan, 1971; Jensen et al., 1976; Foster, 1977; Hall, 1980). Their ability to survive under metal stress may be due to genetic adaptation or due to changes in physiology resulting from metal exposure. Several tolerance mechanisms have been reported and can be divided between external and internal detoxifying mechanisms. External mechanisms include extracellular release of organic material, metal sequestration in the cell wall and changes in cell membrane

permeability which limit cation uptake. Algal extracellular products might produce an ameliorating effect on metal toxicity by binding to metal ions and thus rendering them nontoxic. Many eukaryotic algae produce organic acids which are weak complexing agents (Rai et al., 1981). They are also known to release hydroxamic acid, which is a strong complexing agent present in natural waters. Steeman-Nielsen and Wium Andersen (1971) have reported that diatoms react to copper by excreting organic matter which binds to copper and partly removes the toxic effect. The filamentous green alga Hormidium fluitans responds to copper stress forming a sheath proportional in size to the concentration of the cation. The alginic sheath is capable of concentrating copper from the surrounding medium in discrete regions (Sorentino, 1985). Spirogyra submargaritata grown on iron-ore tailings binds metal ions within the cell wall (Chan et al., 1981), and Chaetomorpha brachygona from the same area substantially increases the thickness of both the lamellated side wall and the cross wall, thus partially excluding metal ions by binding the metal precipitate within the cell wall (Chan and Wong, 1987). Foster (1977) found that non-tolerant strain of Chlorella vulgaris accumulated 5-10 times more copper than the tolerant strain at comparable external copper concentrations, and concluded that copper exclusion was likely to be the only mechanism of tolerance in this alga. The same mechanism seems to operate in copper tolerant strains of the ship fouling alga Ectocarpus

siliculosus (Hall et al., 1979) and cadmium resistant strains of Euglena gracilis (Bariaud and Mestre, 1984). De Fillipis and Pallaghy (1976) created a zinc-tolerant Chlorella strain in the lab. The development of tolerance was gradual, and led to the reduction of zinc binding sites and the inhibition of a temperature-sensitive component of zinc uptake. These results indicate a physiological development of an exclusion mechanism, but the possibility of genetic adaptation cannot be discounted, because the zinc concentration employed in this study was quite high (Whitton, 1980).

In the absence of exclusion mechanisms toxic cations enter the cytoplasm where several detoxification mechanisms are possible. Copper-tolerant Skenedesmus accumulates copper in intranuclear complexes probably through attachment of the cation to protein ligands (Silverberg et al., 1976). Similar structures are present in Skenedesmus exposed to lead (Silverberg, 1977). For Porphyra umbilicalis the nucleus is also the site for intracellular bound cadmium (McLean and Williamson, 1977). Several studies have indicated that polyphosphate bodies could be implicated in internal detoxification. X-ray dispersive analysis (Sicko-Goad and Stoermer, 1979; Jensen et al., 1982a; 1982b; 1984; Rachlin et al., 1985) has shown that normally found ions are displaced by heavy metals in these structures. Additionally, morphometric studies have demonstrated

significant proliferation in the number and/or volume of polyphosphate bodies following heavy metal treatment (Rachlin et al., 1982a; 1985). Other cellular sites responsible for metal sequestration include membranous organelles (Sicko-Goad and Stoermer, 1979), cytoplasmic tubules (Smith, 1983) and multivesiculate bodies (Sicko-Goad and Stoermer, 1979; Smith, 1983; Chan and Wong, 1987).

In many cases a combination of mechanisms is employed by algae in order to counteract metal toxicity. Mercury tolerance in Dunaliella tertiolecta is partly related to slow rate of mercury accumulation and partly to intracellular detoxification possibly by the precipitation of a highly insoluble mercury compound (Davies, 1976). In Plectonema boryanum heavy metals are sequestered in the cell wall, polyphosphate bodies, intracellular membrane whorls, and in the cytoplasm in the form of granular bodies (Rachlin et al., 1982). Chaetomorpha brachygona manages to grow in the presence of high concentrations of Cd, Cr, Cu, Fe, Pb, Zn, Mn by increasing the thickness of its cell wall and thus decreasing the rate of metal uptake. Furthermore, it binds toxic cations within the cell wall and in intracellular multivesicular bodies (Chan and Wong, 1987). In Skeletonema costatum cytoplasmic tubules, multivesicular bodies and electron dense inclusions in vesicles serve as possible metal sequestration sites (Smith, 1983), whereas in Diatoma tenue polyphosphate bodies, membranous organelles

and multivesicular bodies are involved in internal detoxification (Sicko-Goad and Stoermer, 1979).

In order to examine the development of tolerance in algae, the three chlorophytes employed in this study were subjected to low level copper or cadmium stress. After eight months copper and cadmium treated cultures were challenged with the established EC(50) values for these cations, to determine whether their growth response indicated that some adaptation has taken place. Additionally, the possibility that co-tolerance had developed, was also investigated. Multiple tolerance is found in organisms from habitats where several metals are present in high concentrations i.e Chaetomorpha brachygona. Co-tolerance, to two or more metals, in contrast, is exhibited by organisms coming from an environment rich in only one metal. Tolerance to heavy metals is considered highly specific and co-tolerance an exception (Rai et al., 1981). Hall (1980), however, rightly pointed out that co-tolerance is probably a rare phenomenon because metal tolerant organisms are not examined thoroughly for tolerance to other heavy metals. Bariaud and Mestre (1984) reported that cadmium tolerant Euglena gracilis also shows increased tolerance to cobalt and zinc. Cadmium tolerance is associated with lower cadmium uptake, and the authors suggested that probably the mechanism for Cd, Zn and Co uptake is common. Copper tolerant Ectocarpus siliculosus similarly exhibits significantly greater growth

in cobalt and zinc than the non-tolerant strain (Hall, 1980).

Dunaliella minuta cultures chronically exposed to copper showed a 34% increase in tolerance towards this cation, but exhibited no changes in their growth response towards Cd. Cultures chronically exposed to cadmium were 19% more tolerant towards Cd-ions and also showed 26% increased co-tolerance towards copper. Cu-treated D. salina exhibited extreme sensitivity towards both copper and cadmium. Cd-treated cultures developed complete insensitivity towards the established EC(50) cadmium concentration, but were very sensitive towards copper ions. Chlamydomonas bullosa chronically exposed to copper showed 29% greater sensitivity towards the same cation, but did not change their response towards cadmium. Cd-exposed cultures of the same chlorophyte similarly showed 26% less tolerance towards cadmium, but did not alter their growth response towards copper. The increased tolerance of Cu and Cd-treated D. minuta, of Cd-treated D. salina, and the increased co-tolerance of Cd-treated D. minuta indicate that long term exposure to low cation stress leads to the development of either a new physiological mechanism, or the increased efficiency of an existing one, enabling these organisms to better resist further cation stress. The possibility of genetic adaptation is argued against by the fact that the metal concentrations used for the long term exposure appear too low to exert a strong selective pressure.

Cu-treated *D. salina* and *C. bullosa*, and Cd-treated *C. bullosa* showed increased sensitivity to these cations, while Cu-treated *D. salina* also showed increased "co-sensitivity" towards cadmium. Morphometric data obtained from these chronically stressed cultures showed that ultrastructural changes took place in response to metal stress. Additionally, many studies have shown that algae can accumulate metals proportionally (Riley and Roth, 1971) or in excess of the environmental concentration (Mance, 1987; Livett, 1988). So it is possible that toxic cations entered the cells and at least some of them were rendered non-toxic by internal detoxifying mechanisms. The subsequent sublethal metal challenge overwhelmed the cell defense, and since some of the sequestration sites were already occupied, due to chronic exposure, more metal ions attacked vulnerable cellular structures, severely interfering with normal metabolic processes. Additionally, chronically treated *C. bullosa* cells had significantly thinner cell walls, which presumably was a less effective barrier to metal uptake, and thus contributed to metal sensitivity. Further studies are needed to examine whether the above scenario represents accurately the events that lead to increased sensitivity.

The only case where increased co-tolerance was observed was in Cd-treated *D. minuta* cultures. The fact that co-tolerance did not develop in Cu-treated *D. minuta* confirms that tolerance to one heavy metal does not necessarily confer co-tolerance to others. The co-tolerance

and "co-sensitivity" results that were obtained with Cd-treated D. minuta and Cu and Cd-treated D. salina respectively, can also be explained in terms of metal interaction. The chronic low level exposure and the subsequent sublethal challenge with the other cation is essentially an experiment in metal interaction, only the second metal is added after eight months. The observed results with D. salina, that is severe population reduction, could be due to synergistic copper and cadmium action. This is in agreement with the growth results obtained after short term, sublethal, simultaneous metal exposure. Similarly, the results obtained with Cd-treated D. minuta, secondarily exposed to copper, could be due to the antagonistic action of the cations on the growth of this chlorophyte. They are also congruent with the results of the short term, sublethal, simultaneous metal exposure. The fact that Cu-treated cultures do not respond in the same fashion as the Cd-treated cultures after the addition of the second cation does not negate the validity of this explanation. As has been noted before, the order of metal introduction and the metal concentrations used are important factors affecting the interaction of toxic cations.

Regardless the results obtained from the long term study, both D. minuta and D. salina are able to survive in the presence of high copper concentrations, and the latter can even withstand high cadmium concentrations. Dunaliella species have generally been shown to be tolerant to heavy

metals (Mandelli, 1969; Erickson et al., 1970; Overnell, 1975; Davies, 1976; Pace et al., 1977; Lustigman et al., 1985) and to chlorinated hydrocarbons (Menzel et al., 1970; Harding and Phillips, 1978). The exact mechanism involved in heavy metal tolerance is not known, however, several possibilities have been suggested. Gimmler et al. (1981) excluded differential copper uptake as the source of copper resistance in D. parva. They proposed that resistance could be the result of copper binding to the increased amounts of salt-induced SH-groups and to copper complexation by glycerol. Davies (1976) attributed the mercury tolerance of D. tertiolecta to both the slow rate of mercury accumulation and to internal detoxification possibly by the precipitation of a highly insoluble mercury compound. X-ray microanalysis of Cd-treated D. bioculata revealed the displacement of Ca and P from intravacuolar precipitates, probably polyphosphate bodies, and their replacement by cadmium. Additionally it demonstrated the presence of cadmium and sulfur in electron dense condensed material within the vacuoles. Comparison of the Cd/S ratio of the vacuolar content with the ratio found in an inorganic compound and in a metallothionein, indicated that it was similar to the latter. Heuillet et al., (1986) proposed a two step detoxification process in D. bioculata. An initial cytosolic cadmium binding by a metallotheionein-like protein, and a final sequestration of this protein in the vacuolar system.

The sensitive species of this study, C. bullosa, was also not devoid of possible sequestration sites. Cations could have been bound in polyphosphate bodies or on membranous organelles. Membranous organelles are usually found within vacuoles, and can be described as concentric masses of membranes. They have been observed in several algal species (Trezzi et al., 1964; Marano-LeBaron and Izard, 1972; Sicko-Goad and Stoermer, 1979; Hoshaw and Maluf, 1981). Their role in normal cell is not known, however, Sicko-Goad and Stoermer (1979) reported that their numbers increased in Diatoma tenue cells exposed to lead. Similarly, Rachlin et al. (1982a) detected the presence of membrane whorls, structures comparable to membranous organelles, in Plectonema boryanum exposed to Co, Zn, Hg, Cd, Ni and Cu, and suggested that the lipids and proteins of these membranes may tie up excess quantities of intracellular heavy metals. These results are consistent with the findings of the present study, where the percentage of cells carrying membranous organelles increased under all experimental conditions. Additionally, an increase in the number of cells with intranuclear vacuoles was observed after short term copper treatment. The membranes of these vacuoles appeared to be several layers thick, and it is suggested that they represent an early stage in the membranous organelle development.

Examination of internal and external algal defenses indicates that the very same mechanisms of toxic ion action

that can impair normal membrane function and change cell membrane permeability, can also serve to bind metals to non critical proteins and other organic compounds, thereby mitigating their toxic effects (Davies, 1976; Rachlin et al., 1982; 1983). Organic exudates and membranous organelles are two cases in point. The former are released by diatoms due to the increased permeability of their cell membranes caused by cation stress. However, the released material binds metal ions externally, thus prohibiting further metal uptake. The latter present a great surface area where metals can bind to membrane sulphydryl groups, and thus reduce the number of cations free to interact with vulnerable internal sites. Membranous organelles would be most efficient at sequestering metals with high affinity for sulphydryl groups such as mercury and copper, but less efficient towards metals with lower -SH affinity, like cadmium.

The dual role of some toxicity mechanisms that also serve in algal detoxification illustrates the difficulty of constructing a biochemical model to account for metal toxicity in all algae. Observed metal effects are the results of one to several toxicity mechanisms that affect various cellular processes, interacting with one or several detoxifying mechanisms. The net result of this interaction depends on the cellular sites and physiological processes affected, as well as on the adequacy of cellular defenses to counteract metal stress. Ultimately, it rests on the

biochemical profile of the species and on its genetic makeup.

APPENDIX I

Colby's Formula

$$E = XY/100$$

E = the expected population density of the alga as a percentage of the control

X = the population density of the alga as a percentage of the control when exposed to metal X

Y = the population density of the alga as a percentage of the control when exposed to metal Y

Hall's Modified Formula

$$I = (Nt - No)/No \,/\, (Nt - No)/No$$

I = the Index of Tolerance

Nt = the algal population at the end of the 96-h period

No = the algal population at the beginning of the testing period

The values of the numerator represent the metal challenged cultures, while the values of the denominator represent the control cultures.

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