THE EFFECTS OF COPPER, ZINC AND CADMIUM ON THE PIGMENTS AND PHOTOSYNTHESIS OF BLACK SPRUCE (*Picea mariana* (Mill.) B.S.P.) SEEDLINGS

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Karen E. Watt ©

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ABSTRACT

Keywords: heavy metals; HPLC; fluorescence; red edge; oxygen evolution

The purpose of this study was to quantify the effects of heavy metals on pigments and photosynthesis in four and a half month old black spruce (*Picea mariana* (Mill.) B.S.P.) seedlings. Photosynthetic pigment analysis by HPLC and reflectance red edge methods were compared to chl *a* fluorescence and net oxygen evolution measurements to assess their effectiveness in diagnosing plant stress. Copper, zinc and cadmium were applied as 0, 10 and 20 mM $Cu(NO_3)_2$, Zn $(NO_3)_2$ and $Cd(NO_3)_2$, for 2½ weeks. Response to the treatments was examined by measuring pigment content, reflectance red edge, F_v/F_m, net oxygen evolution, net water content and needle element content. No treatment differences were found for pigment content or red edge. In contrast, F_v/F_m of seedlings treated with 20 mM Cd was significantly lower than all other treatments and net oxygen evolution decreased in metal-treated seedlings compared to untreated control seedlings. Net water content did not differ between treatments. Needle element content did not change, except for increases in Cu, Zn and Cd in the seedlings treated with these elements. It was concluded that pigment content was unaffected by the treatments but that photosynthetic efficiency (i.e. oxygen evolved) decreased. Possible explanations include inhibition of enzyme activity by Cu^{2+}, Zn^{2+} and Cd^{2+}, or overloading of defense mechanisms, such as phytochelatins. The use of HPLC analysis of pigments and remote spectroradiometry may prove useful in assessing seedling quality.

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1.0 INTRODUCTION

1.1 *Heavy Metals as Pollutants*

Forests in proximity to sources of industrial pollution have been in visible decline for many years (Tiller 1989). In addition to the effects of nutrient deficiency, water stress, increased O_3 and acid rain, the presence of heavy metals in contaminated soils can have detrimental effects on the growth and development of vegetation.

Heavy metals have been defined as metals with a specific weight higher than 5 g/cm^3 or having an atomic number above 20 (Barceló and Poschenrieder 1990). This encompasses many elements, not all of which are toxic at concentrations found in nature. The heavy metals most often considered environmental pollutants include Bi, Cd, Cu, Ni, Pb, Sb and Zn (Tiller 1989).

Toxic levels of heavy metals in soil occur as a result of both natural and anthropogenic causes. Natural sources of heavy metals include windblown dusts, volcanoes, forest fires and mineral deposits. Anthropogenic sources of metal pollution are of greater concern and include industrial pollution, agricultural fertilizers and pesticides, municipal wastes and use of sewage sludge for soil amelioration (Tiller 1989).

1.2 *Heavy Metals and Plant Metabolism*

Heavy metals affect plants in a variety of ways: reduction in shoot and root growth and biomass, reduction in plant water content and decreased transpiration, decreased leaf chlorophyll content and photosynthetic rate, modification of chloroplast

ultrastructure and metabolism, chlorosis, increased lateral root formation, thickening of root tips and root discolouration, changes in enzyme activity, changes in cell permeability, disturbance in nitrogen metabolism, and interference with nutrient uptake and metabolism (Goetz *et al.* 1983; Clijsters and Van Assche 1985; Balsberg Påhlsson 1989; Punz and Sieghardt 1993). Visible symptoms aid in the diagnosis of heavy metal toxicity on vegetation. These include alteration of pigmentation, premature needle drop or complete defoliation, decreased amount of new growth, root stunting and thickening, shallow rooting, chlorosis, and necrosis (Malhotra and Blauel 1980; Heale and Ormrod 1982; Heale and Ormrod 1983). Heavy metal uptake and effects will vary depending on species, genotype, plant part, growth conditions and rooting depth (Tiller 1989).

Many studies have been done on the effects of heavy metals on the growth of agricultural crops and vegetables. Contaminated garden soil or fields where plants are grown for human consumption are of great concern due to the possibility of metals being transported through the food chain (Pip 1991; Boon and Soltanpour 1992; Gagné and Létourneau 1993). Studies using *Phaseolus vulgaris* found that exposure to nickel (0-500 μM), zinc (0-30.6 mM) or cadmium (0-712 μM) resulted in decreases in growth and pigment levels, changes in chloroplast metabolism and increases in the capacity of some enzymes (Van Assche *et al.* 1988; Krupa *et al.* 1993). Rascio *et al.* (1993) found that cadmium-treated (10-250 μM) maize plants (*Zea mays*) exhibited decreased shoot length, root length, chlorophyll content, oxygen release and alterations in chloroplast ultrastructure. It was concluded that cadmium inhibits PSII activity and photosynthetic apparatus functionality. Wheat plants *(Triticum aestivum*) grown on copper enriched ore

bodies or in the presence of toxic levels of manganese (0.91-9.1 mM) displayed inhibited growth and chlorosis, as well as decreases in chlorophyll levels, total soluble protein, sugars and starch, lipids, and fluorescence parameters (Ohki 1985; Lanaras *et al.* 1993).

Of particular interest, with regards to forest decline, are studies on trees. Field studies have been conducted to examine the effects of various forms of pollution on the growth and development of nearby trees. Concentrations of heavy metals are generally highest in trees growing closest to the source of pollution and are inversely related to distance from the source (Malhotra and Blauel 1980; Bramryd 1981; Lukaszewski *et al.* 1988). One of the best examples of this is the nickel smelter in Sudbury, Ontario, whose emissions of sulphur dioxide and heavy metals have severely affected an area of nearly 500 km^2 around the smelter (Tiller 1989).

In addition to pollution, vegetation growing over mineral deposits is affected by heavy metals. Most studies find that mineralization causes vegetation to have higher reflectances in the visible wavelengths of the spectrum. This effect is caused by destruction or lack of synthesis of chlorophyll (Horler *et al.* 1980).

Many greenhouse studies have examined the effects of heavy metals on seedlings grown in nutrient solutions. Studies involving *Pinus strobus* L. and *Picea glauca* (Moench) Voss seedlings found decreases in plant growth after exposure to copper (0-100 ppm) and nickel (0-10 ppm) for 12 weeks (Lozano and Morrison 1982). The exposure of *Picea abies* (L.) Karst to zinc (0.1-100 μM), cadmium (0-15 μM) or mercury (0-0.5 μM) for five weeks resulted in reduced needle chlorophyll content, water content and CO_2 uptake. These reductions were due to inhibition of iron transport and stomatal closure,

respectively (Schlegel *et al.* 1987). Heale and Ormrod (1982, 1983) studied a variety of woody plant species and their susceptibility to copper and nickel, applied at concentrations of 2 to 20 ppm for 40 days. Classic toxicity symptoms were observed but there was much variability in response within and between species. It was suggested that any individuals exhibiting resistance to heavy metal toxicity should be considered for the development of genetically resistant populations.

1.3 *Study Objectives*

The objective of this study was to quantify the effects of copper, zinc and cadmium treatments on the pigment content, net oxygen evolution, fluorescence and red edge in *Picea mariana* (Mill.) B.S.P. seedlings. Copper, zinc and cadmium were chosen due to their abundance in nature and prevalence in polluted areas. A further goal was to compare the effectiveness of high performance liquid chromatography (HPLC) analysis of pigments and remote spectroradiometric measurements of reflectance to standard techniques, such as fluorescence and net oxygen evolution, for monitoring of seedling stress. If effective, these new methods may broaden the scientific options available for monitoring decline in forest vigour and possibly aid in prospecting for heavily mineralized areas.

2.0 LITERATURE REVIEW

2.1 *Plant Photosynthetic Pigments*

Photosynthetic pigments, chlorophylls and carotenoids, play an important role in the overall metabolism of plants (Goodwin and Mercer 1983). The main role of chlorophyll in photosynthesis is the absorption of visible light. Chlorophyll absorbs light in the blue and red regions of the spectrum with wavelength peaks at 428 and 660 nm for chlorophyll *a* (chl *a*), and 452 and 642 nm for chlorophyll *b* (chl *b*) (Lichtenthaler 1987). Carotenoids are classified as the oxygen-free carotenes and the xanthophylls (Lichtenthaler 1987). β-carotene protects chl *a* from damage in high light conditions and may play a role in light absorption (Goodwin and Mercer 1983). The xanthophylls, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin and lutein are also thought to aid in photoprotection (Demmig-Adams and Adams 1992). Carotenoids absorb light in the blue region of the spectrum, with three characteristic wavelength peaks occurring between 400 and 500 nm (Ruban *et al.* 1993). Chlorophyll *a* is considered a major pigment, whereas chlorophyll *b* and the carotenoids are classified as accessory pigments (Lichtenthaler 1987).

2.2 *Plant Stress and Chlorophyll a/b Ratios*

Photosynthetic pigment content and relative composition are sensitive to changes in a plant's environment, hence pigment content is often measured to assess the vigour of a plant. From these measurements, ratios can be calculated and used as indicators of a

plant's environmental conditions. The chlorophyll *a/b* ratio, for example, tends to be 3:1 under favourable conditions but changes dramatically in response to stress (Lichtenthaler 1987). Monge *et al.* (1987) found that sunflower (*Helianthus annuus*) and peanut (*Arachis hypogaea*) plants grown under iron deficiency showed a significant increase in chlorophyll *a/b*. *Hygrophila polysperma* subjected to salt or nutrient stress showed significantly higher chlorophyll *a/b* ratios than either control or cropped plants (Kovach *et al.* 1992). Cucumber (*Cucumis sativus*) plants treated with high light and low temperatures exhibited a greater decrease in chlorophyll *a* compared to chlorophyll *b*. This was attributed to chlorophyll *a* bleaching and the breakdown of the light harvesting complex (Öquist *et al.* 1987). The variation in values of chlorophyll *a/b* illustrated above depends on, not only the immediate environmental conditions, but also on the developmental history of the plant (Venator *et al.* 1977).

Studies of trees often report contents of chlorophyll *a* and *b*, rather than ratios. Edwards *et al.* (1990) found that *Pinus taeda* seedlings exposed to twice ambient O_3 showed significant increases in chlorophyll *a* and chlorophyll *b* compared to ambient and subambient O_3-treated seedlings. However, *Pinus ponderosa* seedlings exposed to long-term elevated CO_2 (greater than 425 μL/L CO_2) showed reductions in chlorophyll *a* and chlorophyll *b* concentrations (Houpis *et al.* 1988). A study of *Picea abies*, in forest decline areas of Germany, found that pigment dynamics of chlorotic trees were similar to that of undamaged trees. Although the chlorotic trees exhibited much lower extractable chlorophyll and carotenoid concentrations, the chlorophyll *a/b* ratio remained unchanged (Köstner *et al.* 1990). Similarly, Wild *et al.* (1993) found significant reductions in the

chlorophyll content of damaged spruce trees compared to undamaged trees.

2.3 *Plant Stress and Carotenoids*

Carotenoid content and ratios are also useful indices of stress and have been used to characterize forest damage and decline (Lange *et al.* 1987; Wolfenden *et al.* 1988; Weikert *et al.* 1989). The carotenoid ratio of particular interest is the ratio of xanthophylls and their role in the xanthophyll cycle. The xanthophyll cycle is composed of the light-dependent conversions of violaxanthin to antheraxanthin to zeaxanthin (Figure 1).

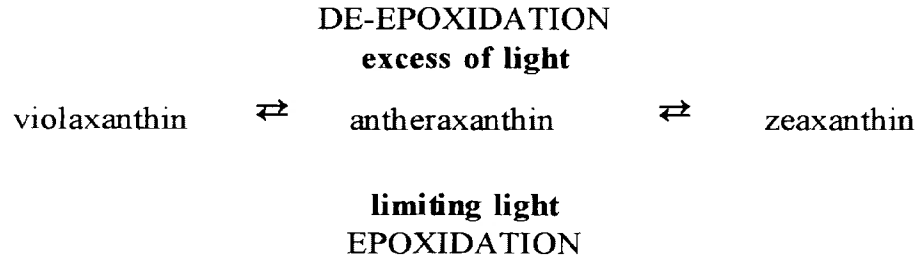


Figure 1: The xanthophyll cycle involving the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin and the epoxidation of zeaxanthin to violaxanthin via antheraxanthin. [From: Demmig-Adams (1990)]

When light levels are excessive, the result is the conversion to zeaxanthin; in non-excessive light levels, violaxanthin predominates (Demmig-Adams and Adams 1992). Zeaxanthin is formed through the de-epoxidation of violaxanthin via antheraxanthin, catalyzed by a de-epoxidase. The conversion of zeaxanthin to antheraxanthin and violaxanthin is facilitated by an epoxidase (Demmig-Adams 1990). The xanthophyll cycle is thought to assist in the dissipation of excess energy not used in photosynthesis. Many

studies support the role of the xanthophyll cycle in the photoprotection of photosystem II (PSII). A study using *Populus balsamifera, Hedera helix* and *Monstera deliciosa* exposed to high light (500 to 1500 μmol photons m^{-2} s^{-1}) resulted in the formation of zeaxanthin and a corresponding decrease in violaxanthin (Demmig *et al.* 1987). Demmig-Adams *et al.* (1989) exposed leaves of *Rhizophora mangle* to an excess of light (120 photons m^{-2} s^{-1}) and chilling temperatures (5°C). Leaves that had been pre-illuminated and, hence, contained high levels of zeaxanthin, recovered within 1 to 3 hours when returned to pre-treatment conditions (low light, 25°C). Leaves that had been kept in the dark prior to treatment did not recover at all.

2.4 *Methods of Pigment Analysis*

Quantification of pigment levels in plants has traditionally been done using spectrophotometry (Lichtenthaler 1987). More recently, high performance liquid chromatography (HPLC) has been utilized effectively to achieve accurate determinations of pigment concentrations in higher plants (Krinsky and Welankiwar 1984; Val *et al.* 1986; Monge *et al.* 1987; Siefermann-Harms 1988; Pfeifhofer 1989; Mínguez-Mosquera *et al.* 1991; Almela *et al.* 1992; Craft 1992; Lopez-Hernandez *et al.* 1993), with particular consideration given to *Picea abies* (Hoque 1988; Zellnig *et al.* 1989; Senser *et al.* 1990; Zaerr and Schill 1991). In HPLC, the sample is injected into a column containing a stationary phase and run under high pressure with a stream of mobile solvent (Meyer 1988). Studies of plant pigments most often utilize reversed-phase HPLC. In reversed-phase chromatography, the stationary phase of the column is very non-polar and

the mobile phase is relatively polar. As a consequence, non-polar compounds are eluted later than polar compounds. HPLC can be used as an alternative to spectrophotometry since both are fast, precise and accurate. However, using HPLC, all pigments can be separated and easily quantified after one sample run.

2.5 *Photosynthetic Energy Utilization and Chlorophyll a Fluorescence*

Increased plant stress can be detected as decreased efficiency of absorbed energy conversion to photosynthetic products. Photosynthetic efficiency is defined as the quantum yield of oxygen evolution, which reflects the moles of oxygen evolved per photon absorbed (Goodwin and Mercer 1983). Not all visible light absorbed by pigments is used in photosynthesis. The reaction centres of the two photosystems absorb up to 90% of the excitation energy derived from visible light. This energy drives the photochemical reactions of photosynthesis (Björkman and Demmig 1987). The light energy absorbed by the pigments which is not used in photosynthesis is dissipated as thermal, fluorescent, reflected and transmitted radiation at all times, and particularly during stress (Banninger 1989). Excess light energy is potentially dangerous to the plant and is thought to be dissipated as thermal energy through the xanthophyll cycle (Demmig-Adams and Adams 1992).

A second process of deactivating excited pigments is the chlorophyll *a* fluorescence of PSII. The ratio of the variable fluorescence (F_v) to maximum fluorescence (F_m) from intact plant leaves can be measured and used to evaluate the physiological state of a plant. The potential yield of the photochemical reaction of PSII correlates well with

F_v/F_m. Thus, F_v/F_m can be used as an indicator of photosynthetic efficiency which is defined as the quantum yield of oxygen or carbon dioxide evolution (Krause and Weiss 1991).

Changes in chlorophyll *a* fluorescence kinetics are often used as indicators of stress (Schmuck 1990). Frequently, environmental stresses that affect PSII efficiency lead to a decrease in F_v/F_m (Krause and Weiss 1991). Studies in which plants have been treated with low temperatures and high light levels have observed a decrease in F_v/F_m (Björkman and Demmig 1987; Bolhàr-Nordenkampf and Lechner 1988; DeLucia *et al.* 1991; Georgieva and Yordanov 1993). However, in a study of nickel toxicity in *Phaseolus vulgaris*, no change in F_v/F_m was observed, indicating that the nickel treatments had no effect on PSII reaction centres (Krupa *et al.* 1993).

2.6 *Red Edge and Vegetation Reflectance Spectra*

A third pigment deactivation process is light reflection. Reflected radiation forms the basis of vegetation surveys by remote sensing. Remote sensing utilizes the reflectance spectra of objects (including pigmented vegetation) in the visible and infrared light spectra and translates it into visible wavelengths. This provides a distinctive spectral "signature" for each observed object. Through the use of remote sensing, it is possible to monitor changes in pigment content (Harper 1983). Canopy chlorophyll levels can be estimated by the measurement of red edge. Red edge is the point of maximum slope in vegetation reflectance spectra which occurs between the red and near infrared wavelengths, at approximately 680-740 nm (Curran *et al.* 1991). It is measured by determining the

wavelength at maximum slope or the derivative maxima of the reflectance curve (Curran *et al.* 1990). Much work has been done regarding red edge, chlorophyll content and fluorescence in plants. A positive relationship between red edge and chlorophyll content has commonly been found (Curran *et al.* 1990; Chappelle *et al.* 1991; Curran *et al.* 1991; Moss and Rock 1991). Often, shifts in red edge occur as a result of plant stress. Studies using nitrogen deficiency and toxicity as a stress have reported, that with increased nitrogen, a shift in red edge to longer wavelengths was observed, which indicates increased chlorophyll in the plant (Clevers and Büker 1991; Plummer *et al.* 1991). However, conifers exposed to air pollution and water stress exhibited a shift in red edge to shorter wavelengths. This result was attributed to chlorophyll degradation (Westman and Price 1988).

In addition to red edge shifts, impairments of the photosynthetic process in response to physiological and biochemical changes in the plant often result in an increase in plant total reflectance (Banninger 1989). This has been illustrated in many studies, with damage caused by O_3 treatment (Ustin and Curtiss 1990), lack of ectomycorrhizae (Cibula and Carter 1992), and high soil metal concentrations (Wagner *et al.* 1989).

It is possible to distinguish different stresses and damage to plants using both fluorescence and red edge. The incorporation of both measurements improves interpretation of results (Lichtenthaler and Buschmann 1987; Buschmann and Lichtenthaler 1988; Banninger 1989; Buschmann *et al.* 1991; Ruth *et al.* 1991). The use of F_v/F_m and red edge provides an indication of both photosynthetic efficiency and pigment levels.

3.0 MATERIALS AND METHODS

3.1 *Seedling Production*

Seedlings of black spruce (*Picea mariana* (Mill.) B.S.P.) were grown from seeds obtained from the Ontario Ministry of Natural Resources (OMNR), Ontario Seed Plant in Angus, Ontario (seed source # 003-34-25-0-00; seed lot # 79). Seeds were planted in peat moss/vermiculite (1:1) in Ventblocks 165 (10 x 10, Beaver Plastics, Edmonton, Alberta) and grown in a greenhouse maintained at day and night temperatures of 20°C and 15°C, respectively, with a relative humidity of 70% and a photoperiod of 16 hours, supplemented with high pressure sodium lamps, when necessary. Water was initially supplied by misting until germination, at which time seedlings were moved to a greenhouse and hand-watered daily. Fertilizer treatment, using 50 mg L^{-1} N Plant Products Seedling Starter (11-41-8), began two weeks after germination and was applied every second day, alternating with water. After four weeks, fertilizer was changed to 100 mg L^{-1} N Plant Products Seedling Special (20-8-20). Seedlings were irrigated every second day, alternating between water and fertilizer solution. One hundred seedlings were grown in each ventblock. Fourteen ventblocks were planted in June, 1993, and again in January and February, 1994.

3.2 *Treatment*

Following four and a half months growth, seedlings (height ≈ 20 cm) were subjected to three heavy metal treatments. The metals used were copper, zinc and

cadmium, as $Cu(NO_3)_2$, $Zn(NO_3)_2$ and $Cd(NO_3)_2$, respectively, at 0, 10 and 20 mM concentrations, adjusted to pH 5.5 to 7.5 using KOH. One litre of solution was applied to 100 seedlings (one ventblock) every three days for 2½ weeks. Precautions were taken to avoid cross-contamination. Between treatments, seedlings were watered and fertilized as usual.

The treatments were applied as a randomized complete block design (RCBD) with time as the blocking factor. The first block of trees were those sown in June, 1993, the second block in January, 1994 and the third block in February, 1994. Hence, treatment commenced on each block in October, 1993, June, 1994 and July, 1994, respectively. One ventblock, containing 100 seedlings, was considered an experimental unit. Each block contained two replicates of each of the seven treatments (control; 10 mM Cu, Zn, Cd; 20 mM Cu, Zn, Cd), for a total of fourteen experimental units. For pigment analyses, two samples were taken from each ventblock. In the case of fluorescence, individual trees were considered samples. Response variables measured were pigment content, reflectance spectra, fluorescence, net oxygen evolution, net water content, and element content and concentration per dry weight seedling. Measurements were made in the week following treatment.

3.3 *Pigment Analysis*

Five seedlings from each ventblock (experimental unit) were randomly selected and needles were combined for pigment extraction. From these extracts, two samples were analyzed. Pigments (neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin,

chlorophyll b, chlorophyll b', chlorophyll a, chlorophyll a', α-carotene, β-carotene) were extracted from needles and prepared for analysis following standard methods (Davies 1976; Holden 1976; Schmitz *et al.* 1993). Needles of known mass were ground with a mortar and pestle in acetone/methanol (1:4). The extracts were centrifuged for 20 minutes at 7000 x g in a Sorvall RC-5B Refrigerated Superspeed Centrifuge, with SS-34 Rotor (DuPont Instruments, Canada). The supernatant was flash evaporated and the residue was dissolved in a known volume of methanol.

The analysis was performed using a Beckman System Gold High Performance Liquid Chromatograph (HPLC), including a model 125 programmable solvent module, a model 168 UV-Vis diode array detector and a Rheodyne model 7725i injection valve, with a 500 µL loop. Chromatography was performed on a Beckman reversed phase Ultrasphere octadecylsilica (ODS) column (5µm, 4.6 mm X 25 cm). A linear gradient was used, from 85% methanol:15% acetonitrile (HPLC grade, Fischer Scientific, Canada) to 100% methanol over ten minutes, with 100% methanol being maintained for a further 16 minutes. At that time, a linear gradient back to the original condition was conducted over four minutes. Solvents were degassed by sonication for ten minutes prior to use. Solvent flow rate was 2 mL min^{-1} and pigment absorption was monitored at 450 nm.

Absorption spectra of peak wavelengths were obtained to confirm the identity of each pigment (Appendix A). The peak wavelengths obtained were compared with published values (Davies 1976; Goodwin and Mercer 1983; Mínguez-Mosquera *et al.* 1991; Almela *et al.* 1992). In addition, retention times of standards chlorophyll *a*, chlorophyll *b* and β-carotene (Sigma Chemical, St. Louis, MO) were established.

The areas under the peaks of chromatograms are proportional to the amount of compound injected (Meyer 1988). Concentrations were calculated by comparing the peak areas of the unknowns with the peak areas of standard chlorophyll *a*, chlorophyll *b* and β-carotene of known concentration. Dilution factors, injection volume and mass of needles were taken into consideration (Appendix B). The ratios of the concentrations of chlorophyll:carotenoid, chlorophyll *a*:chlorophyll *b* and violaxanthin:zeaxanthin were calculated.

3.4 *Reflectance Spectra and Red Edge*

The reflectance spectra of the seedlings were measured using a Spectron Engineering SE590 Spectroradiometer, provided by the Ontario Ministry of Natural Resources, Provincial Remote Sensing Office (North York, Ontario). For each block, ten randomly chosen seedlings from each ventblock were used for these measurements. Seedlings were placed together to form a solid mat of needles, in order to avoid background interference. Barium sulphate was used as a reference to standardize the light source. From the scans, the derivative maxima of the reflectance curve from 670 to 780 nm (the "red edge") was calculated. Each curve from 670 to 780 nm was defined by a third or fourth order polynomial equation. The zero crossing of the second derivative of each equation provided the appropriate wavelength (red edge).

3.5 *Chlorophyll a Fluorescence*

Fluorescence was measured using a BioMonitor Plant Stress Meter (Umeå, Sweden) according to manufacturer's instructions. A sample of five seedlings from each ventblock was taken and the F_v/F_m of each seedling was measured. Seedlings were dark-adapted for 10 minutes, then exposed to an activation light of 100 μE $m^{-2}s^{-1}$, for two seconds. The ratio of F_v/F_m was recorded.

3.6 *Net Oxygen Evolution and Net Water Content*

Photosynthesis and respiration were monitored, as oxygen evolution and consumption, using a Hansatech Oxygen Electrode (Norfolk, England). Measurements were made at 20°C, using light intensities of approximately 200-400 μmol m^{-2} s^{-1}, under CO_2 saturating conditions. CO_2 was supplied by a capillary matting soaked in bicarbonate. Approximately 0.2 g of needles from randomly selected seedlings from each ventblock were used for measurement (Delieu and Walker 1972; Delieu and Walker 1981). Net oxygen evolution per g dry weight was calculated.

Fresh weights and dry weights (4 hours at 80°C) of needles from randomly chosen seedlings were measured. Net water content was calculated.

3.7 *Element Analysis*

Approximately 1.0 g of needles from randomly selected seedlings from each ventblock were analyzed for element content and concentration using Inductively Coupled Plasma Spectrophotometer Analysis (Thermo Garrell-Ash, ICAP 9000) according to

manufacturer's instructions. Elements quantified included Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, S and Zn. Needles were oven-dried, ground and pre-digested overnight in nitric acid. Nitric acid digestion was completed the following day at 100°C for 3 hours. Samples were cooled and diluted to 5% HNO_3, using nanopure water (Barnstead/Thermolyne Series 550, Dubuque, IA), prior to filtration and analysis by ICP (Havlin and Soltanpour 1980; Boon and Soltanpour 1992).

3.8 *Statistical Analysis*

Response variables and pigment ratios were tested for significant differences using analysis of variance (ANOVA) at $\alpha = 0.05$. Multiple comparisons were done using Duncan's test. All statistical analyses were done using SPSS.

4.0 RESULTS

4.1 *Pigment Analysis*

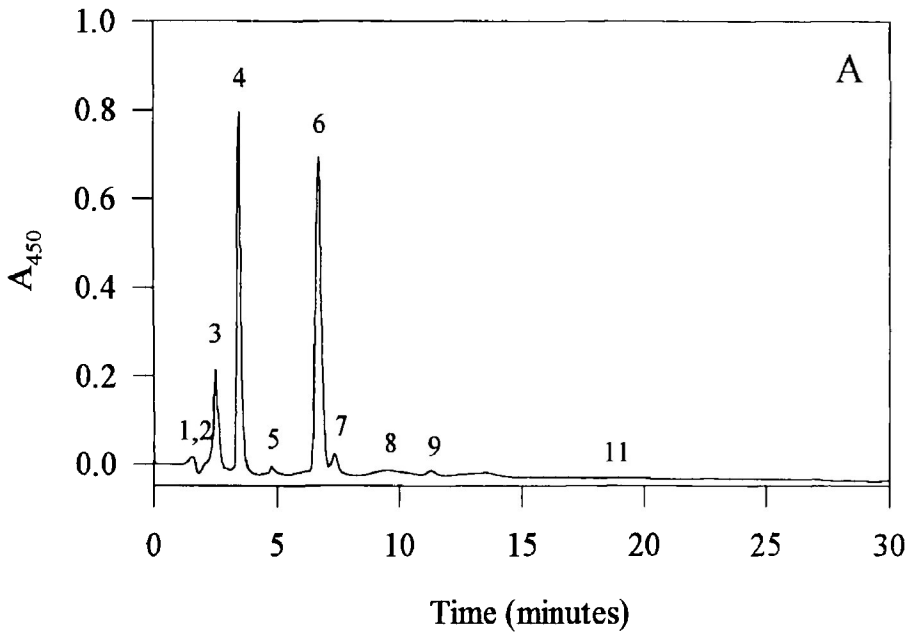
Chromatograms produced from HPLC analysis of pigments are shown in Figure 2. Each chromatogram is the mean of two replicates and two samples for Blocks 2 and 3 by treatment. Block 1 data were not available for pigment analysis due to technical problems. From the chromatograms, pigment concentrations were calculated and standardized, and analyzed by ANOVA. The expected mean squares (EMS) table for this design indicated no test for blocks, hence, only treatment and block by treatment tests were available (Appendix C).

The standard error of the mean was large in many cases. This can be attributed to the fact that means were calculated based on only four values. Hence, the large standard errors are a reflection of wide differences in replicates. These large standard errors may cause significant differences in means to be undetected.

The results of the ANOVA of pigment content revealed no significant differences, with the exception of neoxanthin content. Neoxanthin exhibited significant treatment differences ($p=0.001$). The metal-treated seedlings all displayed significantly lower neoxanthin levels than the control seedlings, based on a Duncan's test (Appendix D). There was no difference in neoxanthin between metal treatments.

No significant difference in pigment content between treatments was found for all other pigments (Appendix D). Total chlorophyll content displayed slightly higher levels for zinc-treated seedlings, although these differences were not significant (Figure 3).

Figure 2: Mean chromatograms of pigments, by treatment, before standardization on the basis of dry weight: A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd (B, C and D not shown since they are similar to A). Peaks correspond to: 1=neoxanthin, 2=violaxanthin, 3=antheraxanthin, 4=lutein, 5=zeaxanthin, 6=chlorophyll *b*, 7=chlorophyll *b*', 8=chlorophyll *a*, 9=chlorophyll *a*', 10=α-carotene, 11=β-carotene.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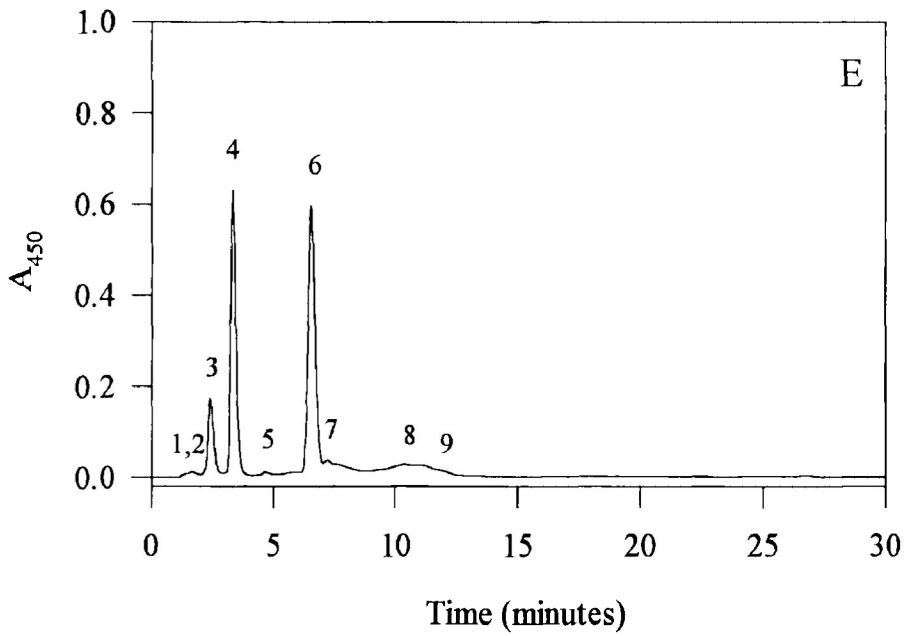
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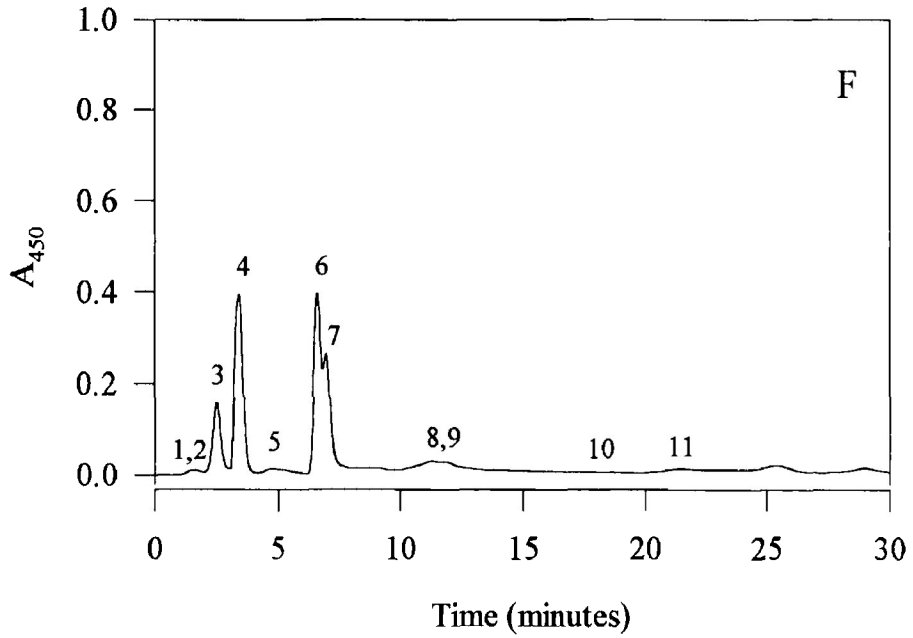
25
30
Time (minutes)


F
1.0
0.8
0.6
0.4
0.2
0.0
A_{450}
1,2
3
4
5
6
7
8,9
10
11
0
5
10
15
20
25
30
Time (minutes)

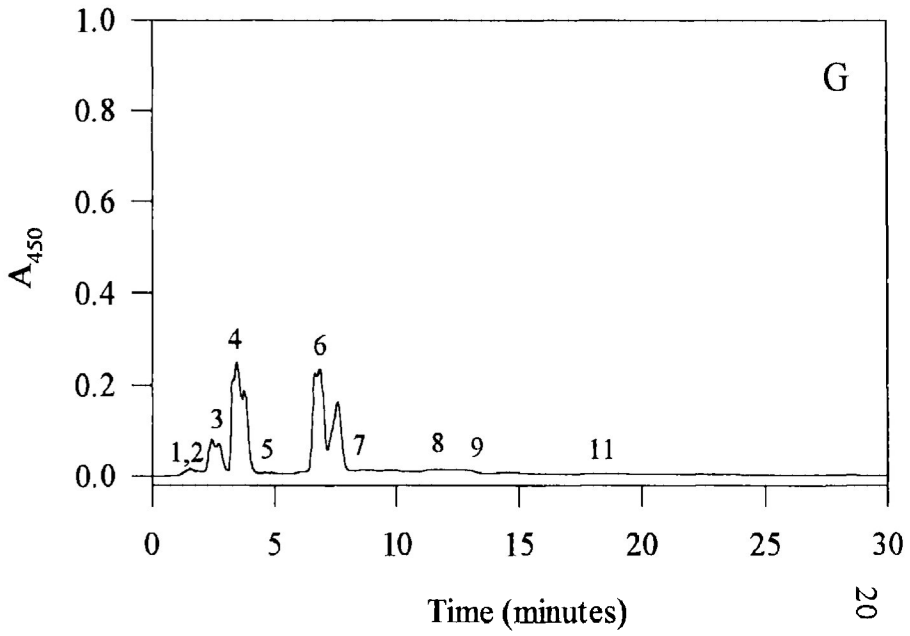
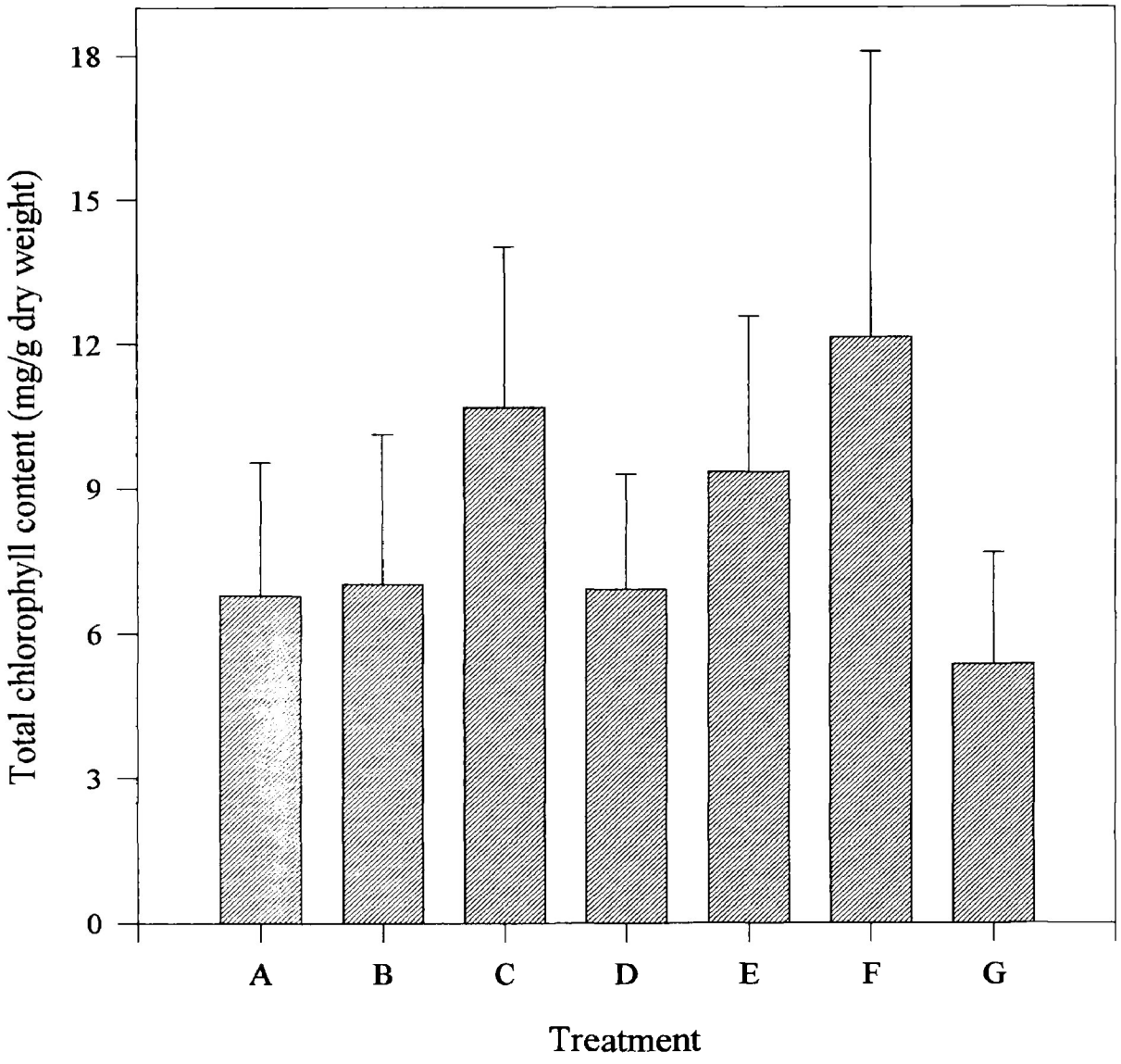

G
1.0
0.8
0.6
0.4
0.2
0.0
A_{450}
1,2
3
4
5
6
7
8
9
11
0
5
10
15
20
25
30
Time (minutes)

Figure 3: Means and standard errors of seedling total chlorophyll content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).


Total chlorophyll content (mg/g dry weight)
18
15
12
9
6
3
0
A
B
C
D
E
F
G
Treatment

Total carotenoid contents were not different (Figure 4), nor was the ratio of chlorophyll to carotenoid (Figure 5), although the ratio exhibited results similar to total chlorophyll. Slightly greater values of chlorophyll *a/b* were shown for seedlings treated with 10 mM Zn and 10 mM Cd (Figure 6), but these effects were not significant. The ratio of violaxanthin to zeaxanthin appeared higher for control seedlings than for the metal treatments but the difference was obscured by the variation in the controls (Figure 7).

4.2 *Reflectance Red Edge*

The calculated red edge values for all treatments over the three blocks showed no significant treatment effects. Means and standard errors of red edge for each treatment are shown (Figure 8).

4.3 *Fluorescence*

According to the ANOVA, treatment and block by treatment interaction effects of F_v/F_m were significantly different ($p<0.0005$). Over all blocks, only the 20 mM Cd treatment was lower than the control. Block 1 seedlings demonstrated the greatest decrease. Means and standard errors of F_v/F_m for seedlings from each treatment are displayed (Figure 9).

Figure 4: Means and standard errors of seedling total carotenoid content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

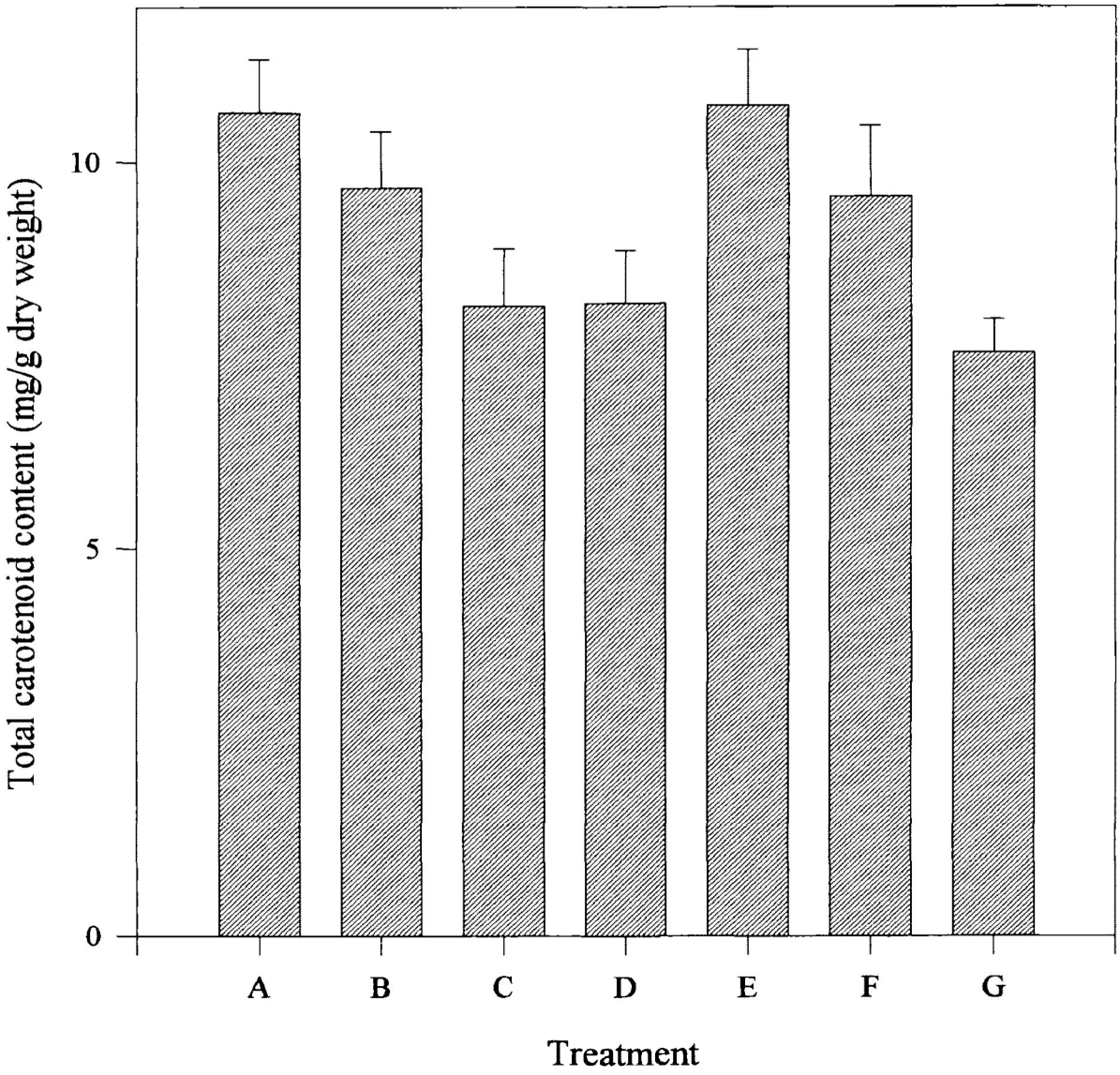

Total carotenoid content (mg/g dry weight)
10
5
0
A
B
C
D
E
F
G
Treatment

Figure 5: Means and standard errors of the ratio of chlorophyll to carotenoid by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

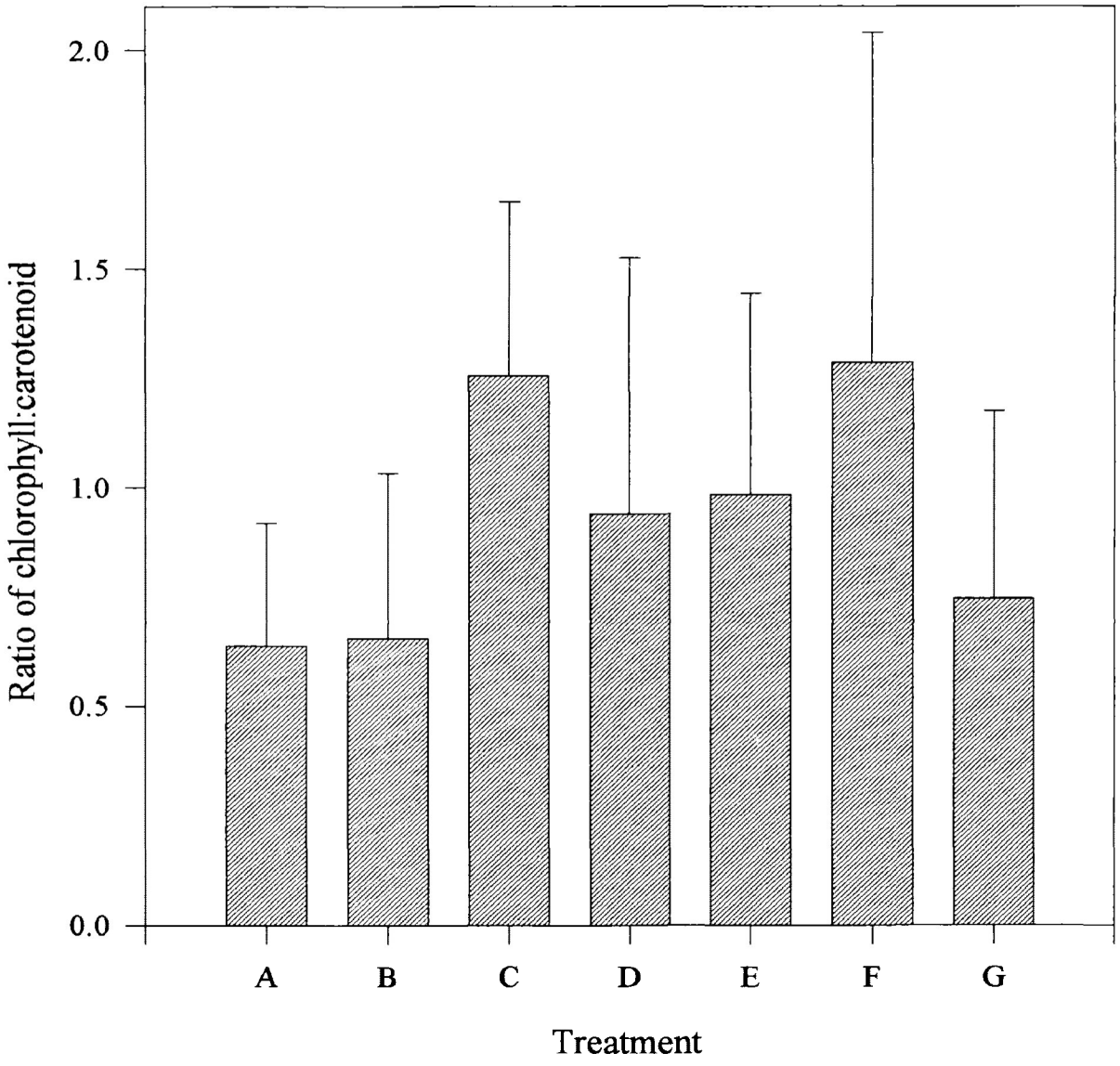

Ratio of chlorophyll:carotenoid
2.0
1.5
1.0
0.5
0.0
A
B
C
D
E
F
G
Treatment

Figure 6: Means and standard errors of the ratio of chlorophyll *a* to chlorophyll *b* by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

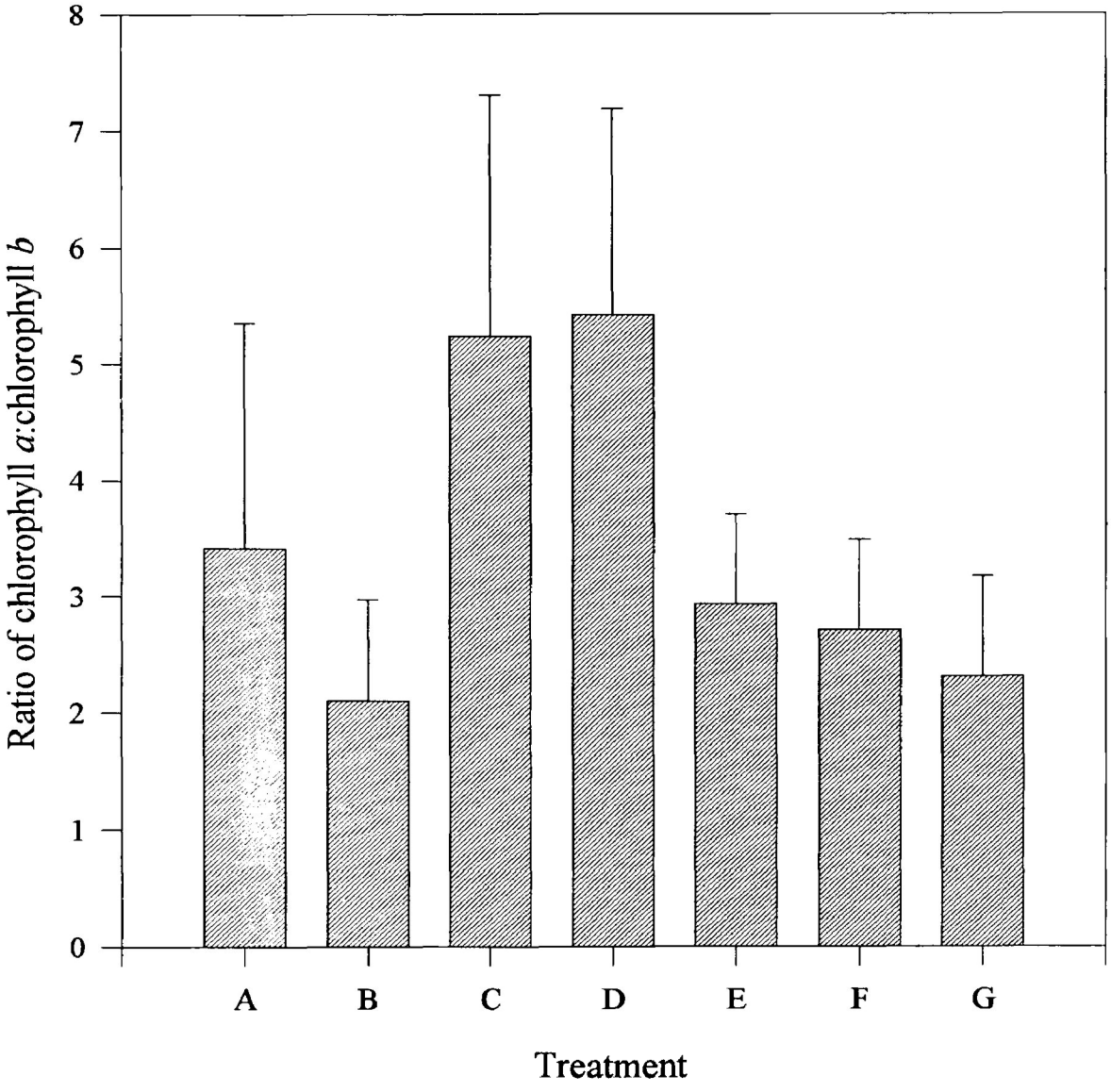

Ratio of chlorophyll a:chlorophyll b
8
7
6
5
4
3
2
1
0
A
B
C
D
E
F
G
Treatment

Figure 7: Means and standard errors of the ratio of violaxanthin to zeaxanthin by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

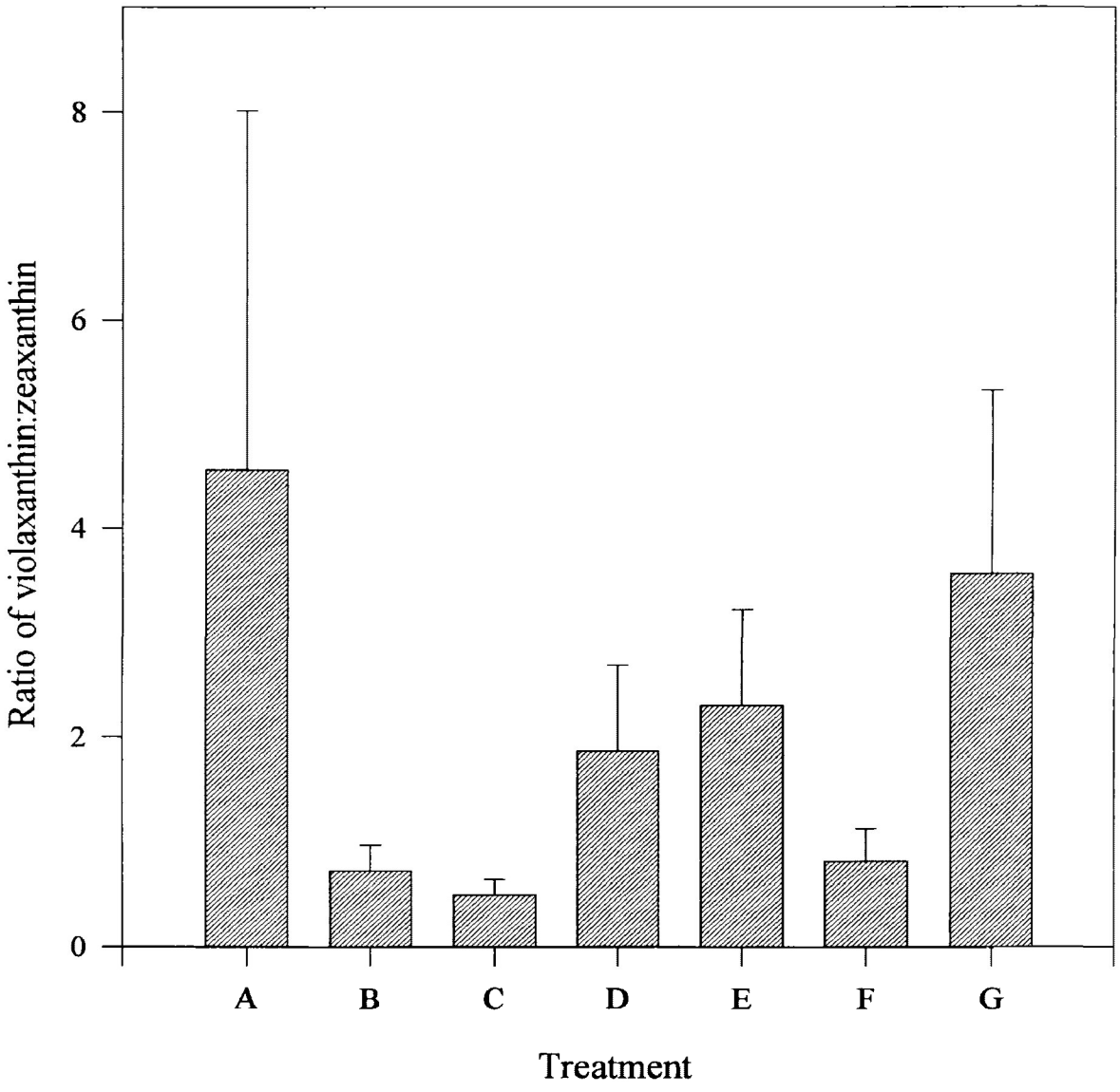

Ratio of violaxanthin:zeaxanthin
0
2
4
6
8
A
B
C
D
E
F
G
Treatment

Figure 8: Means and standard errors of red edge by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

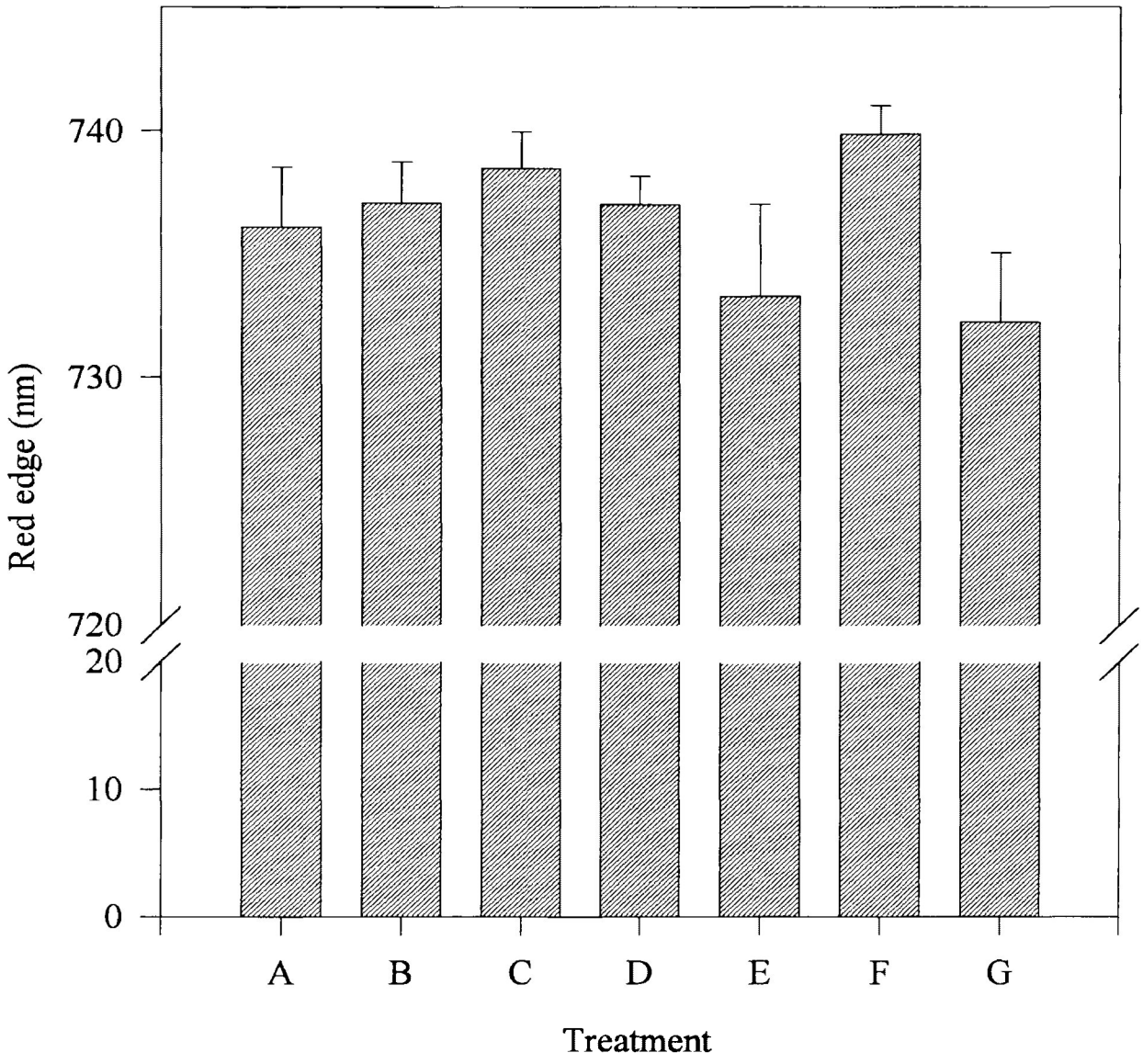
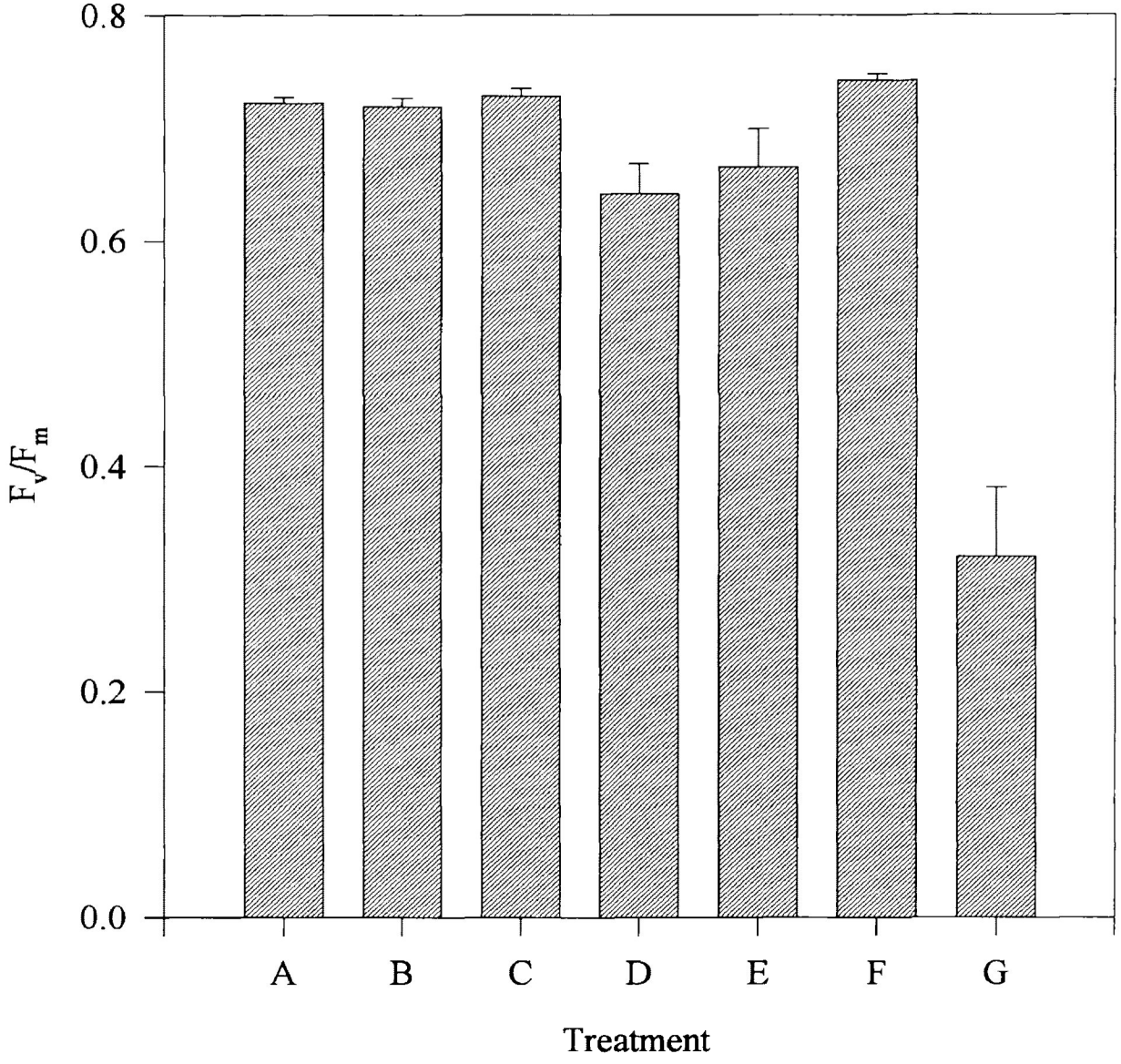

740
730
720
20
10
0
Red edge (nm)
A
B
C
D
E
F
G
Treatment

Figure 9: Means and standard errors of F_v/F_m by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).


0.8
0.6
0.4
0.2
0.0
F_v/F_m
A
B
C
D
E
F
G
Treatment

4.4 *Net Oxygen Evolution and Net Water Content*

Both net oxygen evolution and net water content displayed significant treatment and block by treatment interaction effects ($p<0.0005$). Mean net oxygen evolution of seedlings decreased in response to the metal treatments when compared to the control seedlings, with the greatest decreases occurring in seedlings treated with 10 mM Cd and 20 mM Cu (Figure 10). Net water content of seedlings in Blocks 2 and 3 remained constant over all treatments (Figure 11). Block 1 seedlings, however, displayed great variability over all treatments. Means and standard errors of net oxygen evolution and net water content of needles from each treatment are shown in Figures 10 and 11, respectively.

4.5 *Needle Element Concentration*

The p-values of the ANOVA of needle element concentration are listed in Appendix E. In general, Block 1 seedlings contained higher element concentrations than Blocks 2 and 3. Cadmium-treated seedlings exhibited much higher Cd concentrations than seedlings of other treatments (Figure 12). Similarly, copper- and zinc-treated seedlings had higher concentrations of Cu and Zn, respectively, than seedlings exposed to other treatments (Figure 12). Means and standard errors of needle element concentration for each treatment are shown in Appendix E.

Figure 10: Means and standard errors of seedling net oxygen evolution by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

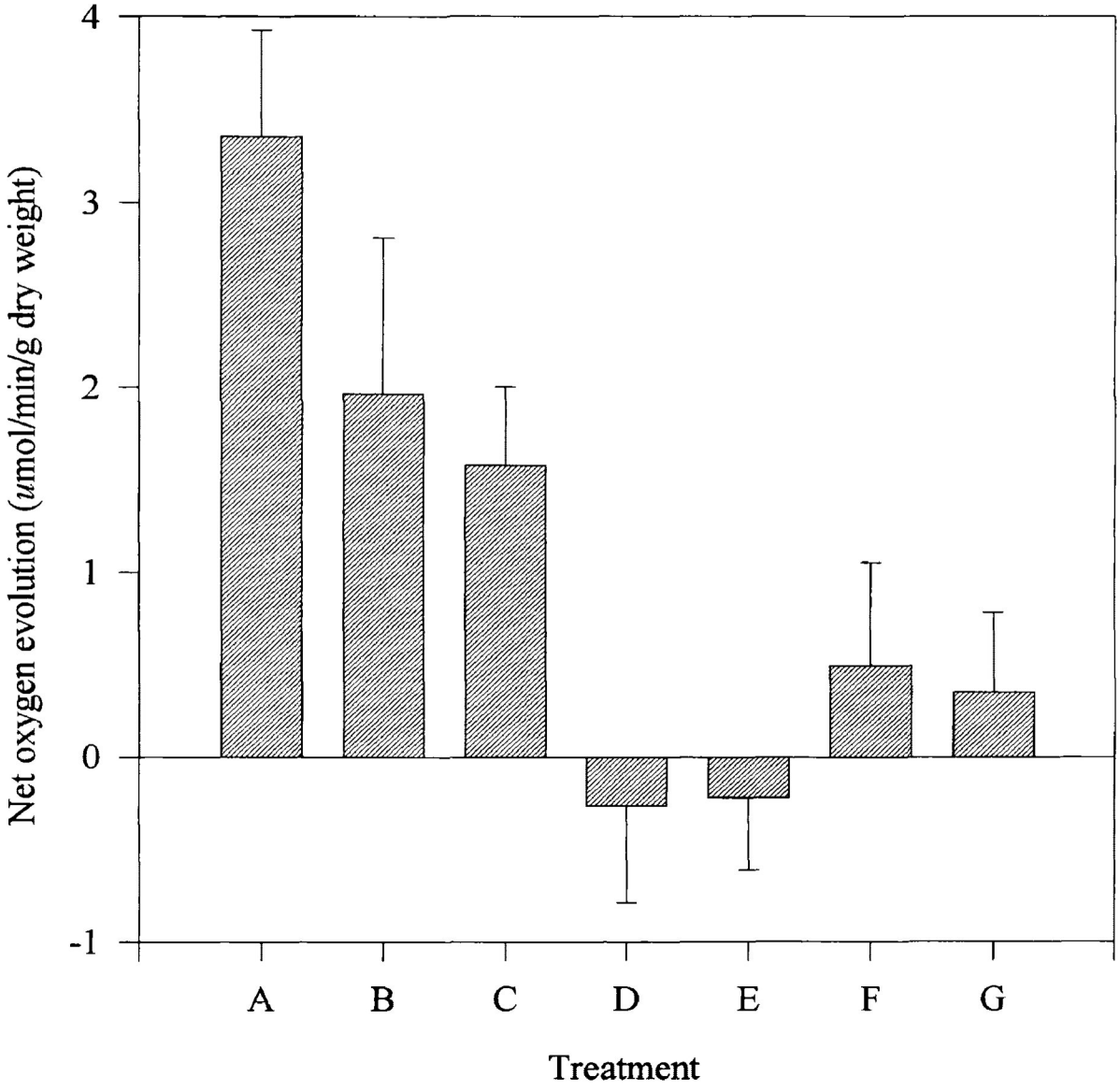

4
3
2
1
0
-1
Net oxygen evolution (umol/min/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure 11: Means and standard errors of seedling net water content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).

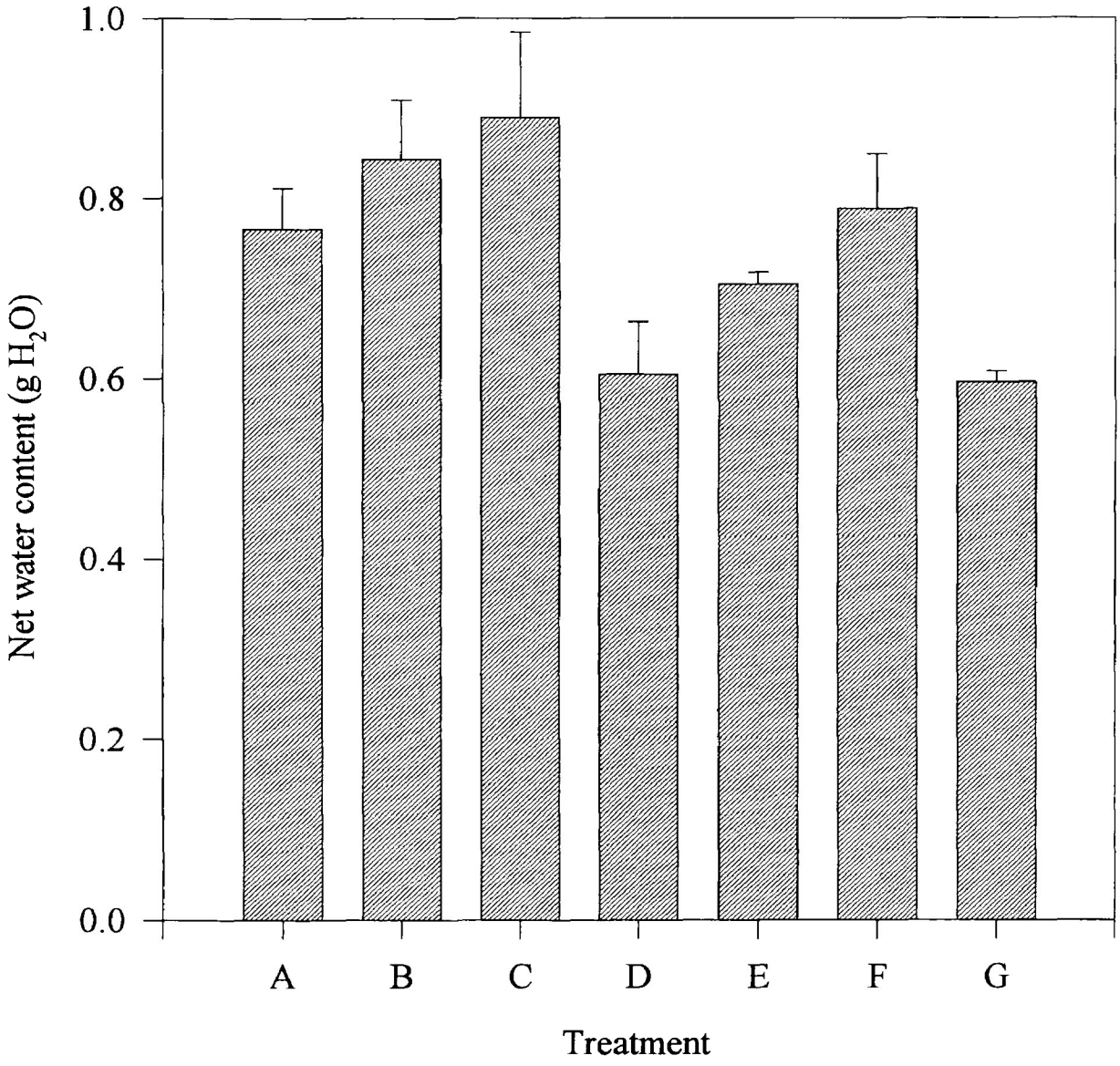
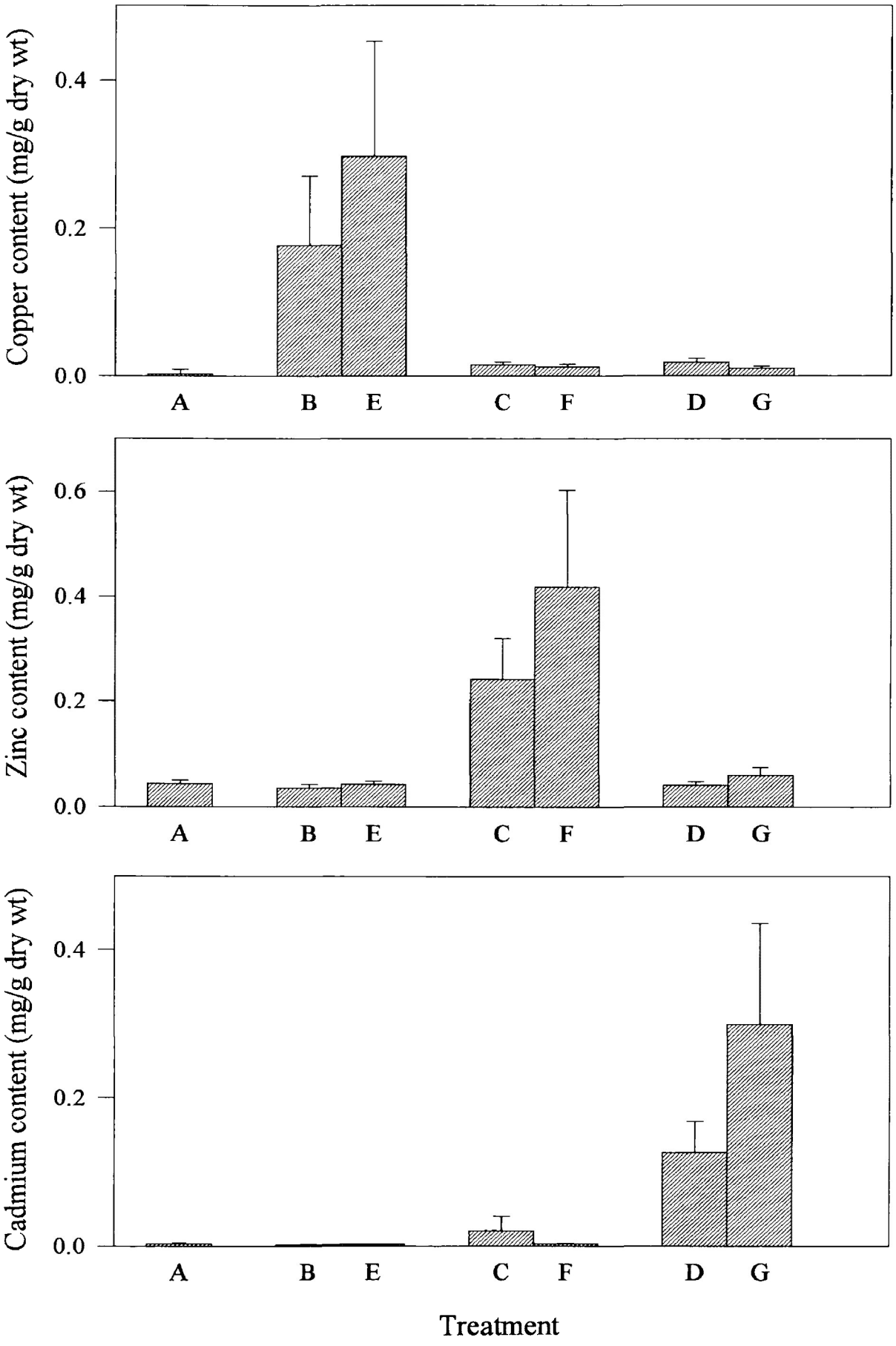

1.0
0.8
0.6
0.4
0.2
0.0
Net water content (g H_2O)
A
B
C
D
E
F
G
Treatment

Figure 12: Means and standard errors of seedling copper, cadmium and zinc content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=10mM Cd, E=20mM Cu, F=20mM Zn, G=20mM Cd).


Copper content (mg/g dry wt)
0.0
0.2
0.4
A
B
E
C
F
D
G
Zinc content (mg/g dry wt)
0.0
0.2
0.4
0.6
A
B
E
C
F
D
G
Cadmium content (mg/g dry wt)
0.0
0.2
0.4
A
B
E
C
F
D
G
Treatment

5.0 DISCUSSION

Significant block by treatment interactions were found in needle element contents (Appendix E). These interactions seem to result from block differences, rather than treatment differences. Block 1 seedlings generally exhibited higher element concentrations. The differences may be attributable to the time of sowing. Block 1 seedlings were sown in June, whereas Blocks 2 and 3 were sown in January and February, respectively. It is likely that differences in growing conditions occurred at the different times of year. However, treatment-related differences were observed in needle copper, zinc and cadmium contents, indicating that these elements were taken up by the seedlings in excess of normal amounts after treatment. Metal-treated seedlings contained higher amounts of the respective treatment metal (Figure 12). Thus, any changes in pigments or photosynthesis may be attributed, at least in part, to the heavy metal treatments.

The heavy metal treatments had no effect on needle pigment content with the exception of neoxanthin. Metal-treated seedlings displayed significantly lower neoxanthin content than the control seedlings (Appendix D). Neoxanthin, like the other carotenoids, is an accessory pigment and is thought to have both light absorption and photoprotective roles (Goodwin and Mercer 1983). Decreases in neoxanthin, or the other accessory pigments, could result in a diminished capacity for one or both of these activities. However, since there were no differences in total carotenoid content or violaxanthin:zeaxanthin ratio, and since neoxanthin comprises a small proportion of total

carotenoids, any reduction in light absorption and photoprotection would be very slight in response to the applied treatments.

High levels of copper, zinc and cadmium are known to reduce chlorophyll content in vascular plants (Balsberg Påhlsson 1989). In this study, however, no change in chlorophyll content was observed. Schlegel *et al.* (1987) found significant reductions in chlorophyll in heavy metal-treated seedlings of *Picea abies*. These seedlings were grown in nutrient solutions with heavy metal concentrations of 0 - 15 μM Cd, 0.1 - 100 μM Zn and 0 - 0.5 μM Hg for five weeks. Our study used much higher concentrations (20 mM) for a shorter period of time (2½ weeks), due to observed needle browning. The needle browning is likely due to the presence of phenolics and does not necessarily indicate pigment changes. It is possible that pigment differences may have been found if the seedlings had been treated with lower metal concentrations and allowed to develop over a longer period of time.

Chlorophyll content has been found to be positively correlated with red edge, meaning that higher chlorophyll levels produce a shift in red edge to longer wavelengths (Horler *et al.* 1982; Curran *et al.* 1990; Chappelle *et al.* 1991; Moss and Rock 1991). This correlation must be established for every forest or tree crop in order to use red edge as an estimate of chlorophyll content. Baseline data is necessary because different "healthy" tree species will produce different red edge values. Correlation analysis was not performed in this study since there was no significant difference in chlorophyll content or reflectance red edge. The fact that red edge values were not significantly different, again, supports the conclusion that the heavy metal treatments had no effect on

the pigment structure of the seedlings within the treatment time chosen in this study.

Although the metal treatments produced no apparent changes in pigment content or composition, other results indicate that some acute physiological or biochemical damage occurred. The ratio of F_v/F_m decreased with the 20 mM Cd treatment indicating a decrease in PSII efficiency (Krause and Weis 1991). This may be due to inhibition of electron transport or reduction in the number of electron acceptors available. Cu^{2+}, Zn^{2+} and Cd^{2+} are known to substitute for Fe^{2+}, Mg^{2+} or Mn^{2+} in some enzymes (Clijsters and Van Assche 1985; Balsberg Påhlsson 1989). Antagonism with ions normally associated with electron transfer reactions is one likely cause of a decline in photosynthetic efficiency. Similar results were found in wheat (*Triticum aestivum* L.) grown in soil containing high levels of copper. The decrease in F_v/F_m was thought to be caused by a decrease in the pool size of electron acceptors and interference with the photochemistry of PSII (Lanaras *et al.* 1993).

Net oxygen evolution also decreased in metal-treated seedlings (Figure 10). Heavy metals have been shown to inhibit gas exchange and photosynthesis by affecting stomatal function, chloroplast structure and function, and enzyme activity (Clijsters and Van Assche 1985). Interference with stomatal function should also result in changes in a plant's water balance (Barceló and Poschenrieder 1990). Since there was very little change in net water content of the needles over all treatments (Figure 11), it would appear that the water balance was not affected by the heavy metals. However, net water content is a gross measurement and without specific stomatal conductivity measurements, conclusions about their status cannot be drawn.

It is possible that chloroplast metabolism may have been affected. Excess divalent cations, such as Cu^{2+}, Zn^{2+} or Cd^{2+}, could affect Mn^{2+} content of the oxygen evolving complex (Goodwin and Mercer 1983).

The inhibitory effects of heavy metals on enzyme activity are well documented. Sulphydryl groups and Fe^{2+}, Mg^{2+} and Mn^{2+}, which are present in many enzymes, can be substituted by Cu^{2+}, Zn^{2+} and Cd^{2+} (Balsberg Påhlsson 1989). Many of the Calvin cycle enzymes have been shown to be inhibited by heavy metals (Goodwin and Mercer 1983; Clijsters and Van Assche 1985; Fitter and Hay 1987; Van Assche *et al.* 1988; Balsberg Påhlsson 1989; Krupa *et al.* 1993). In addition, hydrolytic enzymes, such as ribonuclease, deoxyribonuclease, peroxidase and acid phosphatase, exhibit increased activity in the presence of cadmium (Balsberg Påhlsson 1989). Some hydrolytic enzymes break glycosidic bonds which hold cell wall polysaccharides together (Goodwin and Mercer 1983). As well, increases in these enzymes have been shown to result in premature senescence (Van Assche *et al.* 1988).

Copper, zinc and cadmium are known to increase the permeability of root cell membranes, resulting in nutrient leakage, especially K^+ (Larcher 1980). Damage to roots can also disrupt water relations in a plant (Barceló and Poschenrieder 1990). However, neither net water content nor needle element content (other than copper, zinc and cadmium) resulted in changes. This suggests that seedling water balance was not affected and nutrient leakage was not occurring.

Although no changes were observed in pigment contents or reflectance, changes in F_v/F_m and oxygen evolution were evident. This indicates that the heavy metals did not

affect the pigments of the spruce seedlings or, consequently, their light harvesting abilities, but had an effect on some other component of the photosynthetic pathway. Decreases were found in oxygen evolution for all metal treatments, without a corresponding decrease in F_v/F_m, except for the 20 mM Cd treatment. Since the F_v/F_m ratio reflects the efficiency of electron passage through PSII, and electrons are generally believed to be supplied by the water splitting-oxygen generating complex, a close relationship should exist between the two processes (Björkman and Demmig 1987; Krause and Weis 1991; Lanaras *et al.* 1993). In our experiments, it is possible that the heavy metal treatments may have damaged the function of the water splitting complex. If this were the case, oxygen evolution would decrease but, as long as electrons were being supplied from some other reducing source, such as chlororespiration (Garab *et al.* 1989; Peltier and Schmidt 1991), F_v/F_m may not change.

Evidence suggests that plants may possess mechanisms to modulate metal concentrations between deficient and toxic levels (Rauser 1990). Similar to metallothioneins found in animals, phytochelatins de-toxify excess metals by chelation (Salisbury and Ross 1992). Phytochelatins are small peptides composed of glutathione, 2 to 11 cysteine amino acids and glycine or alanine (Rauser 1990). The amino acid cysteine is rich in sulphur, which is thought to be essential for the binding with metals (Salisbury and Ross 1992). Plants tolerant of metal concentrations in the range of 40 μm to 6 mM have been observed (Gupta and Goldsbrough 1991; De Vos *et al.* 1992). In this study, it seems likely that the seedlings' detoxification mechanisms were overloaded by the extremely high levels of copper, zinc and cadmium, resulting in damage to

metabolism.

The use of HPLC and remote spectroradiometry was effective for the measurement of pigment content and reflectance red edge, respectively. Both methods provided results that were consistent with each other. Both the HPLC and spectroradiometer were easy to use and produced results quickly. Additional long-term exposure experiments using lower metal levels are necessary to confirm the effectiveness of both of these methods. It is likely that lower concentrations of heavy metals over a longer period of time would produce chronic damage to seedlings or long term developmental changes in pigment levels. Damage to trees grown for long periods of time in contaminated soils is well documented (Zedler *et al.* 1986; Wolfenden *et al.* 1988; Köstner *et al.* 1990; Wild *et al.* 1993). The use of these methods may prove invaluable in assessing the extent of damage in such areas. However, it seems apparent from this work that an integrated approach to these methods is necessary for a more complete understanding of the effects of stress on plants.

Although treatment-related effects were not detected using HPLC or red edge, the development of these methods in this study represents an important step in understanding the pigment composition of black spruce seedlings. Information regarding pigment content is essential to the understanding of photosynthesis and energy transfer within plants. Since data on the pigment content of black spruce is limited, the results obtained in this study provide a starting point for future understanding.

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APPENDICES

APPENDIX A

Figure A-1: Absorption spectrum of neoxanthin.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	2.02 - 2.58

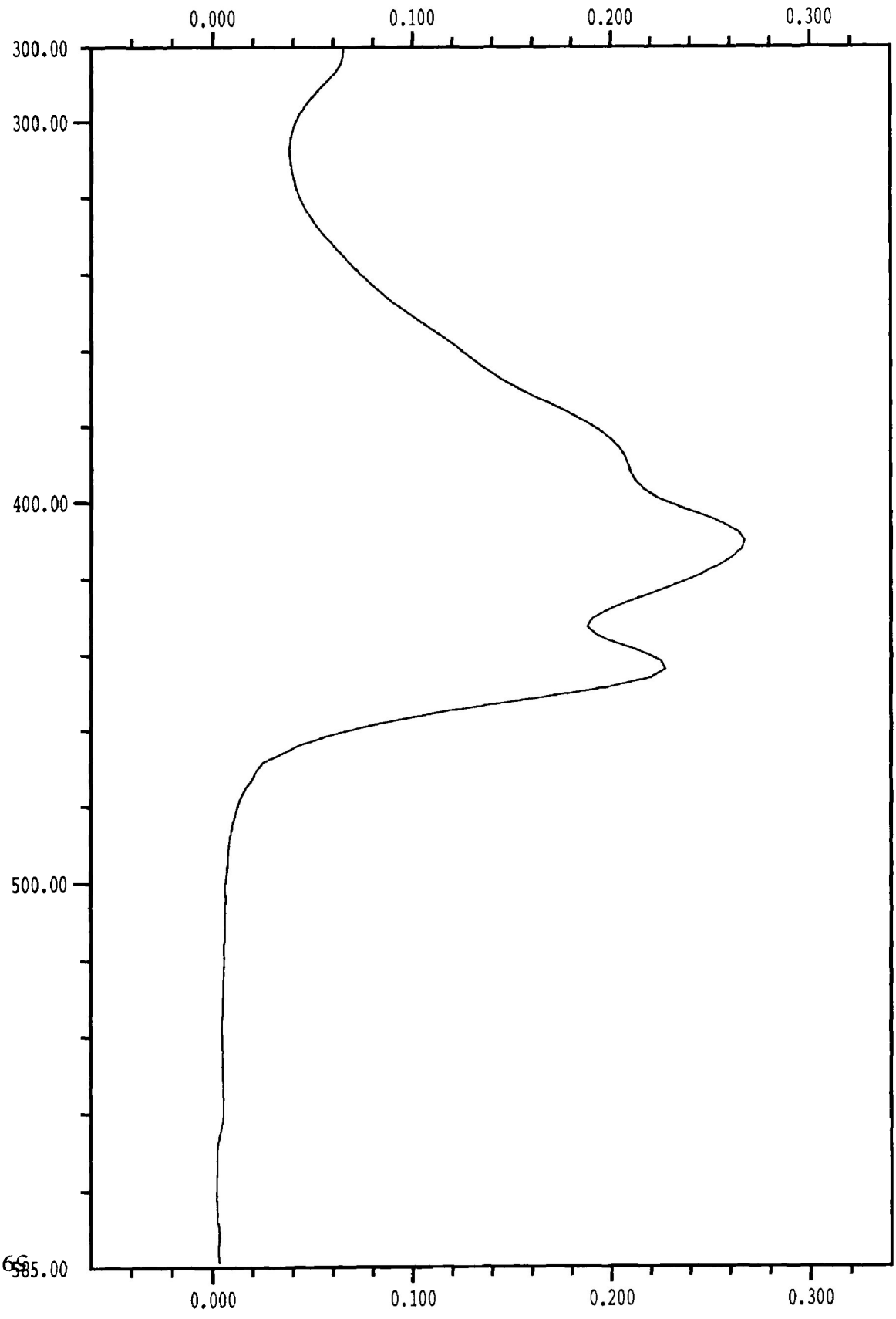

Figure A-2: Absorption spectrum of violaxanthin.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	2.80 - 2.84

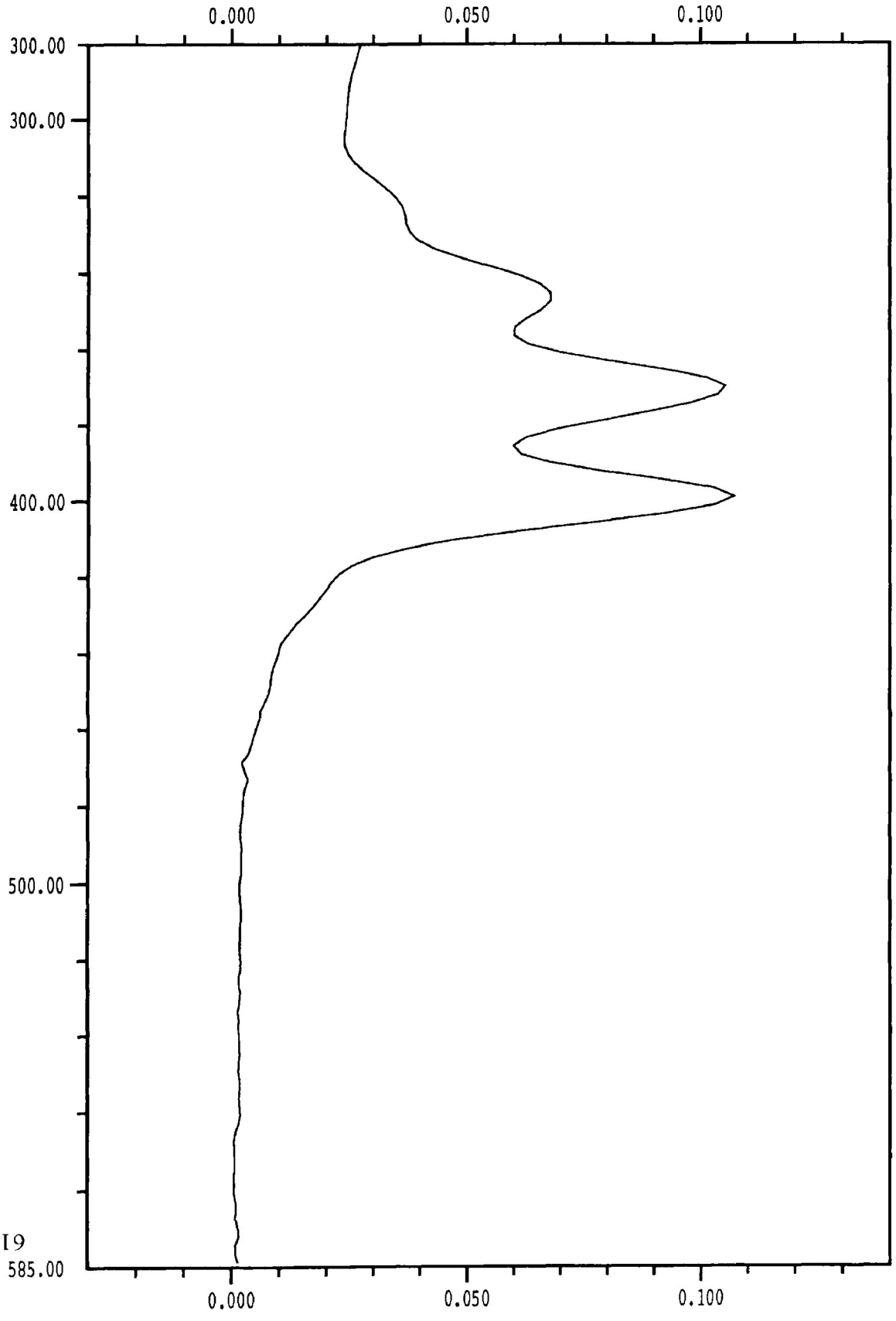

Figure A-3: Absorption spectrum of antheraxanthin.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	2.96 - 3.00

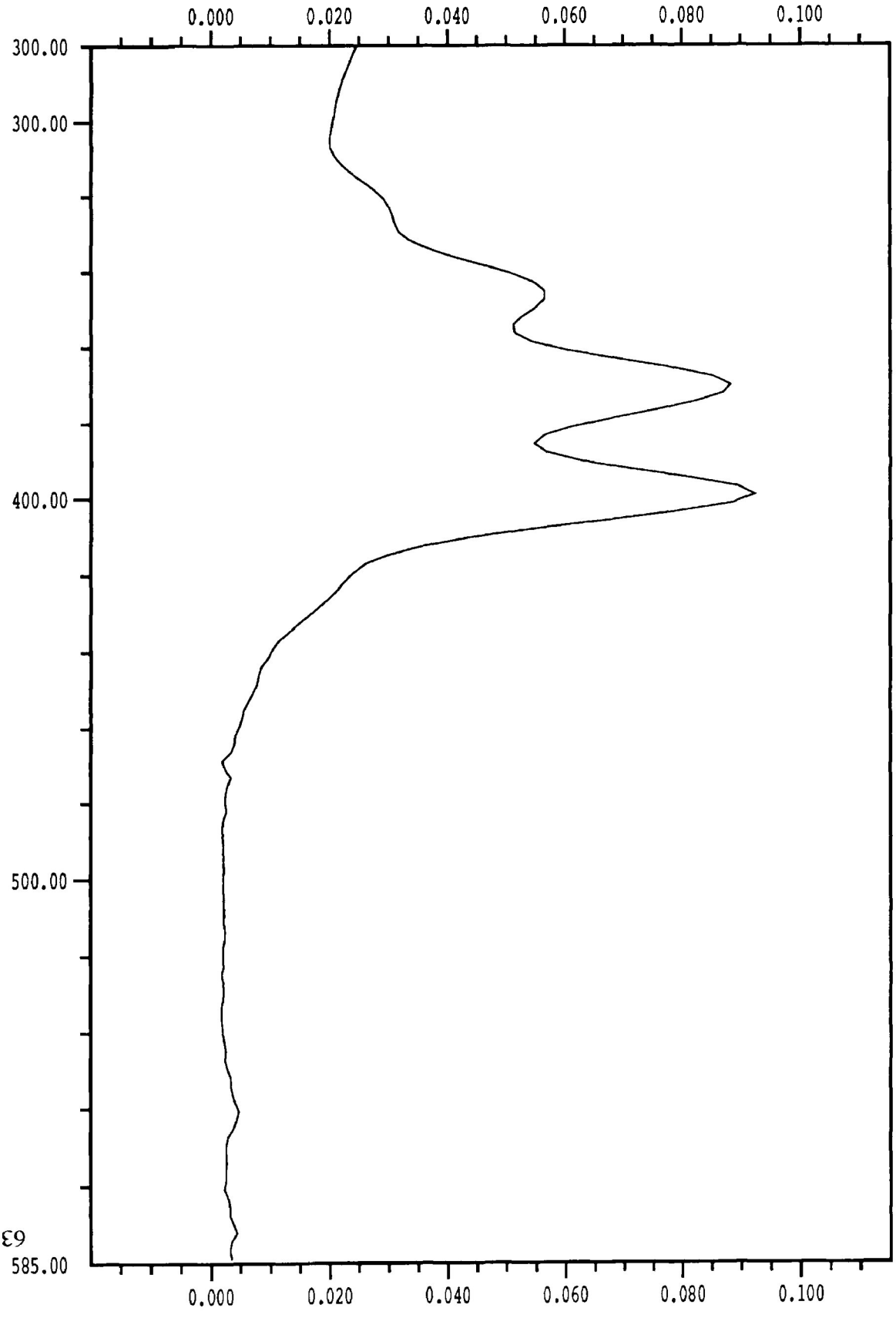

Figure A-4: Absorption spectrum of lutein.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	3.14 - 3.69

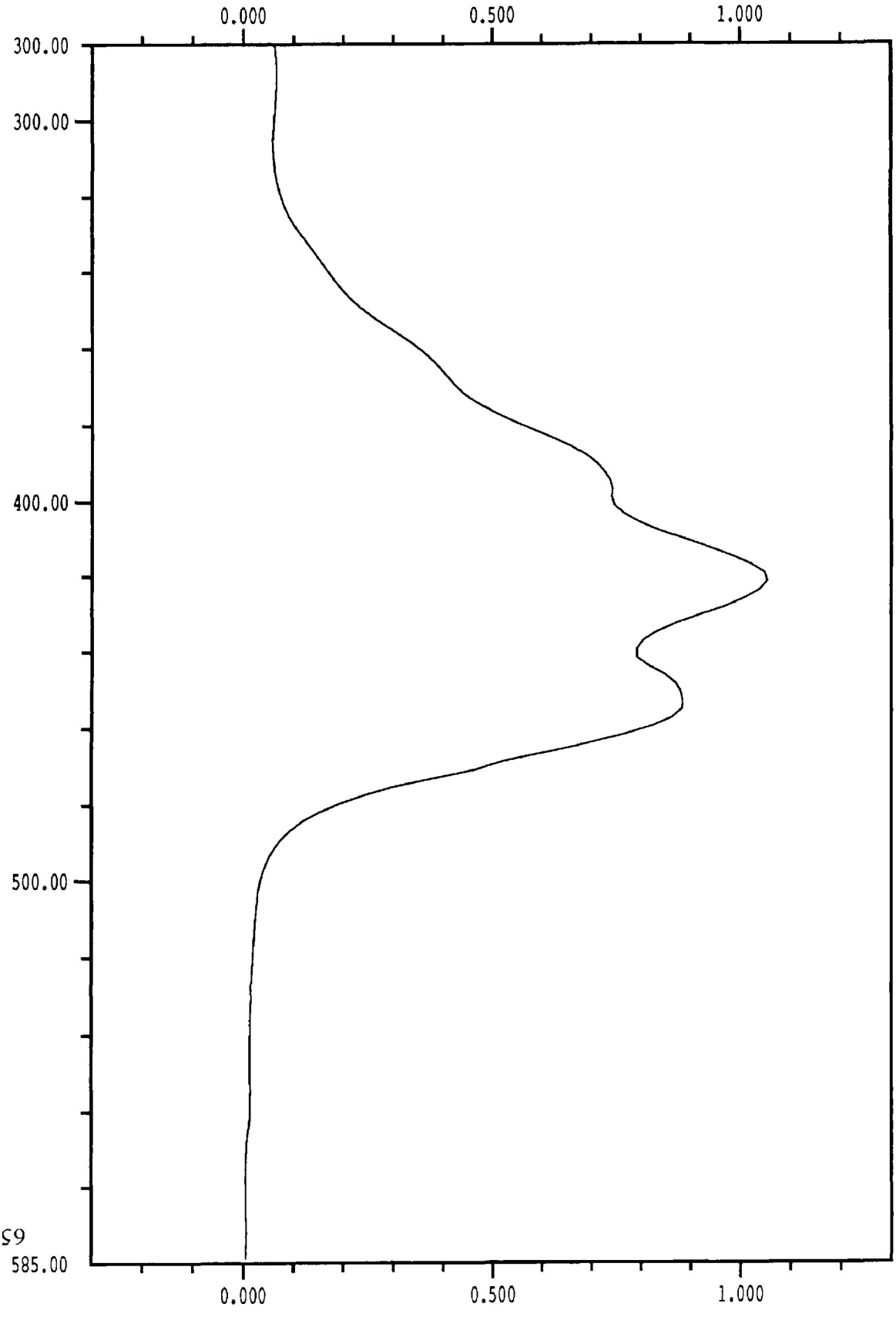

Figure A-5: Absorption spectrum of zeaxanthin.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	4.88 - 5.30

Wavelength

Absorbance

Figure A-6: Absorption spectrum of chlorophyll *b*.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	6.06 - 7.32

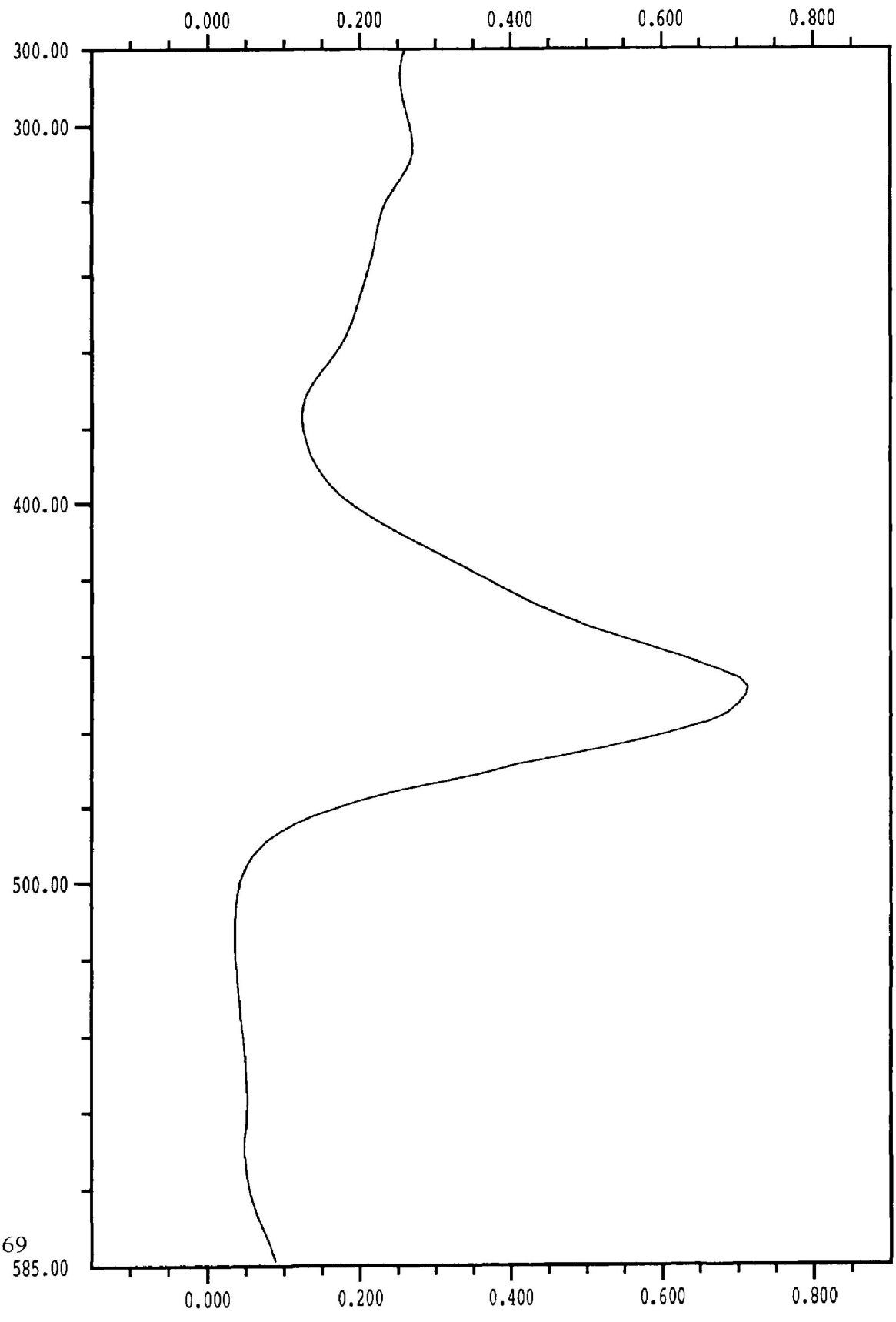

Figure A-7: Absorption spectrum of chlorophyll *a*.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
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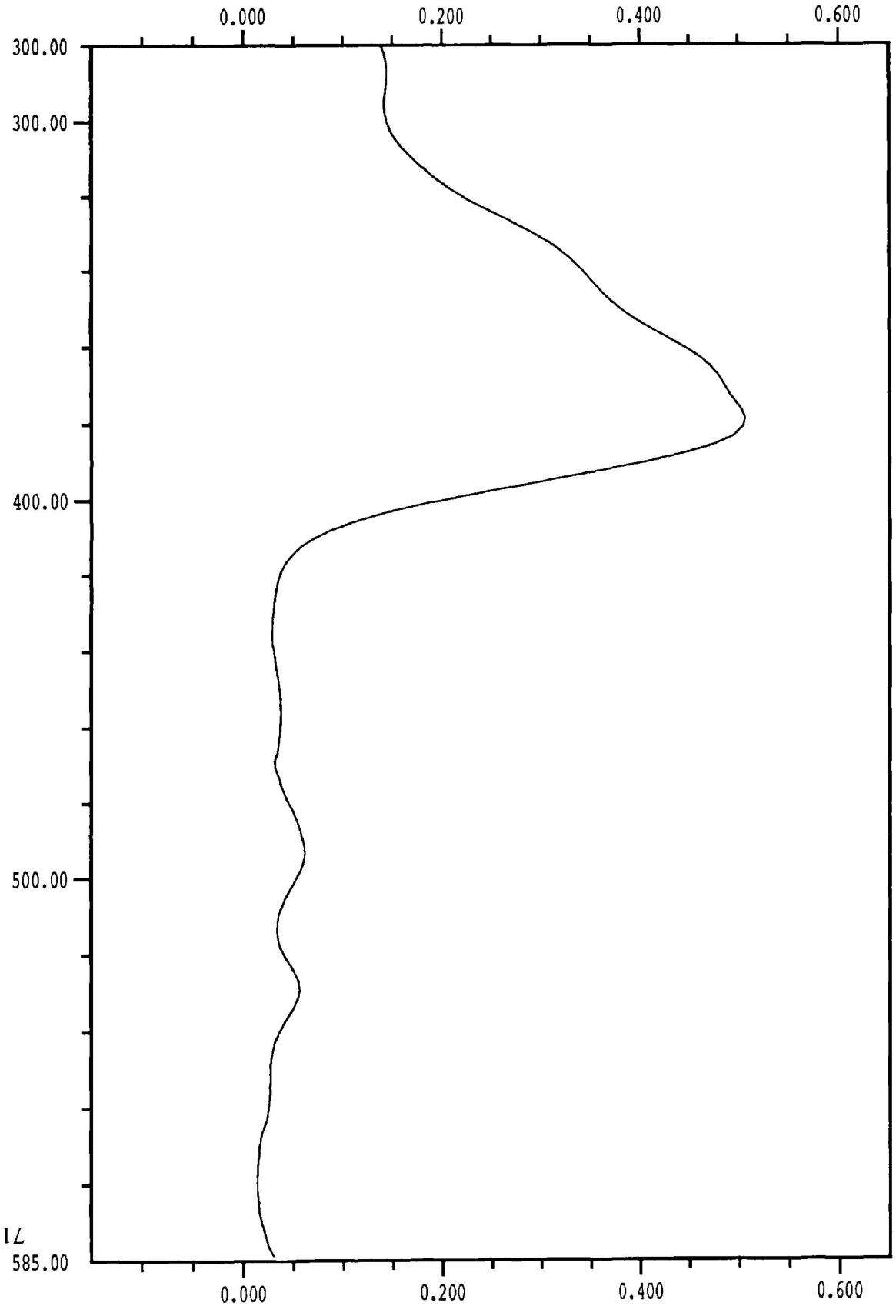
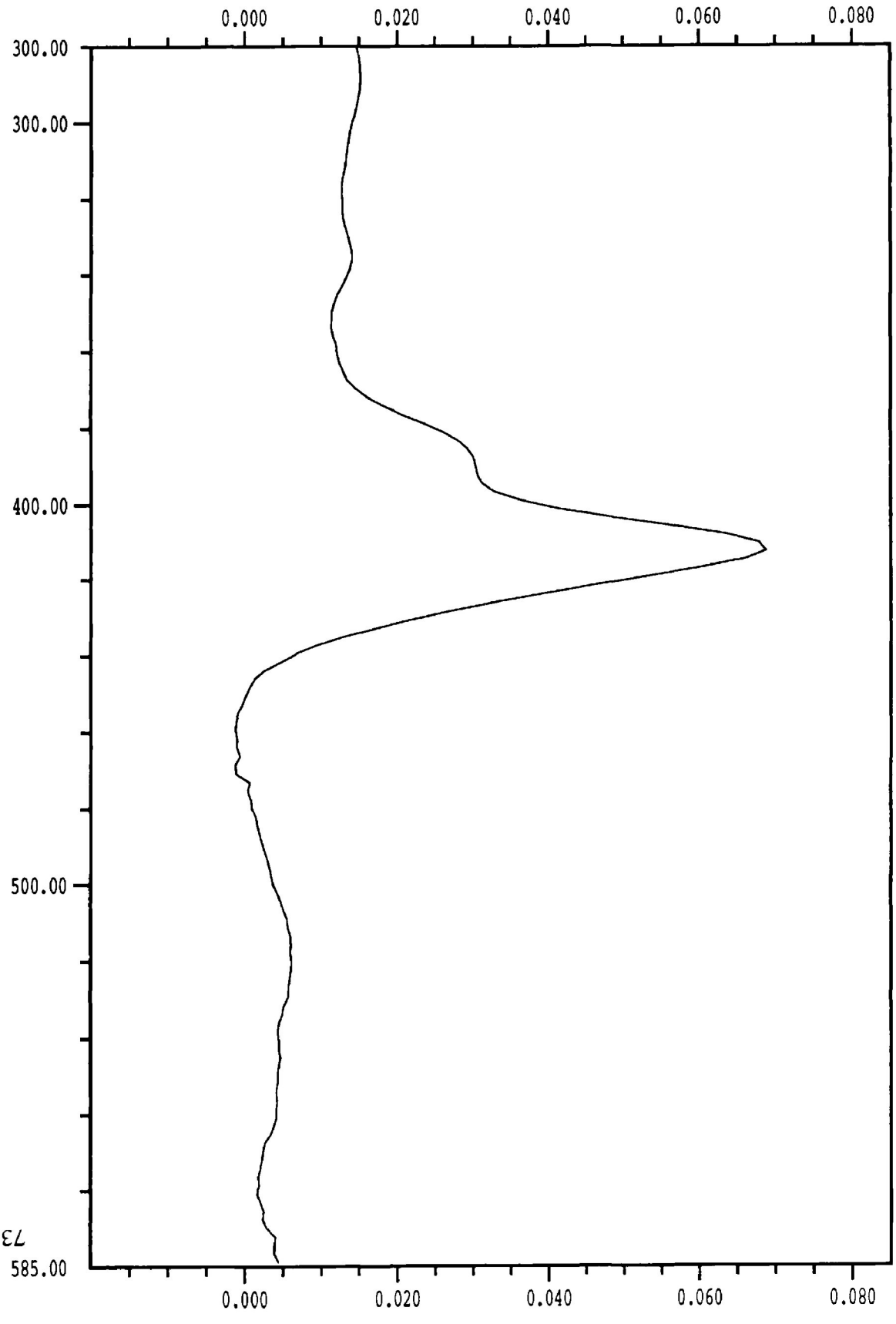


Figure A-8: Absorption spectrum of β-carotene.

SCAN

Nr.	NAME	CHAN	LEV	REP	TYPE	DIRECTORY	LINE TYPE	TIME RANGE
1	B2	A	1	1	Orig	C:\GOLD\SYS1DATB\	———	27.18 - 27.74



APPENDIX B

Table B-1: The areas under the peaks of chromatogram A (Figure 2). Data for the four replicates are shown.

pigment	replicate 1	replicate 2	replicate 3	replicate 4
neoxanthin	12.56	5.25	3.33	3.92
violaxanthin	7.01	4.67	6.05	13.06
antheraxanthin	26.37	27.87	24.71	47.73
lutein	57.12	50.31	68.16	71.93
zeaxanthin	35.36	23.41	6.50	14.17
chlorophyll *b*	468.15	505.73	555.81	1045.54
chlorophyll *b'*	41.17	37.23	53.73	51.02
chlorophyll *a*	3.5	46.11	260.99	144.99
chlorophyll *a'*	0	12.73	0	11.57
α-carotene	0	0	0	134.27
β-carotene	0	0	0	14.58

pigment mass = (area X response factor)*[(extraction volume X dilution factor)/injection volume]*(dry weight)
= (12.56*1.034)*[(7000*1)/500]*(0.297)
=54.00 μg/g dry weight
=0.054 mg/g dry weight

Figure B-1: Formula used to calculate pigment concentrations from peak areas. Data for neoxanthin from replicate 1 of chromatogram A (Figure 2) are used as an example.

APPENDIX C

Table C-1: Model equation and expected mean squares (EMS) table for pigment content.

$$Y_{ijkl} = \mu + B_i + \delta_{(i)j} + T_k + BT_{ik} + \varepsilon_{(ijk)l} + \rho_{(ijkl)m}$$

where:

Y_{ijkl} = the total response of the lth replicate of the kth treatment in the ith block
μ = the overall mean
B_i = the fixed effect of the ith block
$\delta_{(i)j}$ = the random effect of the randomization of the treatments within the ith block
T_k = the fixed effect of the kth treatment
BT_{ik} = the interaction effect of the ith block with the kth treatment
$\varepsilon_{(ijk)l}$ = the random effect of the experimental unit that received the kth treatment in the ith block. The $\varepsilon_{(ijk)l}$'s are assumed to be IID $N(0, \sigma^2)$.
$\rho_{(ijkl)m}$ = the random effect of the mth sample within the lth replicate of the kth treatment within the ith block. The $\rho_{(ijkl)m}$'s are assumed to be IID $N(0, \sigma^2)$.

Source	d.f.	EMS
B_i	1	$\sigma_\rho^2 + 2\sigma_\varepsilon^2 + 28\sigma_\delta^2 + 28\theta(B)$
$\delta_{(i)j}$	0	$\sigma_\rho^2 + 2\sigma_\varepsilon^2 + 28\sigma_\delta^2$
T_k	6	$\sigma_\rho^2 + 2\sigma_\varepsilon^2 + 6\theta(T)$
BT_{ik}	6	$\sigma_\rho^2 + 2\sigma_\varepsilon^2 + 4\theta(BT)$
$\varepsilon_{(ijk)l}$	14	$\sigma_\rho^2 + 2\sigma_\varepsilon^2$
$\rho_{(ijkl)m}$	28	σ_ρ^2

Table C-2: Model equation and expected mean squares (EMS) table for F_v/F_m.

$$Y_{ijkl} = \mu + B_i + \delta_{(i)j} + T_k + BT_{ik} + \varepsilon_{(ijk)l} + \rho_{(ijkl)m}$$

where:

Y_{ijkl} = the total response of the lth replicate of the kth treatment in the ith block
μ = the overall mean
B_i = the fixed effect of the ith block
$\delta_{(i)j}$ = the random effect of the randomization of the treatments within the ith block
T_k = the fixed effect of the kth treatment
BT_{ik} = the interaction effect of the ith block with the kth treatment
$\varepsilon_{(ijk)l}$ = the random effect of the experimental unit thet received the kth treatment in the ith block. The $\varepsilon_{(ijk)l}$'s are assumed to be IID $(0, \sigma^2)$.
$\rho_{(ijkl)m}$ = the random effect of the mth sample within the lth replicate of the kth treatment within the ith block. The $\rho_{(ijkl)m}$'s are assumed to be IID $N(0, \sigma^2)$.

Source	d.f.	EMS
B_i	2	$\sigma_\rho^2 + 5\sigma_\varepsilon^2 + 70\sigma_\delta^2 + 70\theta(B)$
$\delta_{(i)j}$	0	$\sigma_\rho^2 + 5\sigma_\varepsilon^2 + 70\sigma_\delta^2$
T_k	6	$\sigma_\rho^2 + 5\sigma_\varepsilon^2 + 30\theta(T)$
BT_{ik}	12	$\sigma_\rho^2 + 5\sigma_\varepsilon^2 + 10\theta(BT)$
$\varepsilon_{(ijk)l}$	21	$\sigma_\rho^2 + 5\sigma_\varepsilon^2$
$\rho_{(ijkl)m}$	168	σ_ρ^2

Table C-3: Model equation and expected mean squares (EMS) table for red edge, net oxygen evolution, net water content and element content analyses.

$$Y_{ijkl} = \mu + B_i + \delta_{(i)j} + T_k + BT_{ik} + \varepsilon_{(ijk)l}$$

where:

Y_{ijkl} = the total response of the lth replicate of the kth treatment in the ith block
μ = the overall mean
B_i = the fixed effect of the ith block
$\delta_{(i)j}$ = the random effect of the randomization of the treatments within the ith block
T_k = the fixed effect of the kth treatment
BT_{ik} = the interaction effect of the ith block with the kth treatment
$\varepsilon_{(ijk)l}$ = the random effect of the experimental unit thet received the kth treatment in the ith block. The $\varepsilon_{(ijk)l}$'s are assumed to be IID $(0, \sigma^2)$.

Source	d.f.	EMS
B_i	2	$\sigma_\varepsilon^2 + 14\sigma_\delta^2 + 14\theta(B)$
$\delta_{(i)j}$	0	$\sigma_\varepsilon^2 + 14\sigma_\delta^2$
T_k	6	$\sigma_\varepsilon^2 + 6\theta(T)$
BT_{ik}	12	$\sigma_\varepsilon^2 + 2\theta(BT)$
$\varepsilon_{(ijk)l}$	21	σ_ε^2

APPENDIX D

Figure D-1: Means and standard errors of neoxanthin content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd). Bars with the same letter are not significantly different at α=0.05.

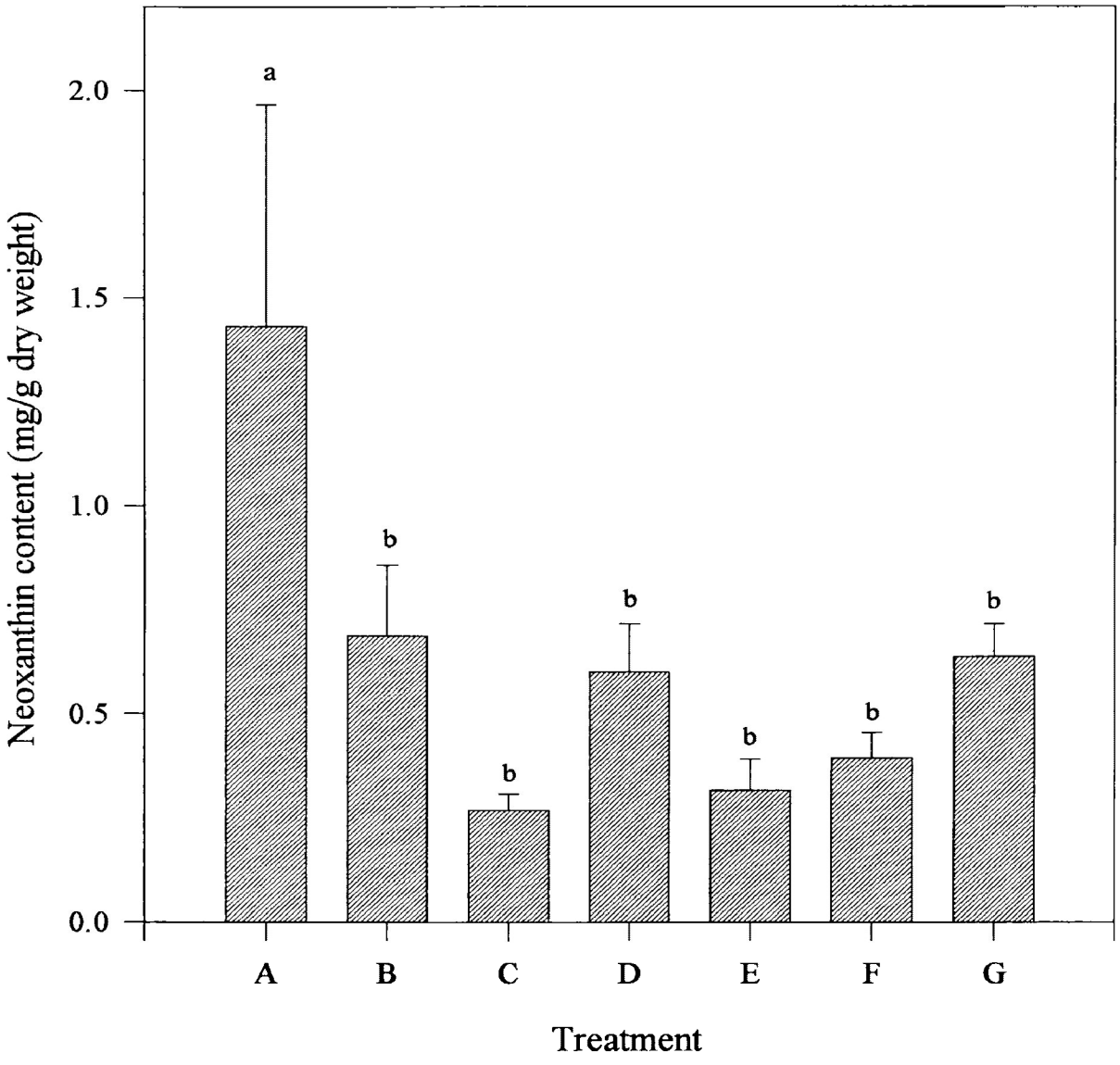

Neoxanthin content (mg/g dry weight)
2.0
1.5
1.0
0.5
0.0
a
b
b
b
b
b
b
A
B
C
D
E
F
G
Treatment

Figure D-2: Means and standard errors of violaxanthin content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

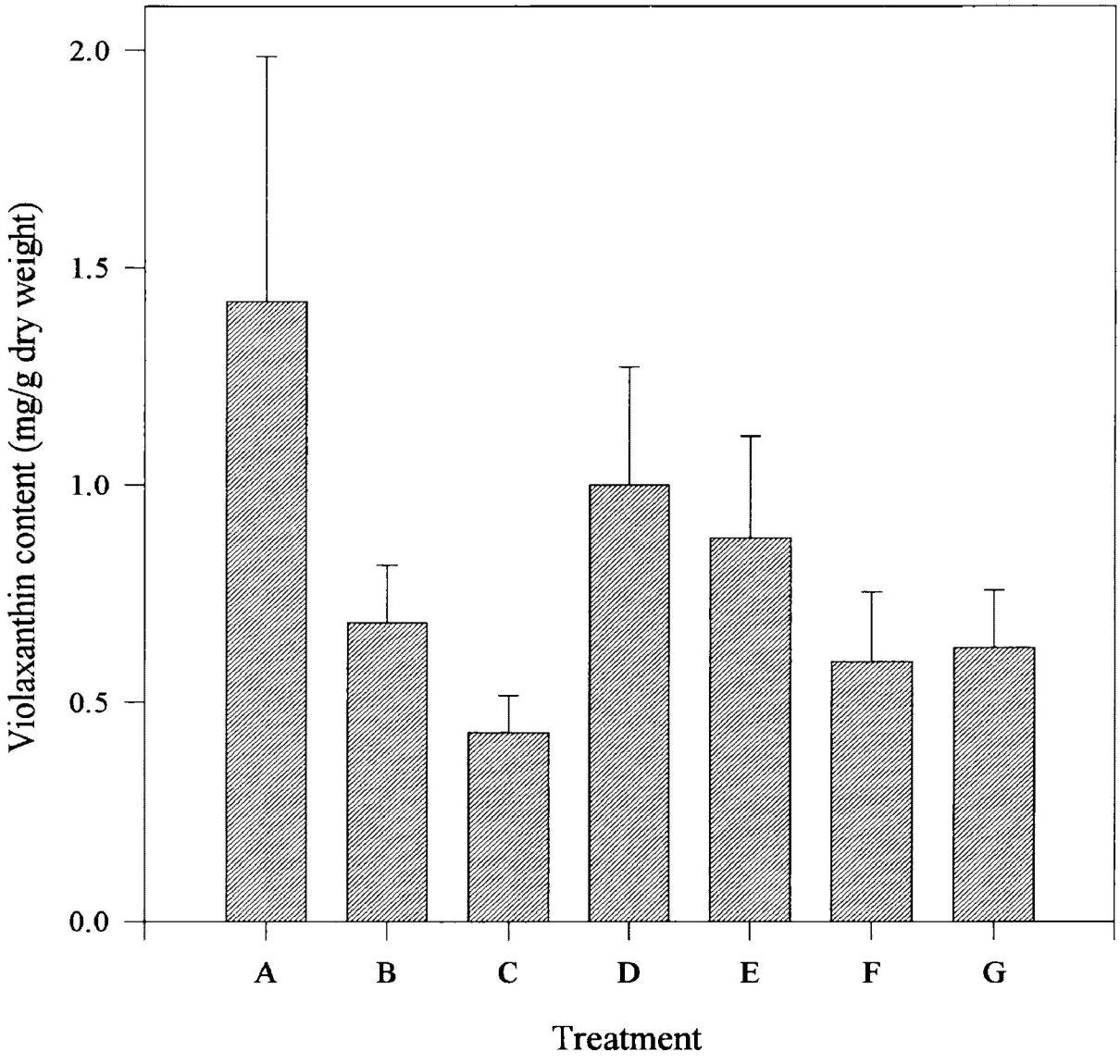

Violaxanthin content (mg/g dry weight)
2.0
1.5
1.0
0.5
0.0
A
B
C
D
E
F
G
Treatment

Figure D-3: Means and standard errors of antheraxanthin content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

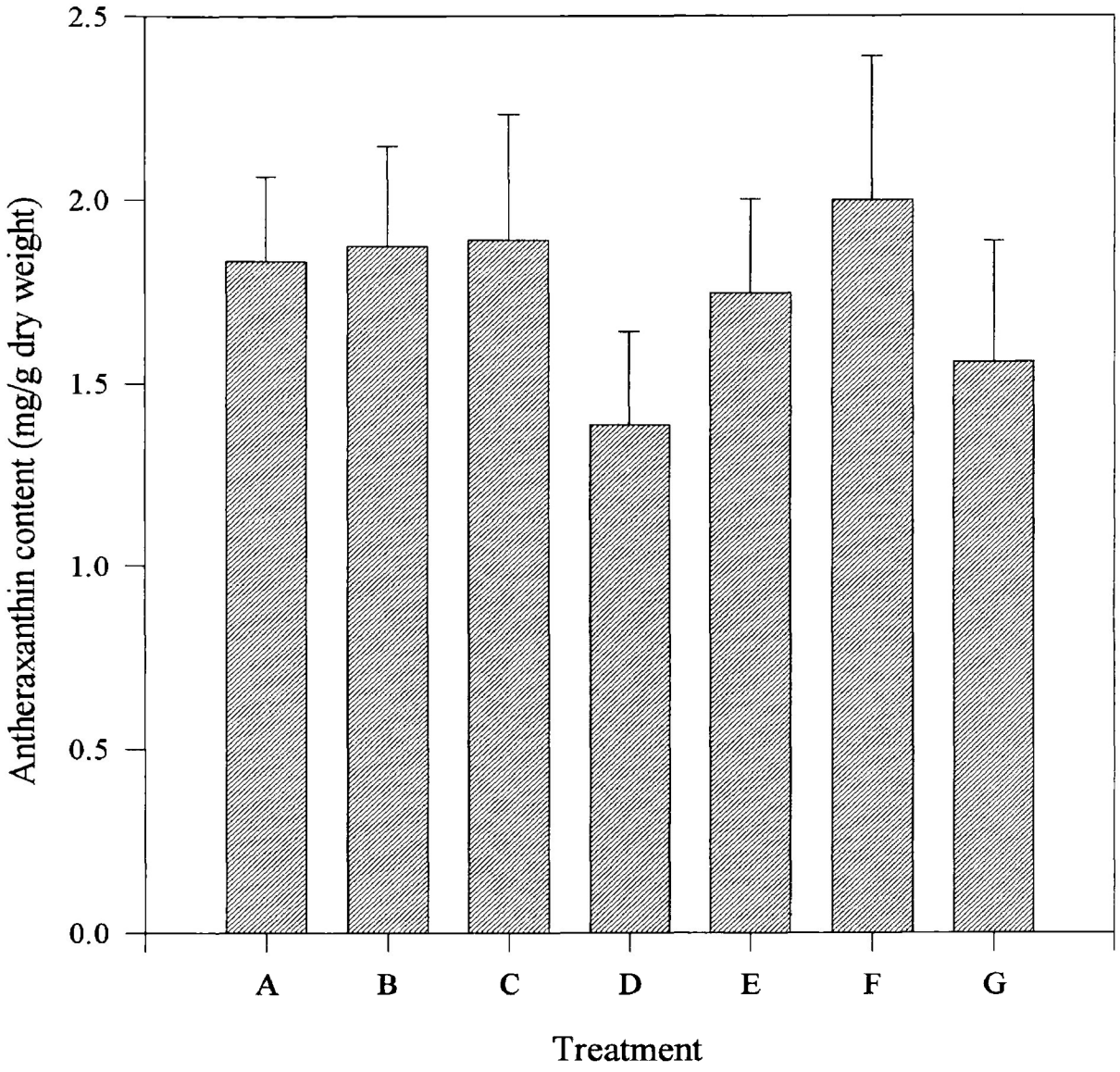

2.5
2.0
1.5
1.0
0.5
0.0
Antheraxanthin content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-4: Means and standard errors of lutein content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

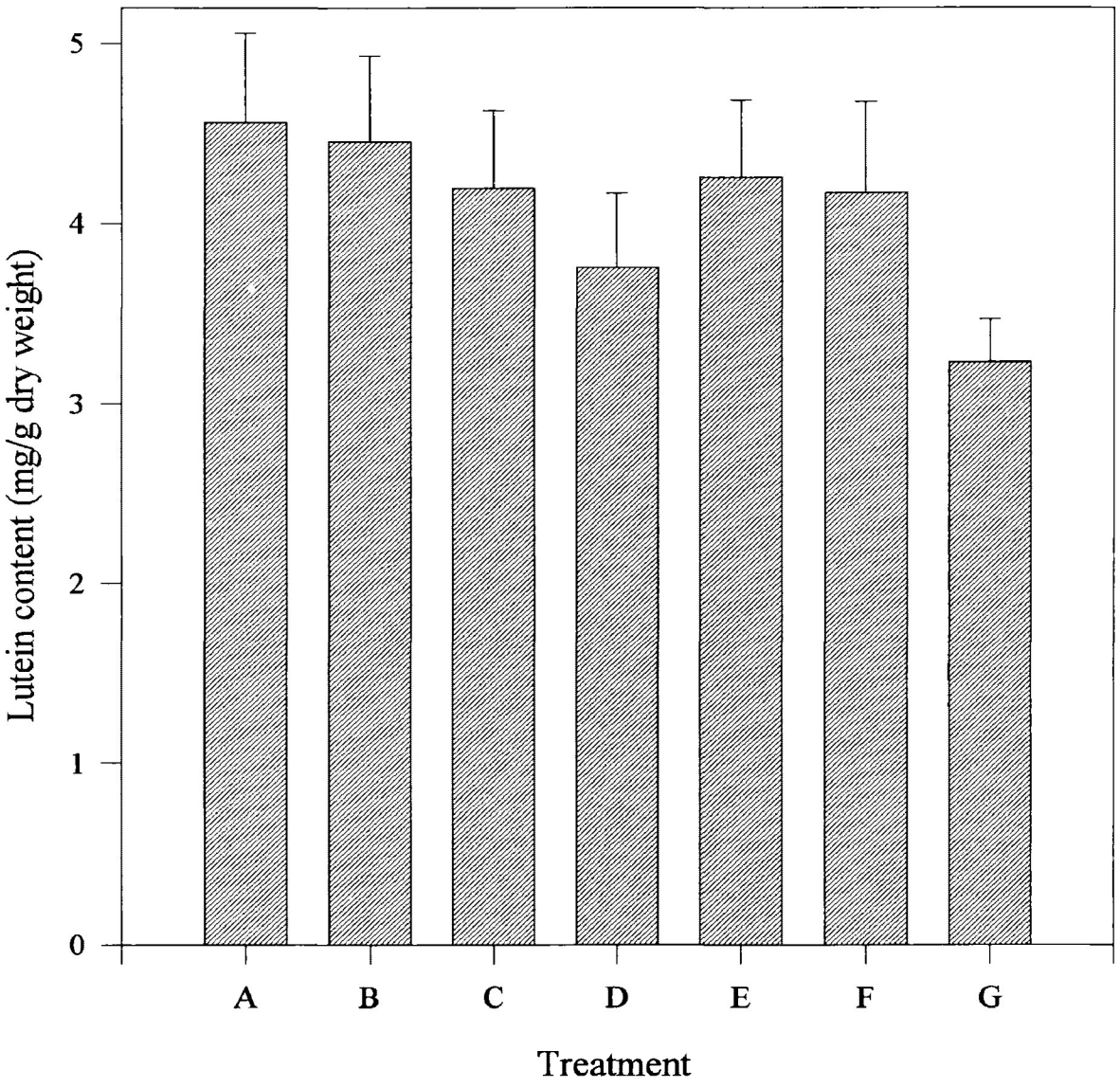

Lutein content (mg/g dry weight)
0
1
2
3
4
5
A
B
C
D
E
F
G
Treatment

Figure D-5: Means and standard errors of zeaxanthin content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

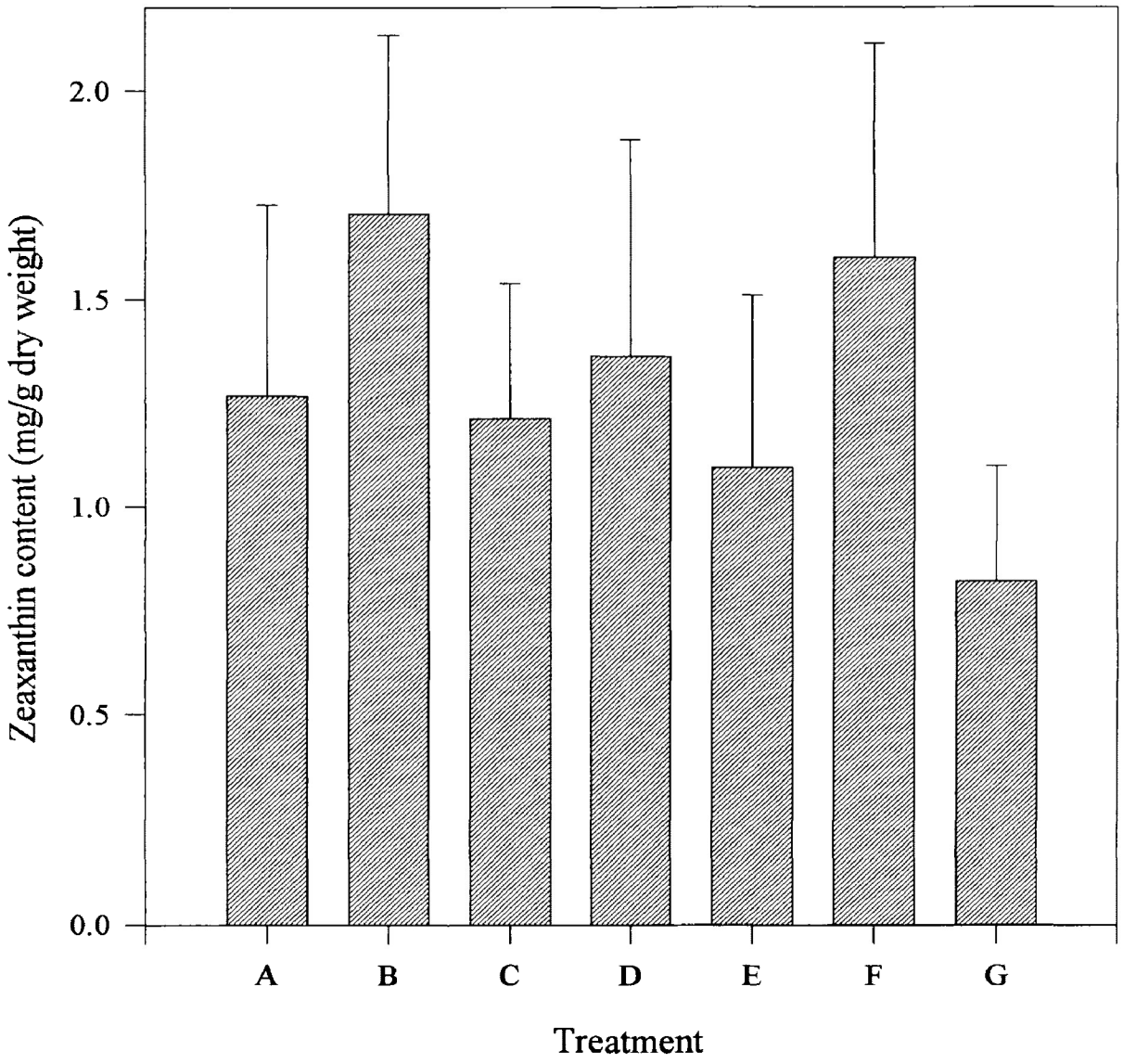

Zeaxanthin content (mg/g dry weight)
2.0
1.5
1.0
0.5
0.0
A
B
C
D
E
F
G
Treatment

Figure D-6: Means and standard errors of chlorophyll *b* content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

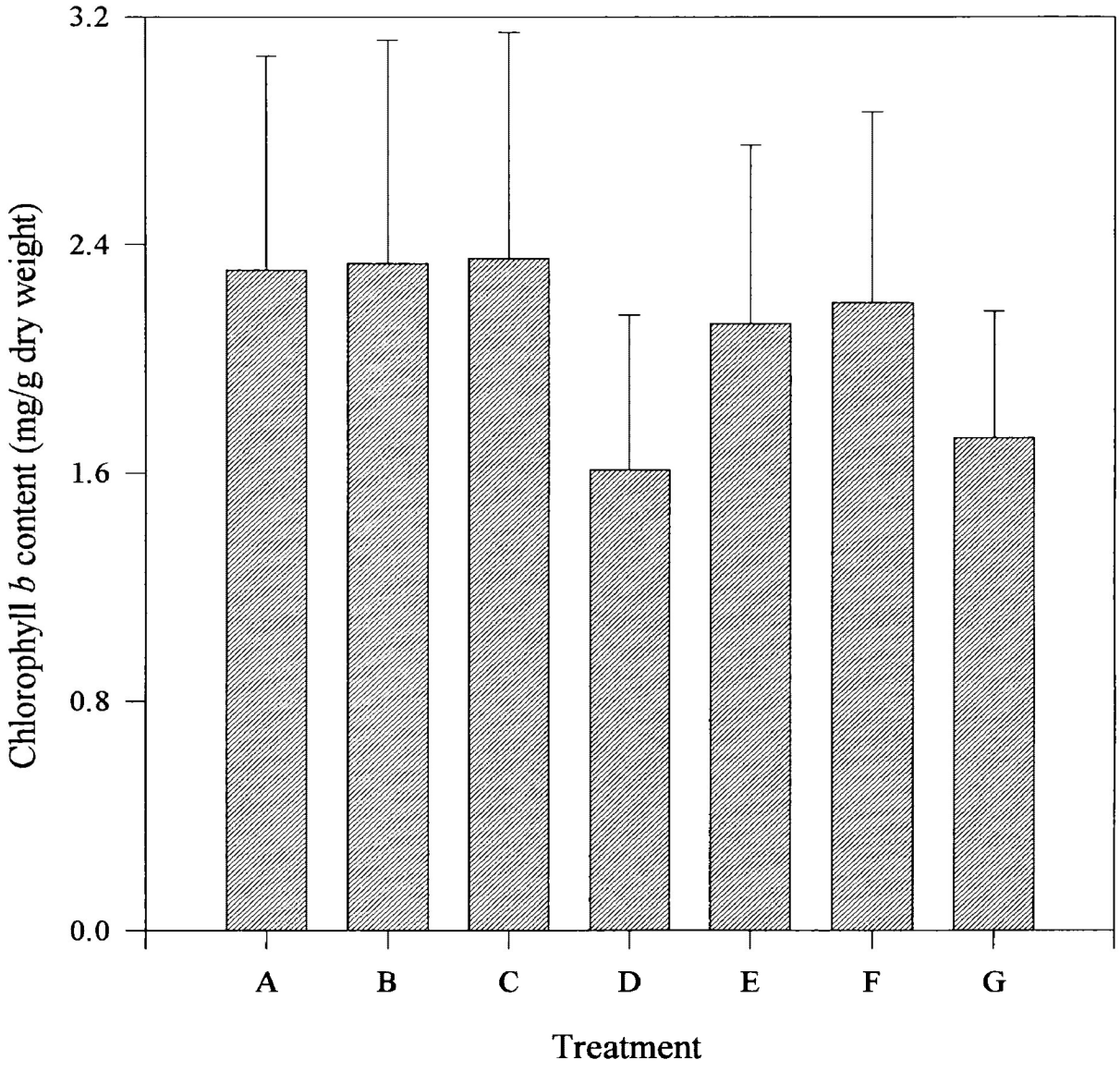

3.2
2.4
1.6
0.8
0.0
Chlorophyll b content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-7: Means and standard errors of chlorophyll *b'* content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

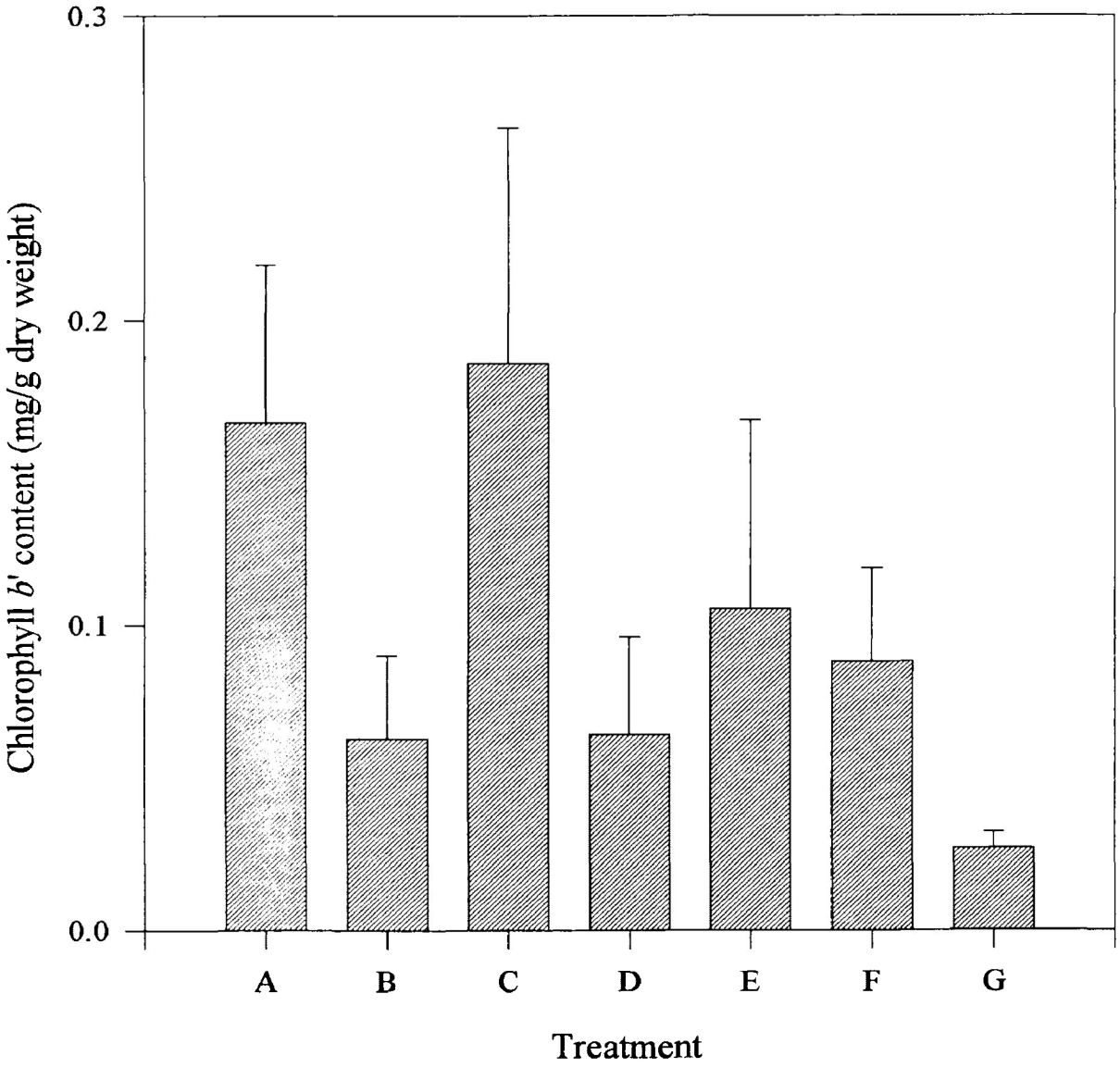

0.3
0.2
0.1
0.0
Chlorophyll b' content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-8: Means and standard errors of chlorophyll *a* content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

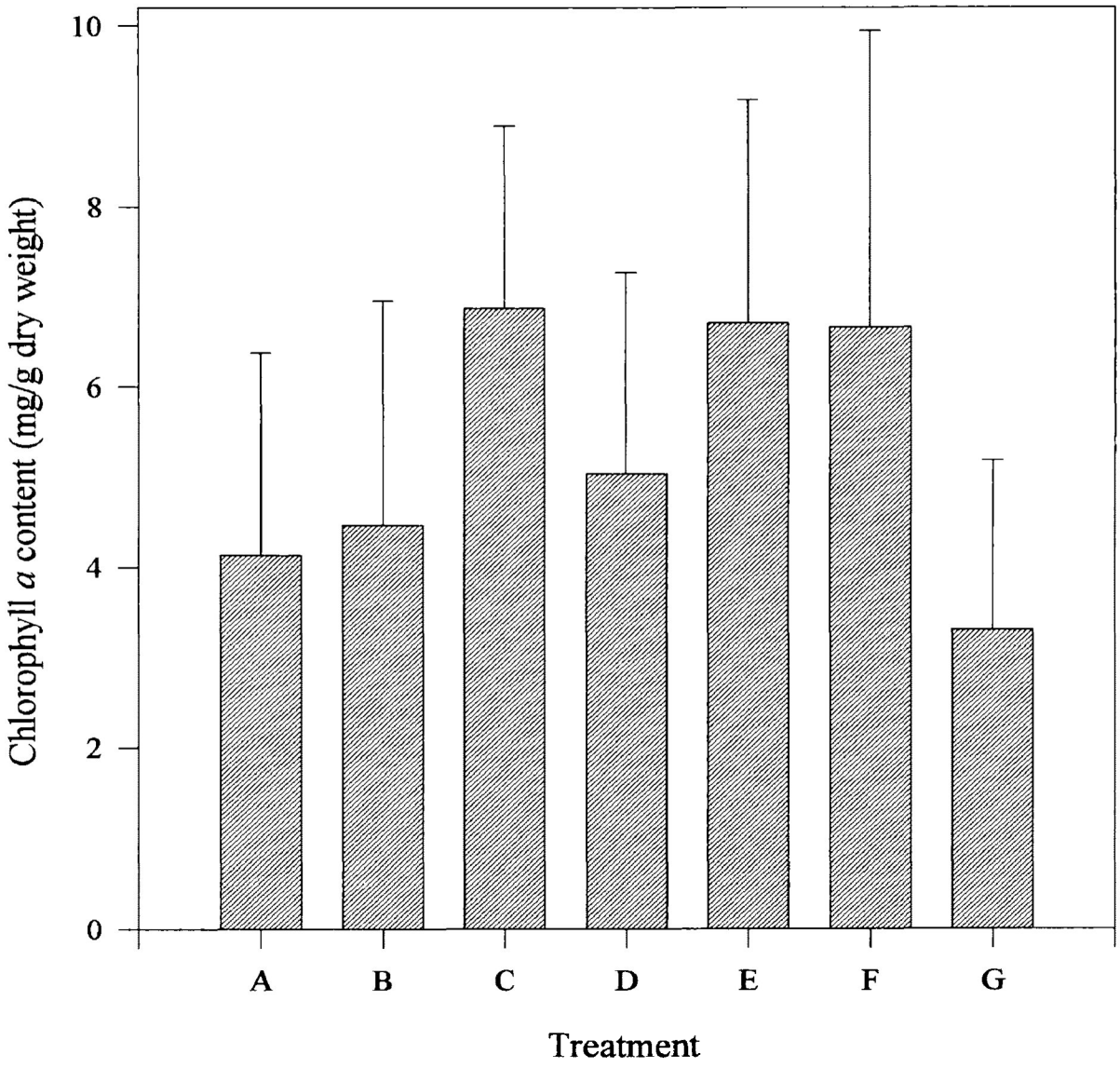

10
8
6
4
2
0
Chlorophyll a content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-9: Means and standard errors of chlorophyll *a* content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

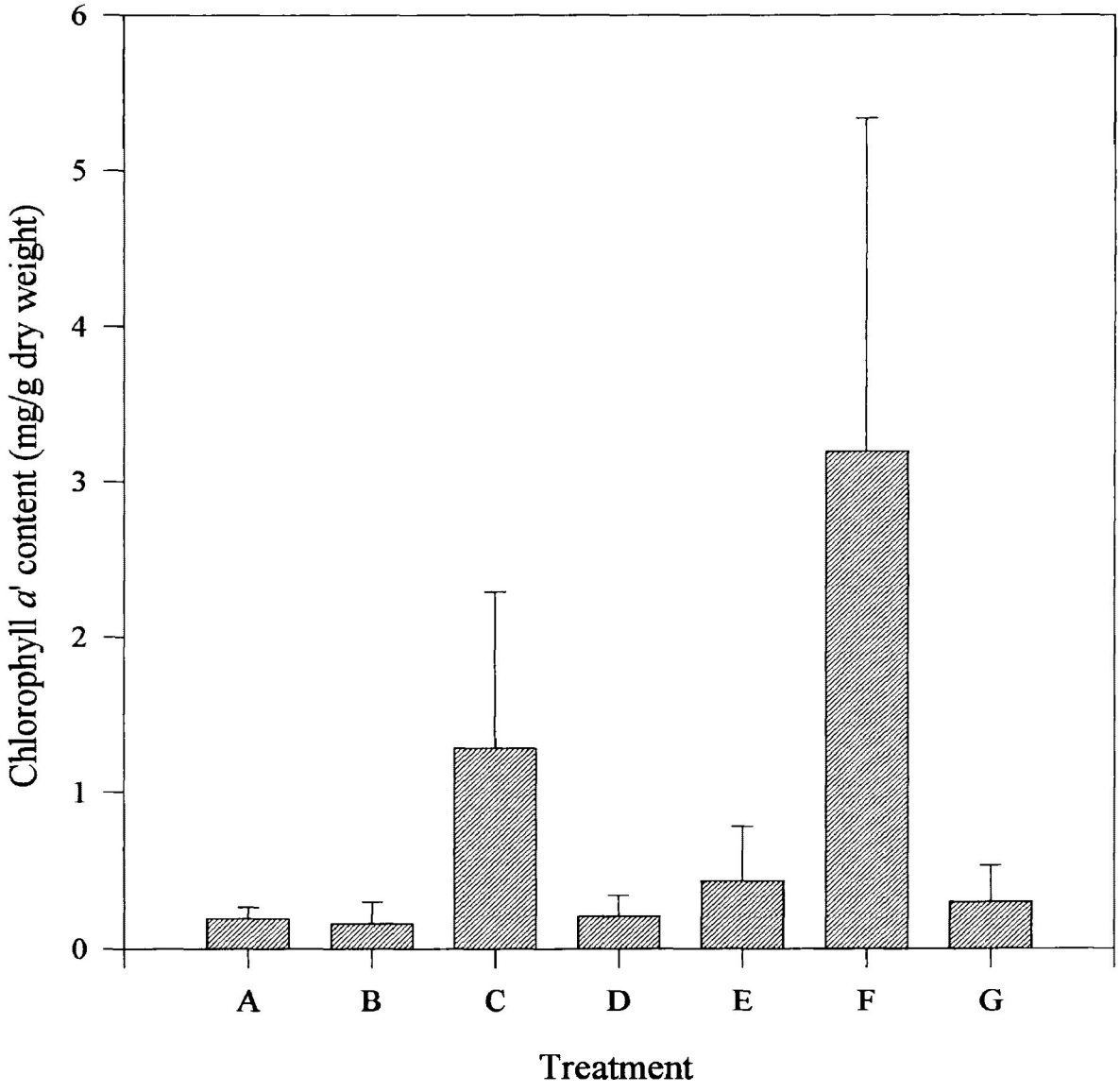

6
5
4
3
2
1
0
Chlorophyll a' content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-10: Means and standard errors of α-carotene content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).

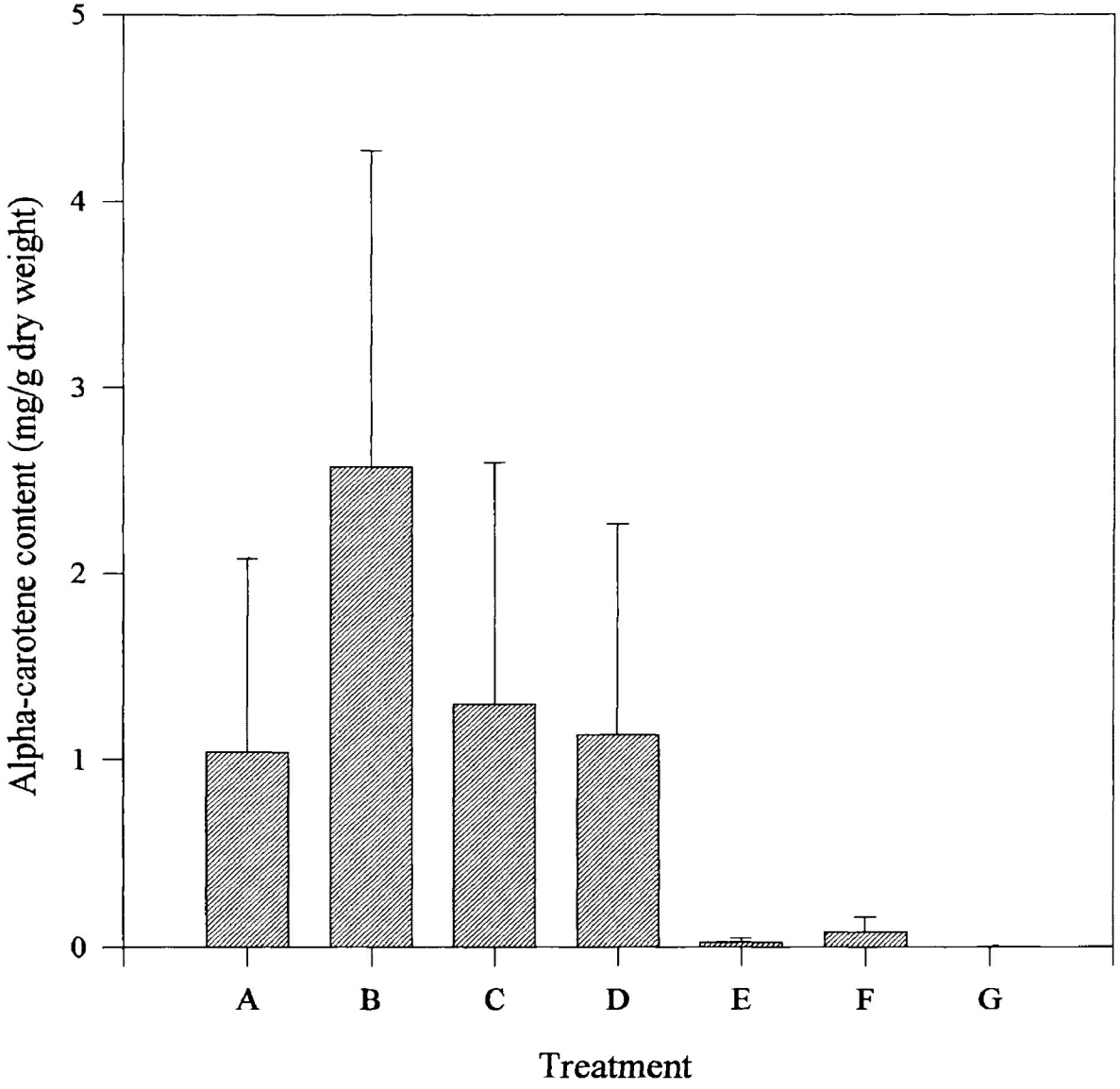
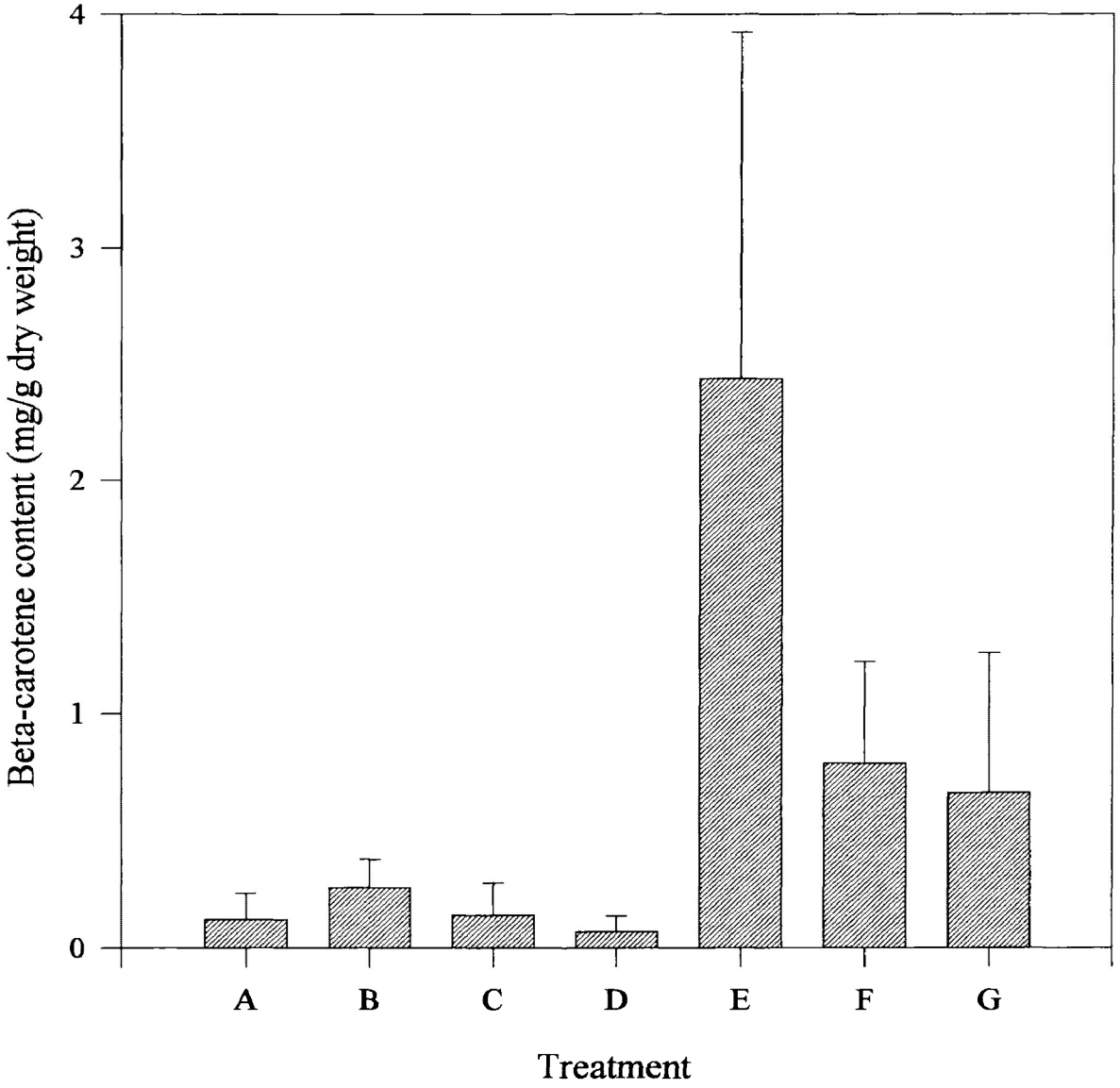

5
4
3
2
1
0
Alpha-carotene content (mg/g dry weight)
A
B
C
D
E
F
G
Treatment

Figure D-11: Means and standard errors of β-carotene content by treatment (A=control, B=10mM Cu, C=10mM Zn, D=20mM Cu, E=20mM Zn, F=20mM Cd).


Beta-carotene content (mg/g dry weight)
4
3
2
1
0
A
B
C
D
E
F
G
Treatment

APPENDIX E

Table E-1: Mean needle element content by treatment.

Treatment	Mean content (mg/g dry weight)										
	Ca	Cd	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
control	2.7803	0.0029	0.0233	0.0952	7.8117	1.2789	0.3037	0.3715	2.2993	1.1603	0.0440
	±0.229	±0.001	±0.006	±0.018	±1.019	±0.154	±0.068	±0.104	±0.435	±0.207	±0.006
10mMCu	2.5358	0.0016	0.1768	0.0976	7.0341	1.1082	0.2526	0.3573	1.9630	1.0685	0.0361
	±0.489	±0.001	±0.093	±0.029	±0.961	±0.171	±0.076	±0.119	±0.345	±0.193	±0.007
20mMCu	2.7991	0.0023	0.2972	0.0628	7.9478	1.3315	0.2774	0.2314	2.1663	1.0659	0.0427
	±0.502	±0	±0.155	±0.004	±0.785	±0.149	±0.063	±0.069	±0.417	±0.196	±0.007
10mMZn	2.8215	0.0205	0.0151	0.0753	7.2760	1.2619	0.2708	0.3148	2.0905	1.0062	0.2426
	±0.436	±0.019	±0.004	±0.016	±0.798	±0.145	±0.057	±0.085	±0.372	±0.186	±0.078
20mMZn	2.3272	0.0026	0.0129	0.0524	7.0377	1.1253	0.1912	0.1443	1.7145	0.8618	0.4187
	±0.475	±0.001	±0.003	±0.003	±1.016	±0.149	±0.043	±0.042	±0.349	±0.149	±0.184
10mMCd	2.9105	0.1261	0.0184	0.0620	8.5583	1.4076	0.2958	0.2534	2.5133	1.0770	0.0419
	±0.574	±0.042	±0.006	±0.008	±0.763	±0.188	±0.071	±0.061	±0.560	±0.234	±0.007
20mMCd	3.0940	0.2992	0.0107	0.0661	9.2914	1.6512	0.3299	0.2339	2.9042	1.0962	0.0601
	±0.878	±0.137	±0.002	±0.011	±1.757	±0.390	±0.111	±0.092	±0.906	±0.335	±0.015

Table E-2: Results of ANOVA of needle element content (p-values are given where significant differences were found).

Element	between treatments	block x treatment interaction
calcium (Ca)	n.s.[1]	p=0.014
cadmium (Cd)	p<0.0005	p<0.0005
copper (Cu)	p<0.0005	p<0.0005
iron (Fe)	p<0.0005	p<0.0005
potassium (K)	n.s.	n.s.
magnesium (Mg)	p=0.006	p=0.016
manganese (Mn)	n.s.	n.s.
sodium (Na)	p=0.002	p=0.023
phosphorus (P)	p<0.0005	p=0.001
sulphur (S)	n.s.	p=0.008
zinc (Zn)	p<0.0005	p<0.0005

[1]n.s. = not significant