Phytochelatins: Biomonitors for Metal Stress in Terrestrial Plants

by

James Edward Gawel

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Signature of Author _____
Department of Civil and Environmental Engineering

Certified by _____
Professor François M. M. Morel
Thesis Supervisor

Accepted by _____
Professor Joseph M. Sussman
Chair, Departmental Committee on Graduate Studies

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Abstract

Anthropogenic emissions of metals have recently been found to be contaminating most of the Earth's surface. Even remote ecosystems far removed from the sources of pollution are being affected. It has been difficult, however, to relate increased metal exposure to greater physiological stress in the biota with metal measurements alone. Metal-binding polypeptides, called phytochelatins, offer both the ability to link metal exposure and physiological stress, and ease of measurement in the field. A few needles or a single leaf is all that is required for detection of phytochelatins in most plants. Because they are produced specifically to bind toxic metals in the cytoplasm, phytochelatins are a direct indicator of intracellular metal stress.

Using this tool for the first time on terrestrial plants, we were able to examine the effects of various sources of metal pollution on trees at different field sites. Our data from the northern Appalachian Mountains of New England indicate that metals in regional air pollution do play a role in red spruce decline. This is the first time a direct connection has been shown between metal stress and forest decline. In Sudbury, Ontario, Canada, we found that current Cu/Ni smelter emissions from the world's tallest stack – designed to alleviate metal toxicity near Sudbury – are depositing elevated concentrations of bioavailable metals more than 20 km away, subsequently causing metal stress in the trees. In contrast, high concentrations of metals in the soils near the "superstack" seem to have little effect on the trees. We also tested the ability of phytochelatin measurements to map groundwater contamination near two Superfund sites north of Boston. Our results show that this may be an effective, inexpensive, and easy way to monitor metal mobility in shallow aquifers.

Thesis Advisor: François M. M. Morel
Title: Professor of Civil and Environmental Engineering

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Chapter 1

Introduction

Metal pollution is a burgeoning problem throughout the developed world, brought about by fast-paced industrialization and incomplete knowledge of the potential detrimental effects of poor disposal practices. Human health concerns are paramount, but ecosystem poisoning may also have far reaching repercussions. Metals are also a natural part of the Earth's geology, however, and biota have undoubtedly evolved to be able to deal with certain amounts. Some are even essential for life. Although metal concentrations in the biota can act as biomonitors of metal contamination (Streit and Stumm 1993), the difficulty arises in trying to discern when metals are creating stress in the environment and when they are not. After all, metal pollution would not be pollution if it did not have detrimental effects. Metal concentrations alone cannot tell us whether they are causing harm, so some other indicator of metal stress is required which signals a physiological response to metals in biota. Phytochelatins provide just such a measure in plants.

Phytochelatins were identified in higher plants by Grill et al. in 1985. They are a class of peptides described by the formula (γ-glutamyl-cysteine)$_n$-glycine, where n=1 to 11 [Figure 1.1].

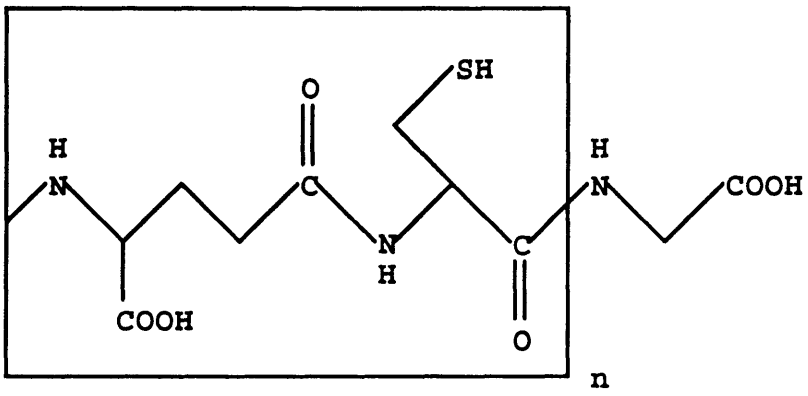

Figure 1.1: Phytochelatin molecule, (γ-glutamyl-cysteine)$_n$-glycine [n=2 to 11].

Phytochelatins are produced in the cytoplasm of plant cells in response to an excess of certain metals (Cd, Cu, Zn, Pb, Ni, Ag, Hg, Sn, Au, Bi, and W) and metalloids (Sb, As, and Te), as well as one non-metal (Se) [Grill et al. 1987]. For simplicity, we refer to this collection of phytochelatin-inducing elements simply as "metals" throughout this thesis. Phytochelatins are formed through the addition of two glutathione (γ-glutamyl-cysteinyl-glycine) molecules, with the loss of one glycine, by phytochelatin synthase, an enzyme activated directly by free metal ions (Loeffler et al. 1989). Termination of the reaction occurs when the enzyme-activating metal is complexed, leading researchers to conclude that a primary role of phytochelatins in plants is detoxification (Grill et al. 1987). Support for this was also provided by Kneer and Zenk (1992), who found that phytochelatins protect plant enzymes from metal poisoning. Because phytochelatins have been found in low concentrations even when metal levels are low, it has been hypothesized that they also play some role in trace metal homeostasis, a view supported by the fact that metal-requiring apoenzymes can be reactivated by phytochelatin-metal complexes (Thumann et al. 1991). In higher plants, however, there has been no demonstration that phytochelatin production increases as metal concentrations decrease. Thus, metal-deficiency probably does not result in higher phytochelatin concentrations.

In phytochelatins the sulfhydryl group of cysteine forms strong bonds with free metal ions. Calculations of stability constants, using data taken from Hancock and Martell (1989), show that this type of bond is strongest for toxic, non-essential metals and weakest for essential trace metals. The progression of the stability of this sulfhydryl/metal bond from strongest to weakest is as follows for thiocyanate ($N{\equiv}C{-}S^-$): $Hg^{2+} > Au^+ > Ag^+ > Bi^{3+} > Cd^{2+} > Pb^{2+} > Sn^{2+} > Cu^{2+} > Zn^{2+}$

$> Ni^{2+}$. Only the last three metals in the series shown above are known to be essential trace elements, thus phytochelatin concentrations may be, more specifically, indicators of stress from toxic, non-essential metals. Stability constants for metal ions with cysteine [strongest $Hg^{2+} > Pb^{2+} > Cd^{2+} > Ni^{2+} > Zn^{2+}$ weakest] and glutathione [strongest $Hg^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Cu^{2+}$ weakest] have not all been determined, but those that are available support this conclusion (Sillén and Martell 1964, Sillén and Martell 1971, Martell and Smith 1974, Perrin 1979, Martell and Smith 1982, Smith and Martell 1989, Morel and Hering 1993). The metalloids As, Sb, and Te, the metal W, and the non-metal Se were not included in any of these compilations.

Further research involving phytochelatins has shown that their concentrations may be particularly good measures of metal stress in higher plants. First, it has been found that they are produced by almost every plant (Gekeler et al. 1989), with the only exceptions being plants that instead produce closely related compounds to serve the same function, such as the plant Order *Fabales* which make homo-phytochelatins having a terminal β-alanine instead of glycine (Grill et al. 1986). Second, it has been shown that increased production of phytochelatins is not a differential tolerance mechanism (Schultz and Hutchinson 1988, Schat and Kalff 1992, de Knecht et al. 1995). In other words, plants with increased metal tolerance do not produce more phytochelatins to bind metals, rather they are able to thrive while producing less than intolerant populations. Finally, prior to our work, the only field measurements published for higher plants have shown higher concentrations of phytochelatins in the roots of seedlings growing on mine tailings than in the same species growing on uncontaminated soils (Grill et al. 1988).

An initial difficulty in measuring phytochelatins in the field was the insensitivity of the absorbance methods then used to quantify them. For example, Grill et al. (1988) were unable to measure phytochelatins in the leaves of the seedlings growing on mine tailings because of their high detection limit. Work by Ahner (1994), however, has produced a fluorometric detection method, modeled after Newton et al. (1981), which is much more sensitive and allows for the low level measurements necessary for field work. For terrestrial plants, this allowed us to measure phytochelatins in single leaf samples, making extensive field sampling more practical. Roots may produce up to two orders of magnitude more phytochelatins per gram of material than foliage when metals are applied to soils (Leita et al. 1991), but roots are difficult if not impossible to sample in mature trees in the field, especially over large geographic scales. In addition root cores can be very damaging to the tree, whereas a few leaves are hardly missed.

Using phytochelatin concentrations in leaves as a measure of metal stress in plants, we examine the interactions of several different types of metal pollution with plants in the vicinity. In Chapter 2 we explore the possibility of a role for metals, as a component of regional-scale air pollution, in forest decline in the mountains of the northeastern United States. In this area, the distribution of metals has already been investigated, but no conclusions could be drawn about their possible harmful effects at that time since other factors were also involved, and their differential effects could not be evaluated separately. We are able to establish that physiological metal stress is directly correlated with forest damage. In Chapter 3 we look at the effects of metals, in current stack emissions and historic soil contamination, on trees in the area surrounding metal-smelting operations in Sudbury, Ontario, Canada. Here the question is one of bioavailability: whether

foliar uptake of current metal emissions or root uptake of high levels of metals in the soil leads to greater plant stress. Finally, in Chapter 4 we have looked at smaller scale metal contamination in the form of subsurface waste sites. Metal wastes from past industrial practices have polluted the groundwater and surface water in Woburn, Massachusetts. We examine the possibility of using phytochelatin concentrations in trees as a monitoring device for determining the extent of metal mobility and migration throughout the watershed. The work included in Chapters 2, 3, and 4 has opened several avenues for future research, a few of which we have begun to follow already. These initial results are included with concluding remarks in Chapter 5.

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Chapter 2

A Role for Metal Stress in Red Spruce Decline in the Northeastern United States Indicated by Phytochelatin Measurements

Introduction

Forest decline was described by Smith (1991) as follows:

> A special and very important type of ambiguous tree disease is termed dieback/decline. It occurs when a large proportion of the trees of a particular species over an area of many square kilometers show symptoms of stress, unusual and consistent growth decreases, or die.

This general phenomenon is more accurately applied to individual species of trees than to the forest in general. In the northeastern U.S., high elevation spruce-fir (or boreal) forests in the northern Appalachian Mountains were diagnosed as exhibiting symptoms of decline beginning in the early 1960's, and *Picea rubens* (red spruce) in particular is the species of concern (Siccama et al. 1982, Johnson and Siccama 1983, Silver et al. 1991). *Abies balsamea* (balsam fir) and *Betula papyrifera* (paper birch), the other two codominant tree species in these forests (Siccama 1974), have been found to be relatively unaffected (Craig and Friedland 1991, Battles et al. 1992) and may even be filling the gap left by red spruce (Friedland 1989). Distinct geographical patterns of red spruce decline have been documented. Damage increases with altitude (Battles et al. 1992) and conditions are worse in high elevation forests in the west – in the Adirondack Mountains of New York and Green Mountains of Vermont – than in the east – in the White Mountains of New Hampshire (Craig and Friedland 1991). Although some researchers have contested the conclusion that red spruce are actually in decline (Hornbeck et al. 1986, Zedaker et al. 1987), a

preponderance of the available data seems overwhelmingly to support that it is indeed a reality (McLaughlin 1985, Johnson and Siccama 1989, Peart et al. 1992).

Differing opinions exist, however, on the causation for red spruce decline, and numerous hypotheses have been put forward to account for the dieback that is threatening the long-term health of the northern montane forests. Suspected agents span the range from natural factors such as insects and drought, to anthropogenic pollutants such as acids and ozone, to complex interactions between humans and nature which may be responsible for an overall decrease in genetic diversity in red spruce (McLaughlin 1985, Klein and Perkins 1988, DeHayes and Hawley 1988). The synchronous nature of the onset of this decline throughout the region, along with the relative geographic scale of the problem has led researchers in the field to disregard biotic factors as primary agents. It is thought that the current episode was probably initiated by unfavorable climate or drought conditions around 1960 (Johnson et al. 1986), but this does not account for the long-term effects documented throughout the Northeast. Extensive research has yet to elucidate just what is responsible for red spruce decline.

Correlations between the occurrence of anthropogenic pollutants and red spruce decline have drawn most of the attention in the ensuing investigations. Deposition of "acid rain", ozone, nitrate, sulfate, and metals has been found to increase with elevation and is generally higher in the west than in the east [Johnson et al. 1982, Friedland et al. 1986, Johnson et al. 1988, Miller et al.1993]. Johnson et al. (1972) showed that these pollutants are carried from the large industrial centers in the Great Lakes region of the U.S. and Canada and deposited in the northern

Appalachians. Orographic clouds formed at high elevations are efficient scavengers of airborne particles, and subsequently deposit these pollutants to the forest canopy and soil (Schlesinger and Reiners 1974, Lovett et al. 1982). The number of days with cloud immersion increases with altitude (Miller et al. 1993, Siccama 1974) permitting higher concentrations of pollutants to be trapped and deposited. Thus, because of physical processes and source locations, patterns of pollutant concentrations in the northern Appalachians generally match patterns of observed dieback intensity. However, this correlation between pollutant concentrations and forest damage does not involve the measurement of direct physiological effects of anthropogenic pollutants on red spruce trees. Therefore, it is difficult to draw firm conclusions regarding red spruce decline causation from these correlations alone.

Our study attempts to bridge the gap between these correlations and actual physiological effects of metals on red spruce trees in particular. Using phytochelatins as a molecular marker of the degree of metal stress exhibited by individual trees, we can avoid problems inherent in trying to extrapolate dose/response curves, derived from greenhouse experiments, using seedlings under artificial growth conditions, to mature trees under field conditions at high altitude. Because phytochelatins are produced by plant cells in response to an excess of toxic metals entering the cytoplasm, increased phytochelatin production signals an increase in metal uptake as well as an increase in the expenditure of energy to deal with it, thus linking physiological response and metal dose in red spruce. Using phytochelatin measurements, we have examined possible correlations between physiological metal stress and red spruce decline as a function of elevation, geographic location, and species differences.

Methods

Sample collection was started in 1993 and continued in 1994 and 1995 with modifications described below. Sampling in 1993 took place on the Whiteface Mountain massif in the Adirondack Mountains of New York. Samples were collected at approximately 700, 900, and 1000 m elevation (using an altimeter set at known benchmarks) starting soon after bud break at the end of June and continued until October, with sampling taking place roughly every 2 to 3 weeks (actual dates are included in Figure 2.2). Using a pole-pruner, foliage samples were collected from opposite sides of the crown of six red spruce trees and three balsam fir trees at each elevation. Current-year needles were pooled from both aspects of each tree for one sample each, and immediately placed in a cryovial in liquid nitrogen at the site to prevent sample degradation. Only trees showing less than 10% needle loss or discoloration in the exposed crown were chosen for sampling to avoid possible differences between damage classes.

After viewing results from 1993, we concentrated our sampling during the month of July only in 1994 and 1995. In 1994 samples were collected once a week for four weeks along the same elevational transect on Whiteface Mountain, New York, as well as at 1000 m sites on eight other mountains: Giant in the Adirondack Mountains of New York; Abraham and Mansfield in the Green Mountains of Vermont; and Monroe, Jefferson, Clay, Boott Spur, and Wildcat E in the White Mountains of New Hampshire [Figure 2.1]. Foliage samples were taken with a pole-pruner from opposite sides of the exposed crown of five visually healthy red spruce and balsam fir trees

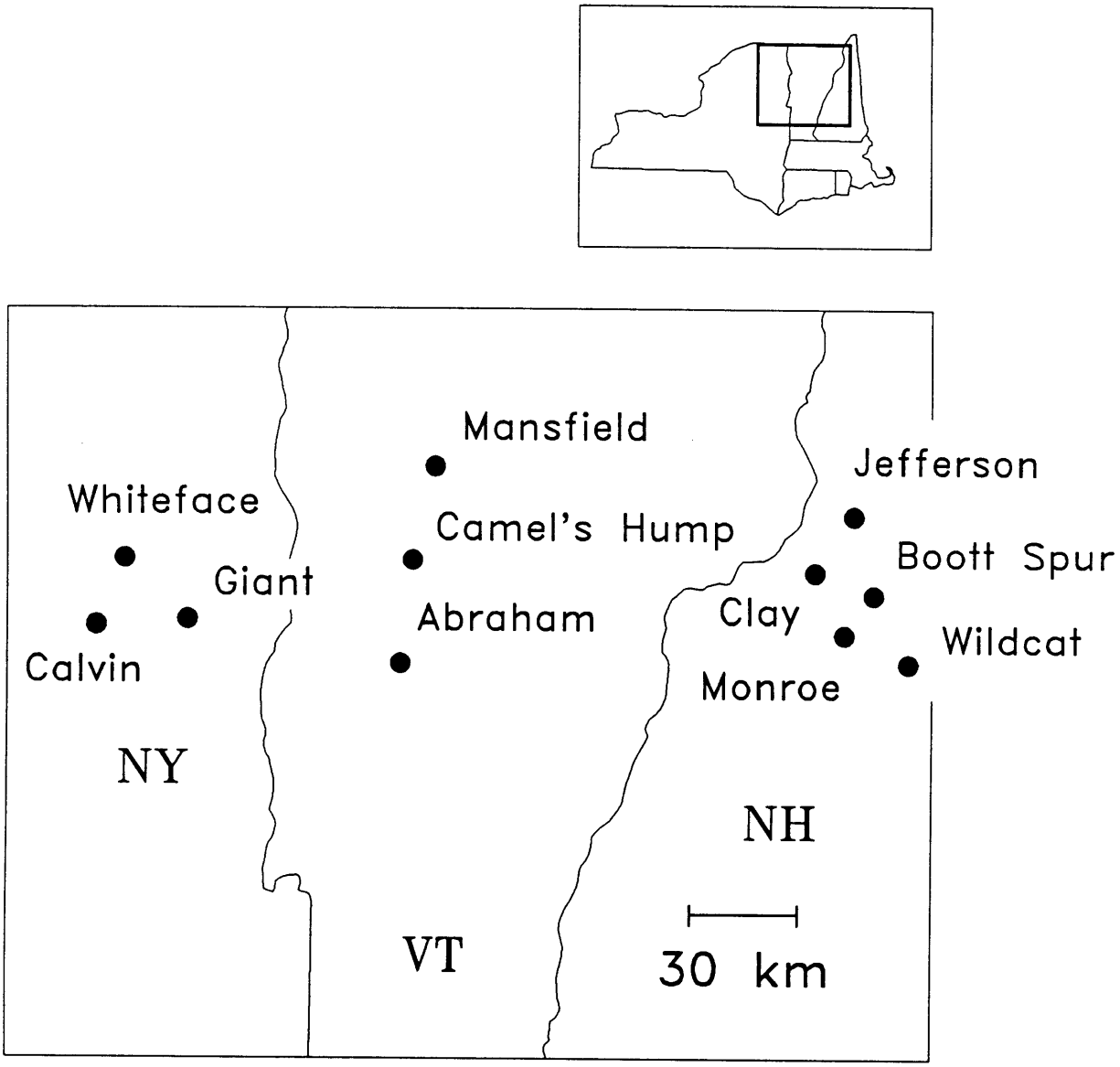

Figure 2.1: Map of mountains sampled in the Northern Appalachians.

at each site, and new foliage pooled for one sample each. These were again placed immediately in liquid nitrogen, which was carried in a portable dewar in a backpack to each site. During the last week of sampling, larger foliage samples were collected for metals analysis from each tree and placed in plastic bags on ice until they could be transferred to a freezer in the lab.

Forest stands sampled on all mountains in 1994, with the exception of Whiteface Mountain, were assessed for the degree of damage they were exhibiting. This was accomplished by establishing a transect following the 1000 m elevation contour at each site. Red spruce trees in point-centered quarters at 25 m intervals were assessed for damage using field glasses and classified as (1) less than 10%, (2) greater than 10% and less than 50%, (3) greater than 50% and less than 100% needle loss or discoloration, (4) standing dead with no needles but fine twigs intact, or (5) standing dead with no fine twigs left. Ten stations along each transect were established, and four red spruce trees assessed at each, for 40 trees in each stand (Craig and Friedland 1991).

Sampling in 1995 was similar to the prior year. Balsam fir was not included this year, but five visually healthy red spruce trees were sampled at the same sites along the elevational transect at Whiteface Mountain once a week for four weeks in July. Four other mountains were included as well - Colvin in New York, and Mansfield, Abraham, and Camel's Hump in Vermont [Figure 2.1] - and five red spruce were sampled over the same period at 1000 m elevation sites. Samples were taken from each tree as described previously and larger foliage samples were again collected during the last week of July from each tree for metals analysis. In addition, at the 1000 m site on Whiteface Mountain, five red spruce trees each, representing the three living damage classes

discussed above, were located and samples were collected during the second week of July as before.

Homogenization of foliage samples in 1993 was accomplished by first pulverizing needles in liquid nitrogen in a ceramic mortar and pestle, then using a ground-glass tube and pestle (Glas-col and Wheaton) hooked up to a variable speed drill motor. This was replaced in 1994 and 1995 by Brinkmann Instruments' Polytron Homogenizer, which required no prior pulverization of the samples. Samples were homogenized on ice in 0.01 M methanesulfonic acid, used as an extractant for phytochelatins. Analytical protocols for phytochelatin quantification, modified from Newton et al. (1981), were developed in our laboratory (Ahner 1994). Homogenates were centrifuged for 30 min at 16,000g and 4°C, and the supernatant decanted and refrigerated prior to analysis. Extracts were derivatized using monobromobimane, a sulfhydryl-specific fluorescent marker, as described in Appendix A, separated using HPLC (Beckman Instruments, Inc.) with a reverse-phase C18 column (Alltech Associates' Adsorbosphere HS C18 5μm), and quantified fluorometrically (Gilson model 121 fluorometer). Phytochelatin standards for the n=2, 3, and 4 chainlengths, synthesized by MIT's Biopolymers Laboratory, were used for calibration of the method. Chainlengths greater than n=4 are not quantifiable because standards cannot be synthesized accurately. Homo-phytochelatins and hydroxymethyl-phytochelatins are also not distinguished by this method. However, in almost all samples taken in the mountains of the northeastern U.S., only the n=2 chainlength was detected. Therefore, measurements reported here include only the n=2 chainlength of phytochelatins. Three different standardization methods were used to determine the variability caused by individual tree physiology. In all years, acid-

soluble protein, as determined using the BCA (bicinchoninic acid) assay (Pierce Chemical Co.), was used as a quantification of cytoplasmic material in the extracts. In 1994 and 1995 wet weight and dry weight measurements were also used to quantify the mass of foliar material used for phytochelatin analysis.

Foliage used for metals analysis was soaked in 0.1 M ascorbic acid for 20 min (after Anderson 1981), then rinsed with Q-water (Millipore) and dried for 3 days at 80°C in a paper bag. This was done to remove external metal contamination from the needles. Digestion of foliage for metals analysis was accomplished using wet digestion procedures similar to those outlined by van Loon (1985). 1994 samples were digested and analyzed by Maria Borcsik at Princeton University. Approximately 1.0 g of dried needles, 10 ml concentrated nitric acid, and 10 ml concentrated hydrogen peroxide were heated in Teflon beakers with Teflon watch glasses to dryness. This digestate was redissolved in distilled water and 5 ml concentrated HCl brought up to 10 ml in a volumetric flask. These samples were analyzed for Cd, Pb, Cu, and Zn using an inductively coupled argon plasma spectrophotometer (Perkin-Elmer 6000). Three replicates for each sample were run for every metal and method blanks were included. Calibration curves were derived daily from diluted metal stock solutions.

Samples from 1995 were digested at MIT using a different procedure. Foliage samples large enough to do so were divided in half, and only one of these soaked in ascorbic acid as previously described in order to assess the usefulness of this procedure. All samples were dried as before. Approximately 1.0 g dried needles, 10 ml concentrated nitric acid, and 2.5 ml concentrated

perchloric acid were heated almost to dryness. The digestate was redissolved in approximately 2% nitric acid brought up to 25 ml in a volumetric flask with Q-water. Samples were analyzed again for Cu using ICP-AES (Thermo Jarrell Ash AtomScan25). Cd and Pb were analyzed using a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer 4100ZL). Analysis of Cu and Pb in NIST pine needles using the digestion methods outlined above for 1995 gave at least 95% and 93% recoveries respectively (Cd is not a NIST certified metal). Method blanks in 1994 (measured using ICP-AES) produced undetectable amounts of all metals, while those in 1995 (measured using AAS) had undetectable amounts of Cd and Cu. Lead concentrations in method blanks reached 15 μg/g dry weight in 1995, still well below the levels found in needle samples.

Results

In 1993, red spruce foliage collected bimonthly at 1000 m showed a distinct and significant ($P < 0.001$) mid-July peak in phytochelatin concentrations normalized to protein [Figure 2.2]. Concentrations in balsam fir, a species not in decline, were significantly lower ($P < 0.001$) during this time, and failed to ever increase above the baseline value. Results from 1994 also show higher phytochelatin values normalized to protein in red spruce [Figure 2.3a] than in balsam fir [Figure 2.4a]. Normalized to dry weight and wet weight, however, baseline concentrations of phytochelatins are higher in balsam fir [Figures 2.4b and 2.4c] than in red spruce [Figures 2.3b and 2.3c]. Protein measurements should be much better indicators of the amount of cytoplasmic material potentially affected by metal poisoning. Phytochelatin production specifically depletes amino acids used for protein synthesis, thus a direct comparison between the amount of total acid-

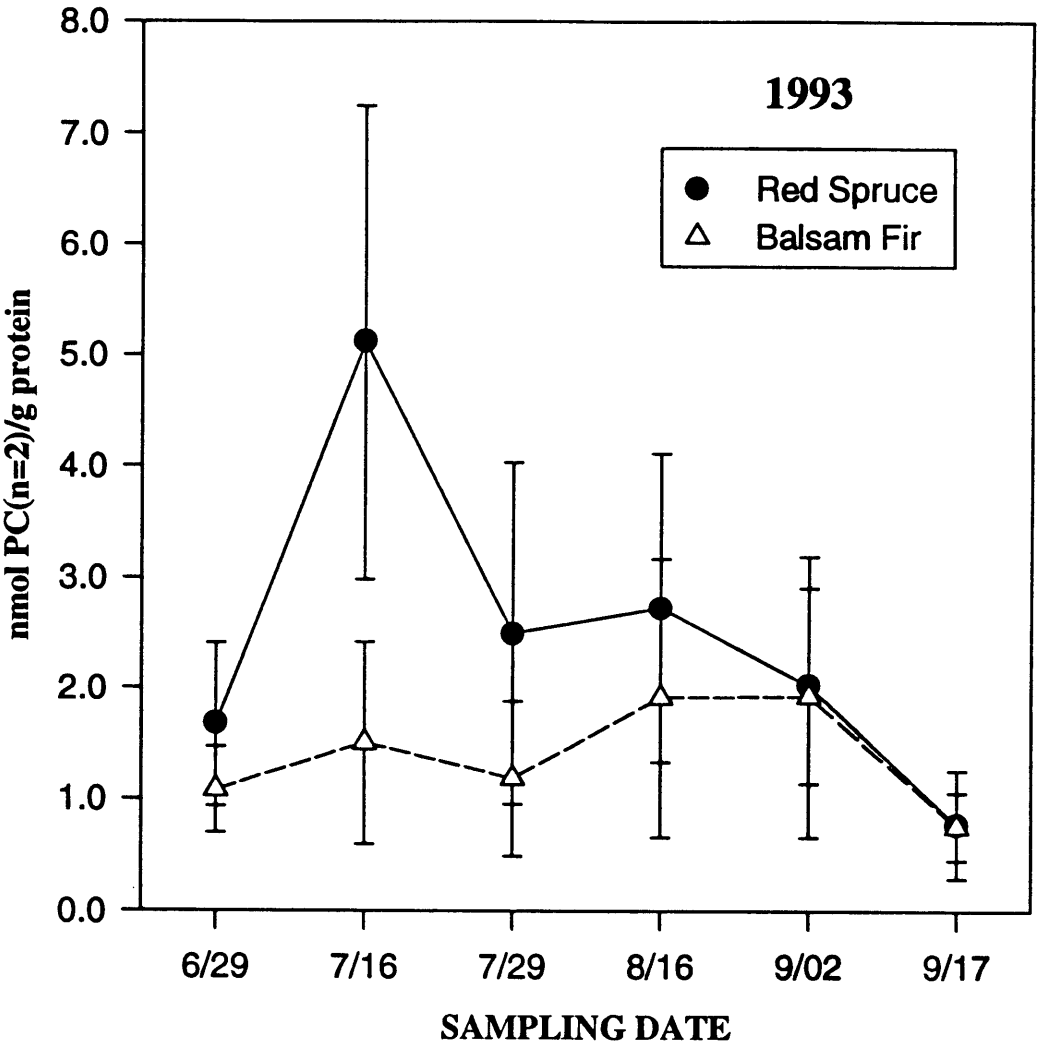

Figure 2.2: Phytochelatin concentrations normalized to protein in foliage taken in 1993 from red spruce and balsam fir trees on Whiteface Mountain, NY.

soluble protein and phytochelatins provides a good indicator of the relative impact of metal stress on plant health. Dry weight and wet weight measurements are much more difficult to use since extraneous intracellular and extracellular structures formed by one species and not the other may unduly influence normalizations and comparisons between species. Thus, phytochelatin concentrations presented in this thesis will primarily use protein measurements for

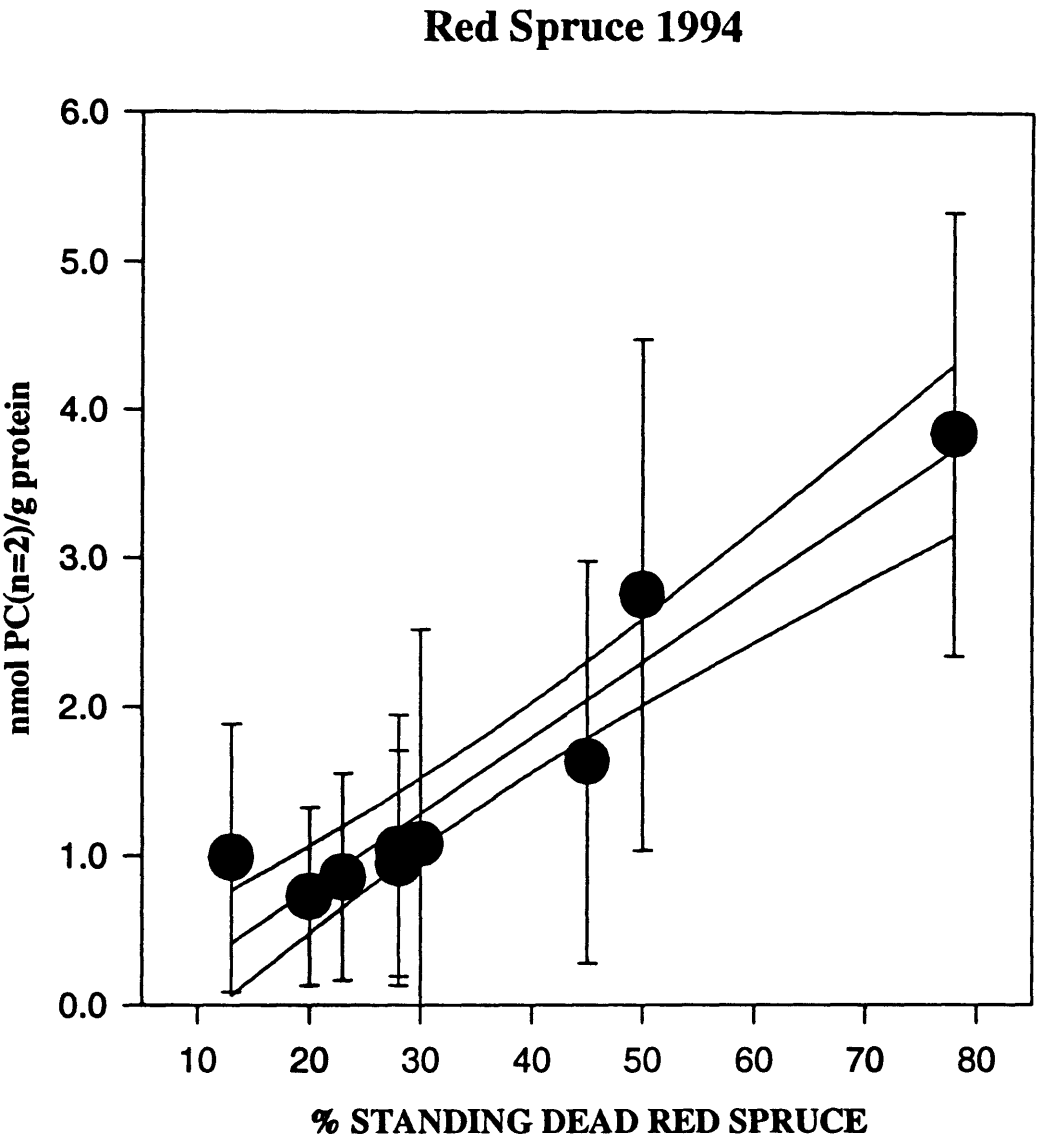

Figure 2.3a: Phytochelatin concentrations normalized to protein, in foliage taken in 1994 from red spruce trees at 1000 m on nine different mountains in the Northeast, compared to percent standing dead red spruce in the stand.

standardization. Normalizations to wet weight and dry weight from 1994 and 1995 not included in this chapter are in Appendix B.

Data from our evaluations of forest stand health for mountains sampled in 1994 and 1995 are

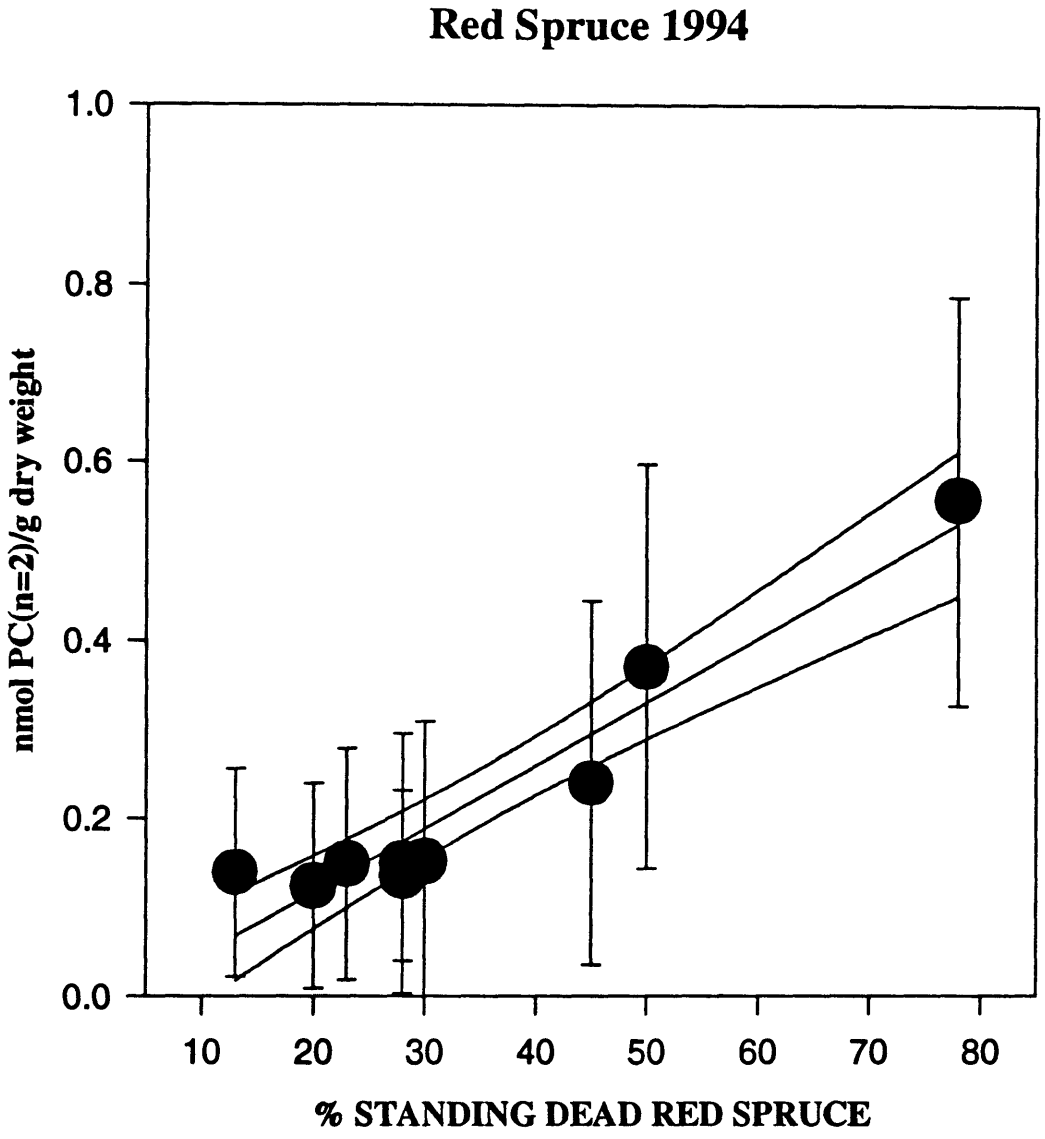

Figure 2.3b: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1994 from red spruce trees at 1000 m on nine different mountains in the Northeast, compared to percent standing dead red spruce in the stand.

given in Appendix B [Table B.1]. Data for Whiteface Mountain were available in Battles et al. (1992), given as standing dead red spruce (4's and 5's) as a percentage of total red spruce density. Damage classifications (1's, 2's, 3's, and 4's only) of red spruce for Whiteface Mountain, Mt. Colvin, and Camel's Hump were obtained from Dr. Wendy Silver (unpublished data summarized

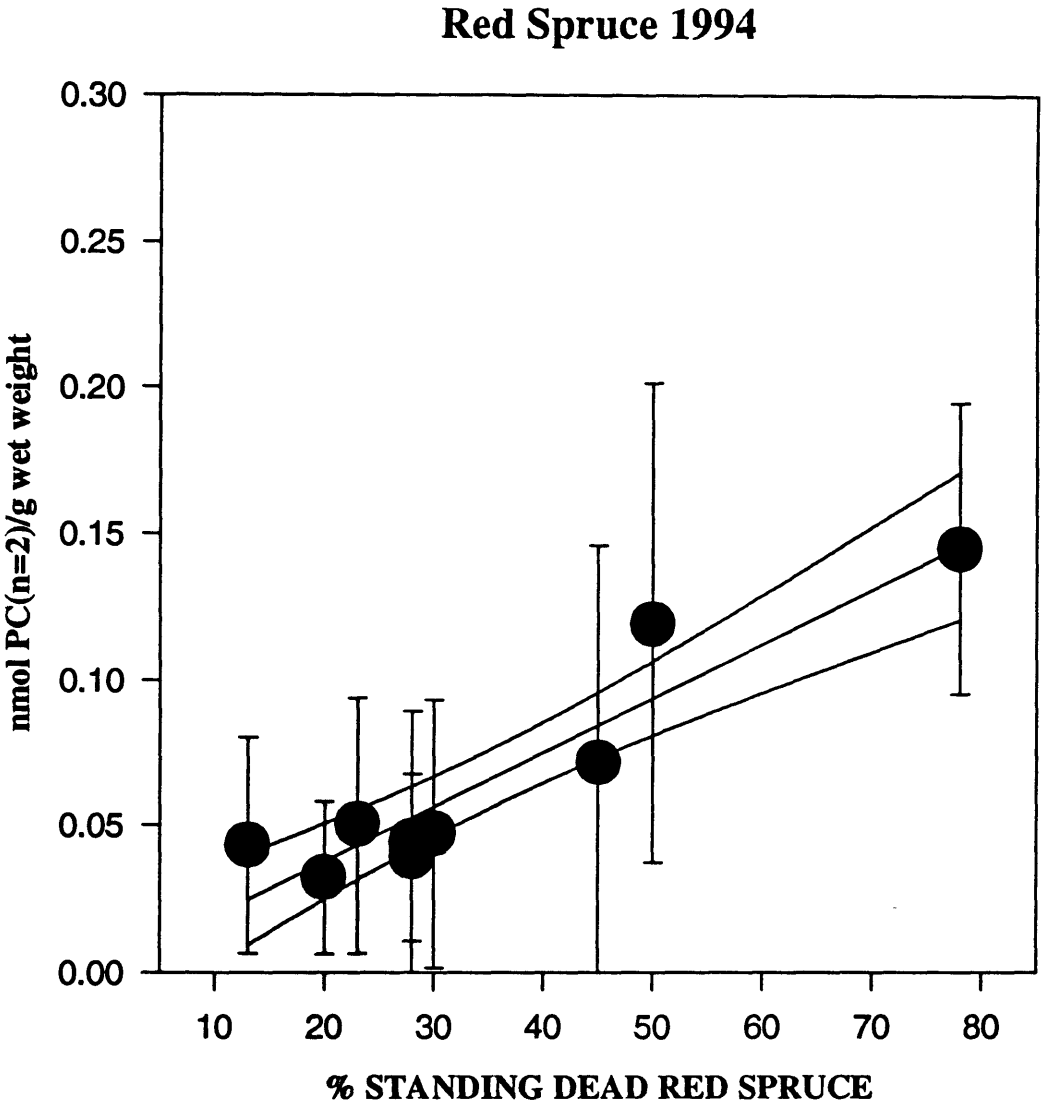

Figure 2.3c: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1994 from red spruce trees at 1000 m on nine different mountains in the Northeast, compared to percent standing dead red spruce in the stand.

in Silver et al. 1991) and Mr. Brad Craig (unpublished data summarized in Craig and Friedland 1991). These data were used to calculate numerical indicators of forest stand health for each site sampled. Assessments carried out in 1994 were used for those sites sampled again in 1995. "Percent standing dead red spruce", as used in Figure 2.3a, was calculated as described above for

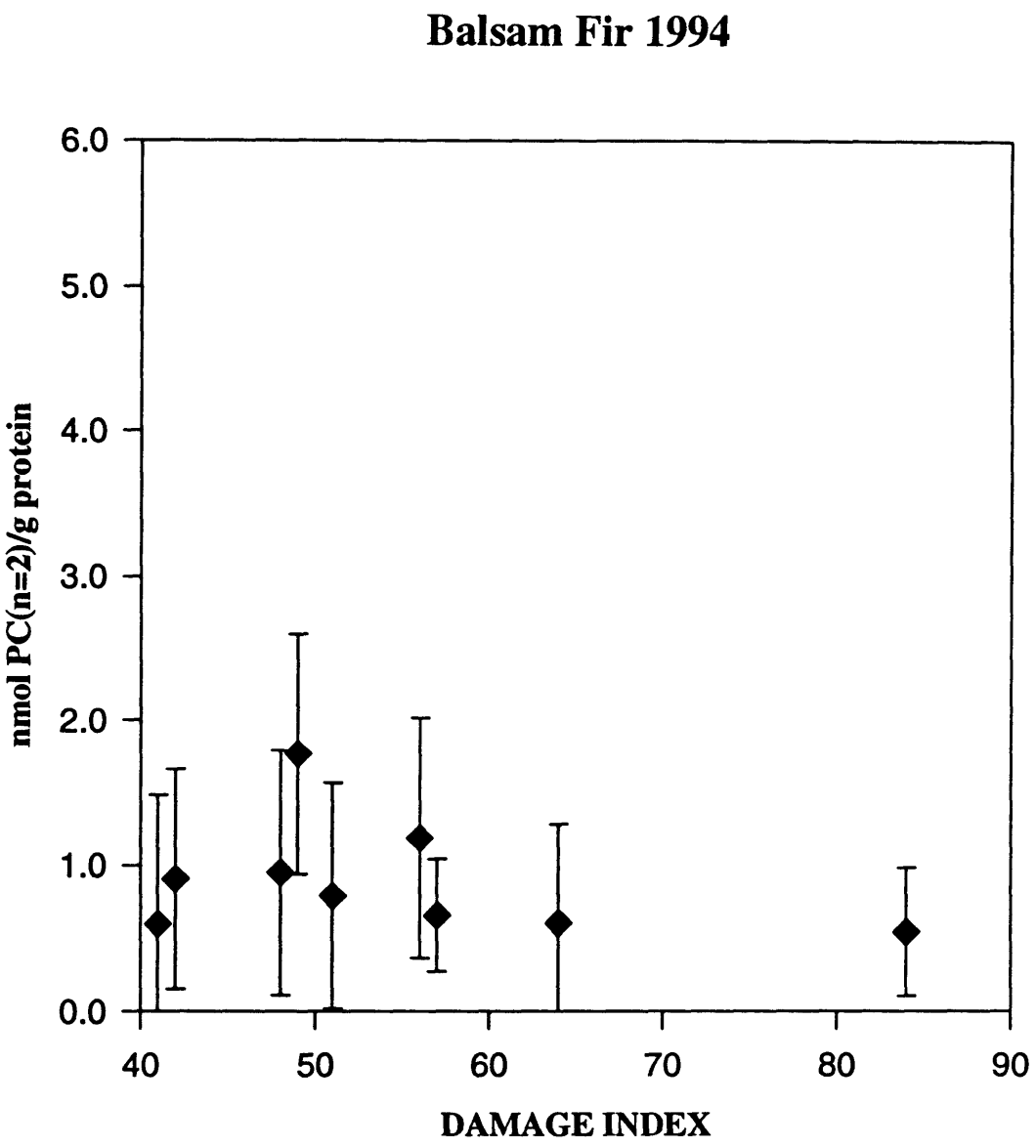

Figure 2.4a: Phytochelatin concentrations normalized to protein, in foliage taken in 1994 from balsam fir trees at 1000 m on nine different mountains in the Northeast, compared to red spruce damage index in the stand.

Battles et al. (1992), and "damage index", as used in Figure 2.4a, was calculated as the sum of all 1's, 2's, 3's, 4's, and 5's (if measured), divided by the highest total possible [i.e. for Mansfield, "damage index" = (6x1 + 7x2 + 9x3 + 9x4 + 9x5)/(40x5) = 64%]. Both of these measures of forest stand health produced similar results when plotted against phytochelatin concentrations in

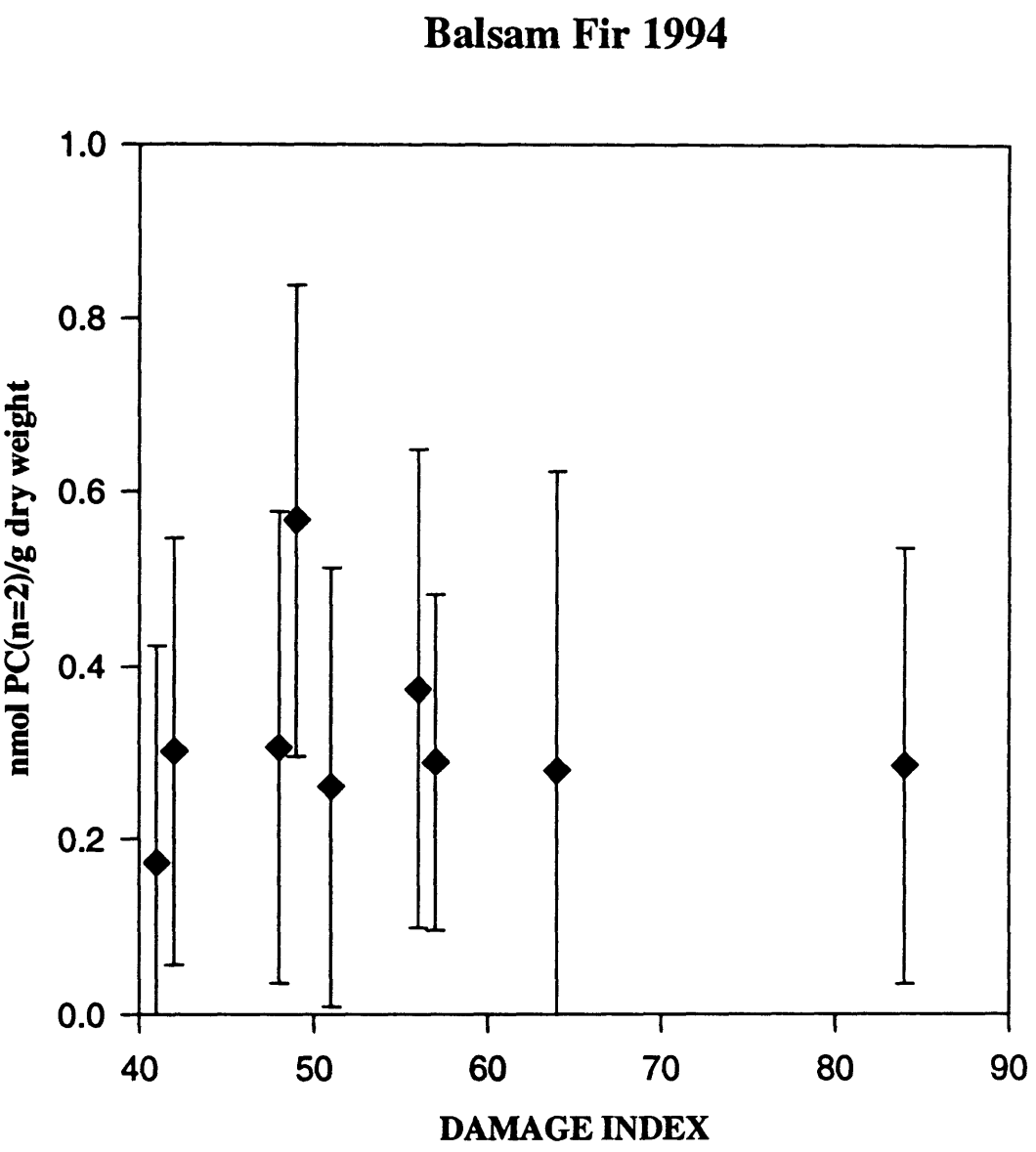

Figure 2.4b: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1994 from balsam fir trees at 1000 m on nine different mountains in the Northeast, compared to red spruce damage index in the stand.

1994 (vs. "damage index" not shown). Only the "damage index" could be compared across all mountains in 1995 due to the nature of Craig's data.

Across all mountains sampled in 1994, a significant ($P < 0.001$) correlation was found between

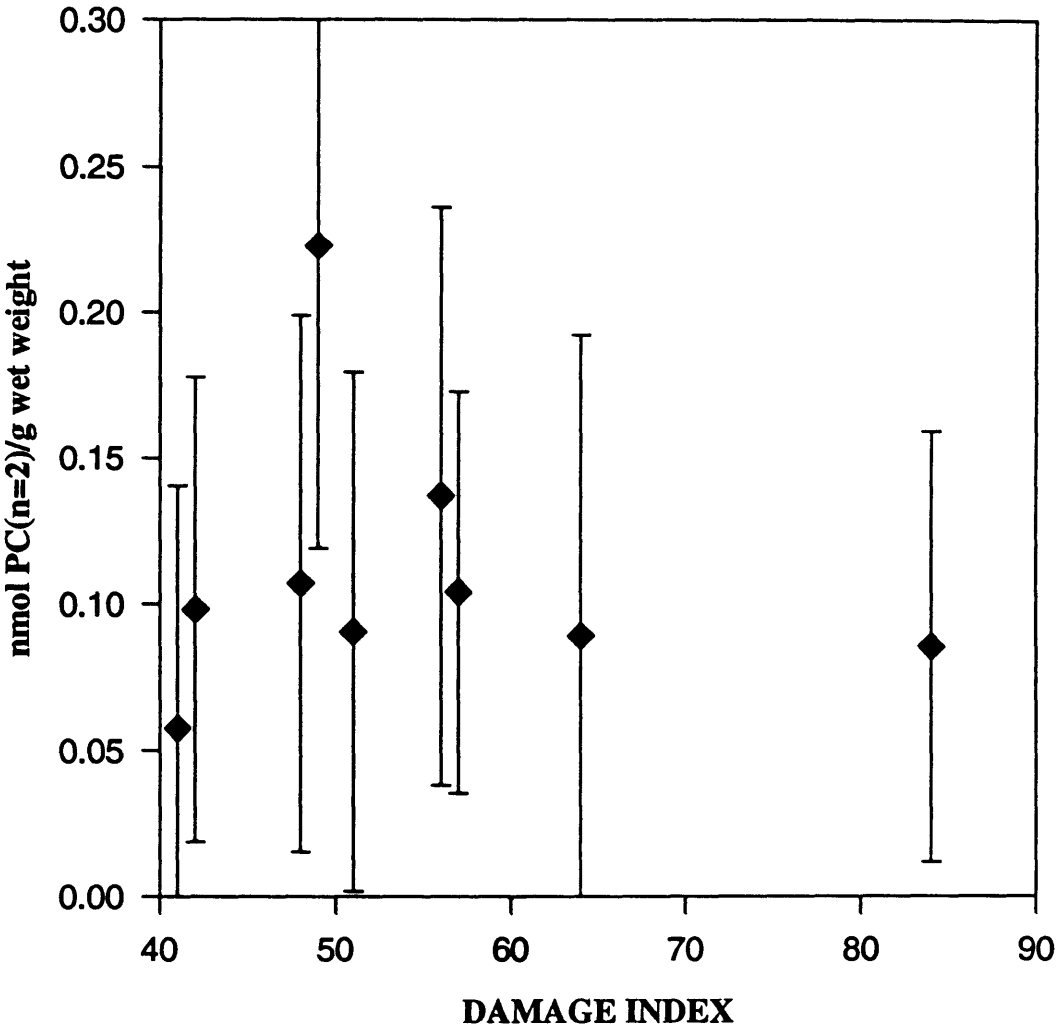

Figure 2.4c: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1994 from balsam fir trees at 1000 m on nine different mountains in the Northeast, compared to red spruce damage index in the stand.

red spruce health indices and phytochelatin concentrations normalized to protein [Figure 2.3a], dry weight [Figure 2.3b], and wet weight [Figure 2.3c]. Additional data were obtained in 1995 to add more high damage sites to those collected the previous year. Figure 2.5, including both 1994 and 1995 data, shows again that the correlation between phytochelatin concentrations and red

spruce health indices is highly significant ($P < 0.001$). It should be noted as well that phytochelatin values in red spruce are highest in the Green Mountains of Vermont, with intermediate values found in the Adirondack Mountains of New York, and the lowest levels in the White Mountains of New Hampshire. Balsam fir, on the other hand, shows no trend in relation to

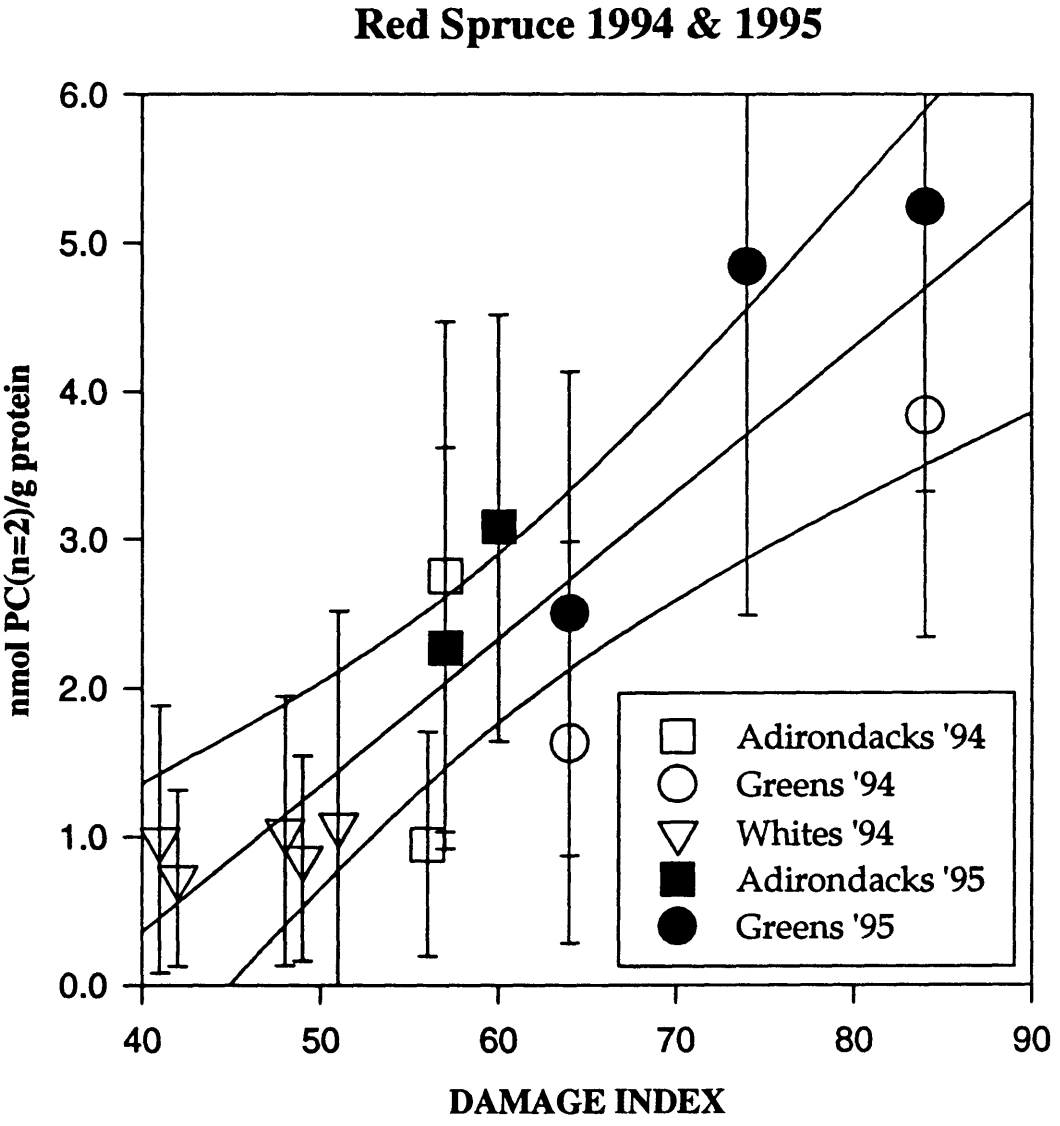

Figure 2.5: Phytochelatin concentrations normalized to protein, in foliage taken in 1994 and 1995 from red spruce trees at 1000 m on eleven different mountains in the Northeast, compared to red spruce damage index in the stand.

red spruce damage or geographical location in the same stands in 1994 [Figures 2.4a, 2.4b, and 2.4c].

Evaluation of the results from the elevational transect in 1993 produced a weak positive correlation ($r^2 = 0.69$) between altitude and phytochelatin concentrations in red spruce during the peak period in mid-July, but showed no consistent trend the rest of the time [Figure 2.6]. In 1994, the same transect that produced only a weak correlation in 1993 produced a highly significant ($P < 0.001$) correlation between elevation and phytochelatin levels, with concentrations again peaking in mid-July [Figure 2.7]. This is plotted with data from Battles et al. (1992) for percent standing dead red spruce versus elevation on Whiteface Mountain collected in 1987, which shows that red spruce damage also increases with altitude on the same mountain. Data from 1995 produced different results along this transect [Figure 2.8], with higher average phytochelatin concentrations found at the 900 m site. This variation from year to year is likely a result of changing climatic factors, which are discussed in the following section. Balsam fir trees sampled in 1993 and 1994 exhibited no significant trend with elevation either year [Figures 2.9 and 2.10].

In using visually healthy trees (i.e. damage class 1) for phytochelatin measurements all three years, we have attempted to show that red spruce damage is preceded by an increase in metal stress. When we actually measure phytochelatin concentrations in red spruce trees in differing damage classes, we find that concentrations are not significantly correlated with damage to the individual tree [Figure 2.11]. Phytochelatin values measured in damage classes 2 and 3 at 1000 m on

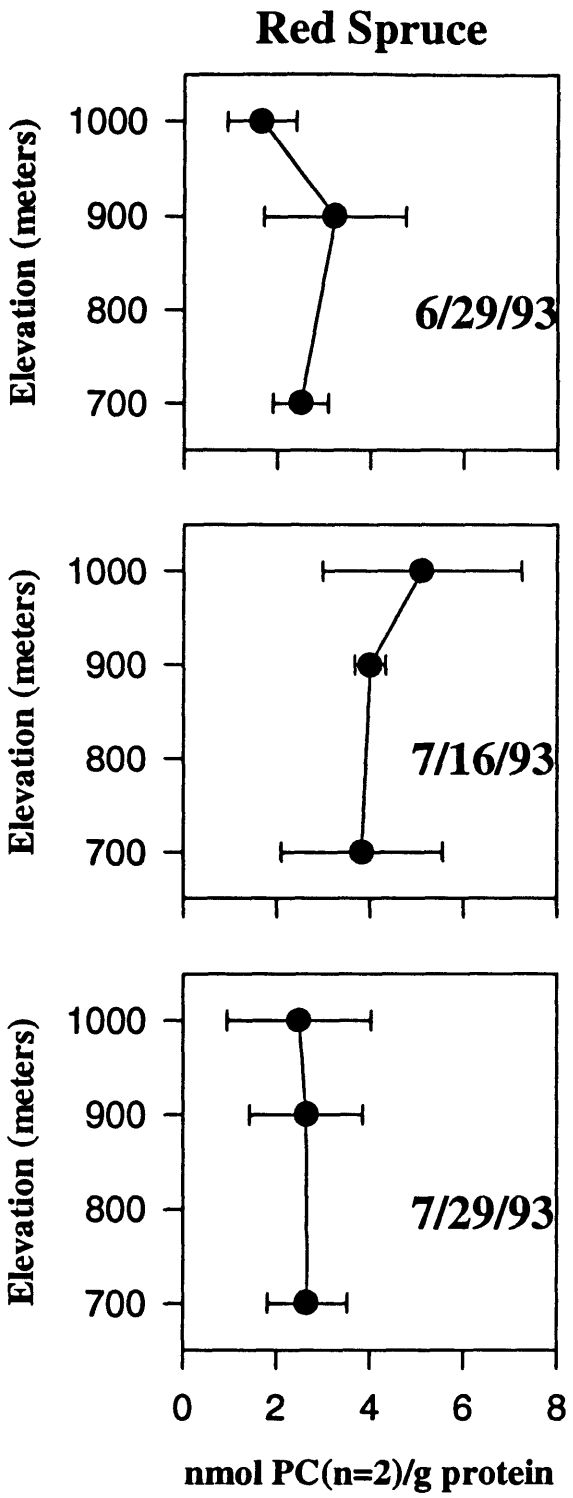

Figure 2.6: Phytochelatin concentrations normalized to protein in foliage taken in 1993 from red spruce trees at different elevations on Whiteface Mountain, NY.

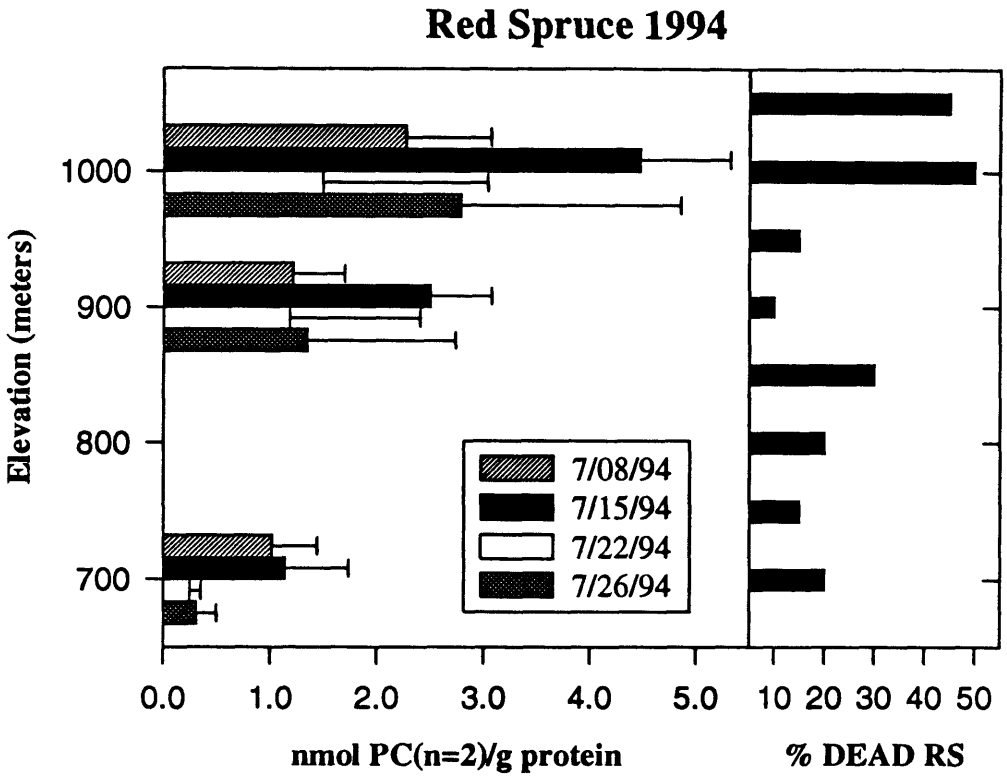

Figure 2.7: Phytochelatin concentrations normalized to protein, in foliage taken in 1994 from red spruce trees at different elevations on Whiteface Mountain, NY, compared to percent dead red spruce (data for percent dead red spruce from Battles et al. 1992).

Whiteface Mountain (3.3 ± 1.4 nmol PC(n=2)/g protein) are not significantly different from phytochelatin concentrations measured in class 1 trees in the same stand (2.4 ± 1.4 nmol PC(n=2)/g protein). In addition, the difference between mean phytochelatin levels in class 1 and class 3 trees is three times smaller than the range of mean phytochelatin concentrations found in class 1 trees at different locations across the region. This shows that increased metal stress is not simply brought about by increasing damage to the trees.

Foliage was also analyzed for metals in an attempt to (1) draw a direct correlation between metal

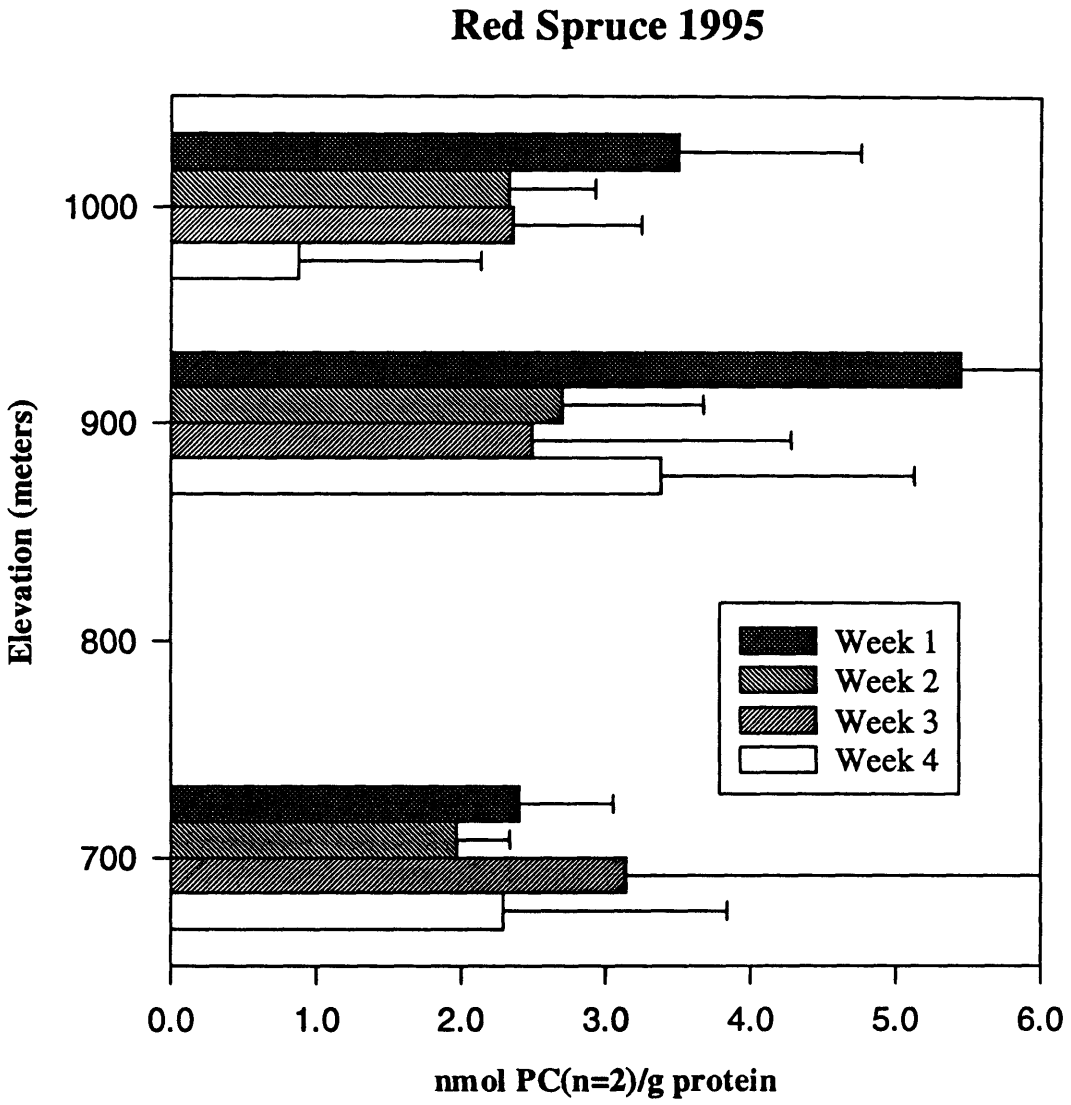

Figure 2.8: Phytochelatin concentrations normalized to protein, in foliage taken in 1995 from red spruce trees at different elevations on Whiteface Mountain, NY.

concentrations and phytochelatin production in the field and (2) try to pinpoint which metal or metals might be responsible for increased phytochelatin production. Data from 1994 [Figure 2.12] and 1995 [Figure 2.13] show significant correlations between cadmium concentrations in red spruce and percent standing dead red spruce and damage index across the region ($P < 0.001$ and $P < 0.001$, respectively). This relationship is even more convincing when the concentrations

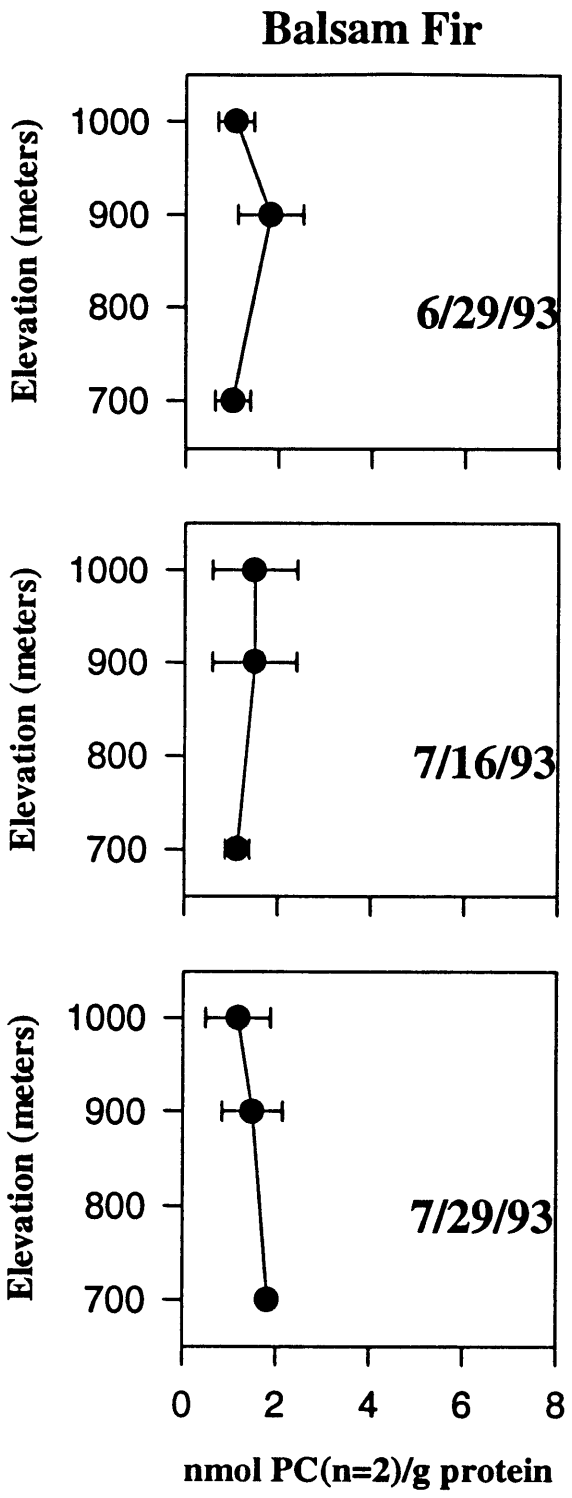

Figure 2.9: Phytochelatin concentrations normalized to protein in foliage taken in 1993 from balsam fir trees at different elevations on Whiteface Mountain, NY.

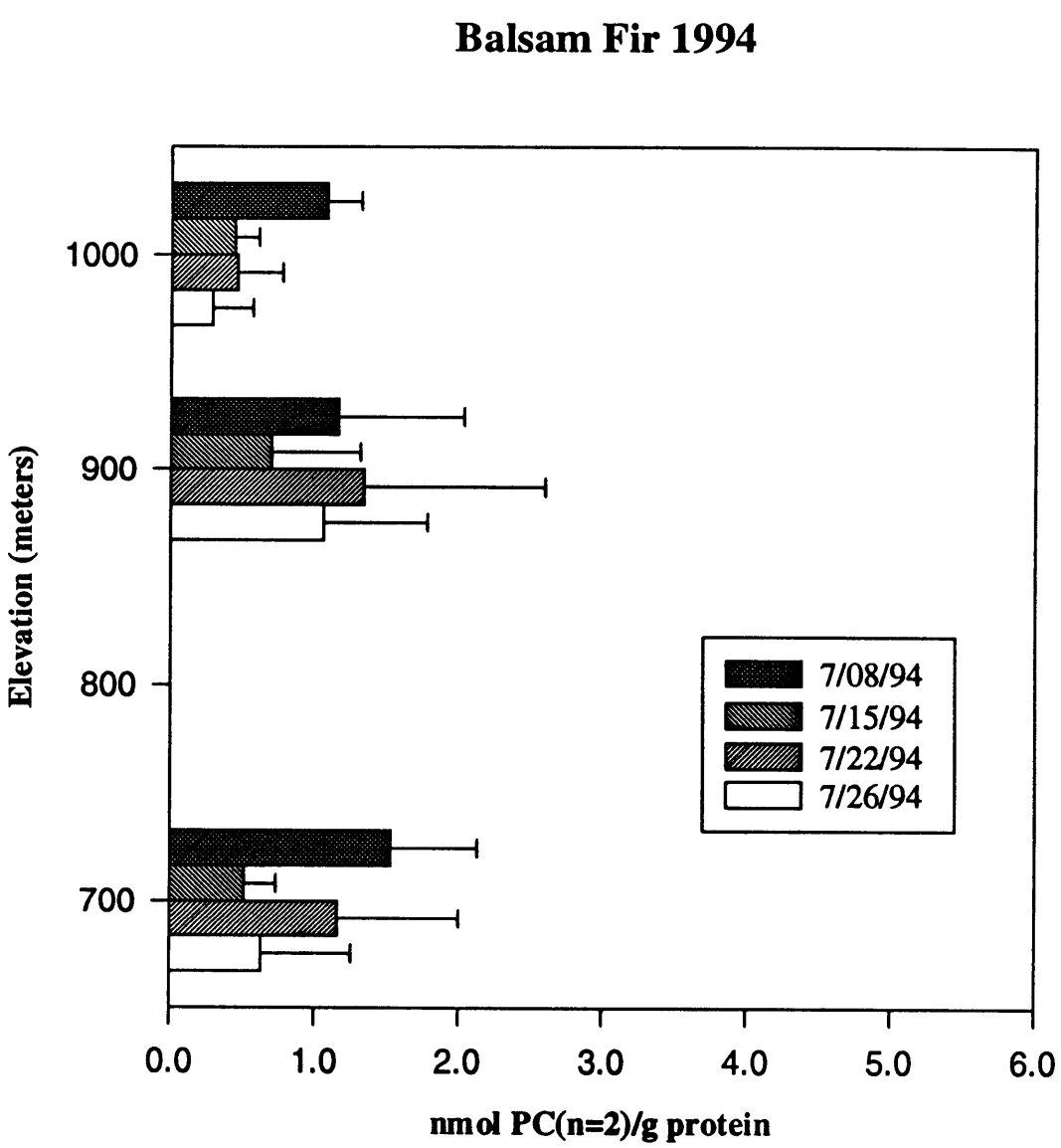

Figure 2.10: Phytochelatin concentrations normalized to protein, in foliage taken in 1994 from balsam fir trees at different elevations on Whiteface Mountain, NY.

from both years are plotted together ($P < 0.001$) [Figure 2.14]. Copper and lead exhibit weaker correlations while Zn shows none. Although lead concentrations appear much lower in 1995, values from 1994 were very near the detection limit of the ICP-AES instrument used, and are therefore suspect. Samples rinsed with ascorbic acid were not significantly different from

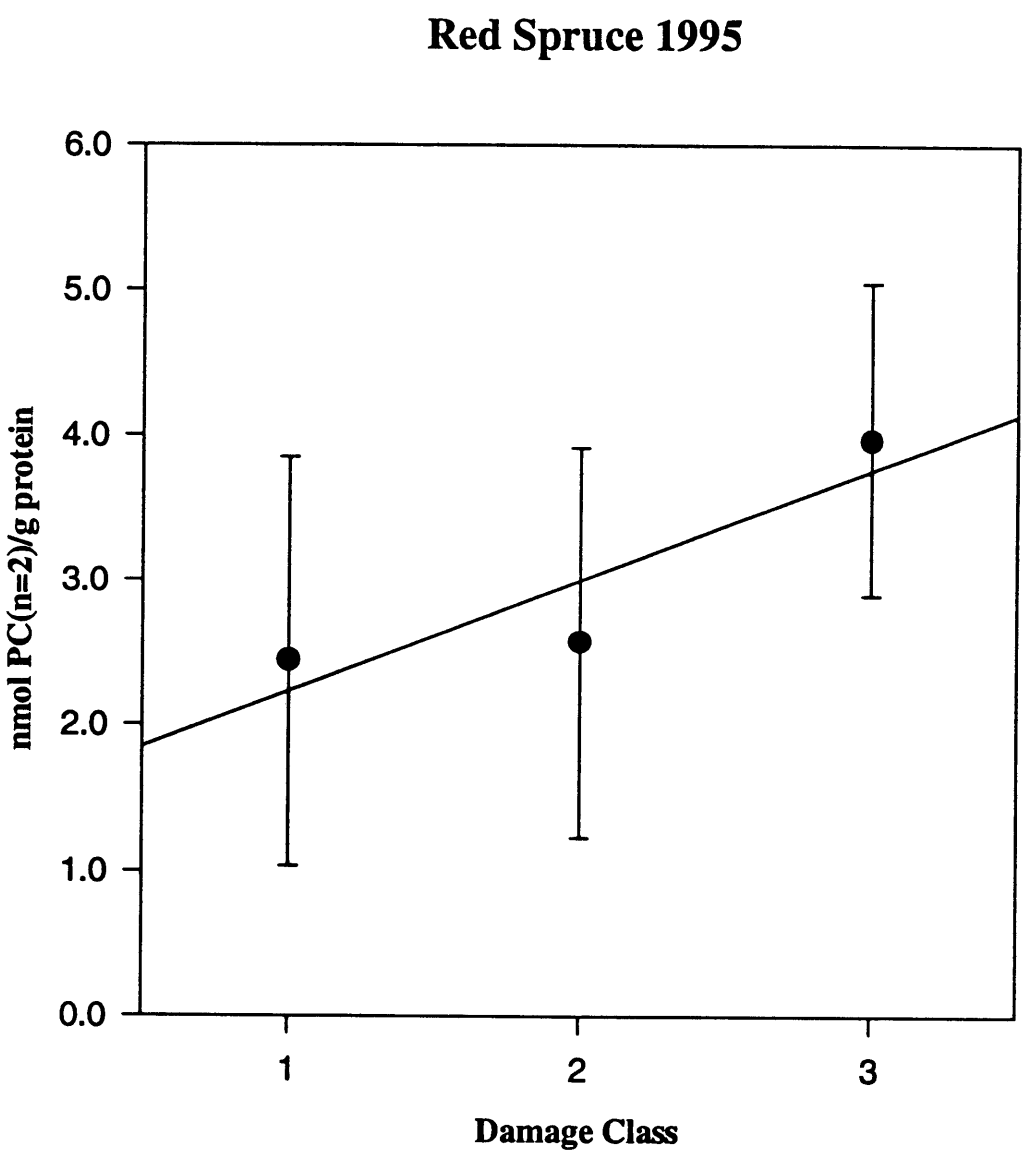

Figure 2.11: Phytochelatin concentrations normalized to protein as a function of damage classification in foliage taken in July, 1995, from red spruce trees at 1000 m on Whiteface Mountain, NY.

unwashed samples and both are included in Figures 2.13 and 2.14. Concentrations in balsam fir for all metals show no correlations with stand damage, and are lower than in red spruce for all metals except Zn [Figure 2.15]. Metal concentrations in red spruce and balsam fir along the elevational transect on Whiteface Mountain, however, showed no correlation with altitude in

either 1994 or 1995 [Table 2.1]. Possible reasons for the discrepancy between phytochelatin concentrations and metal levels with respect to elevation in 1994 are discussed later in this chapter.

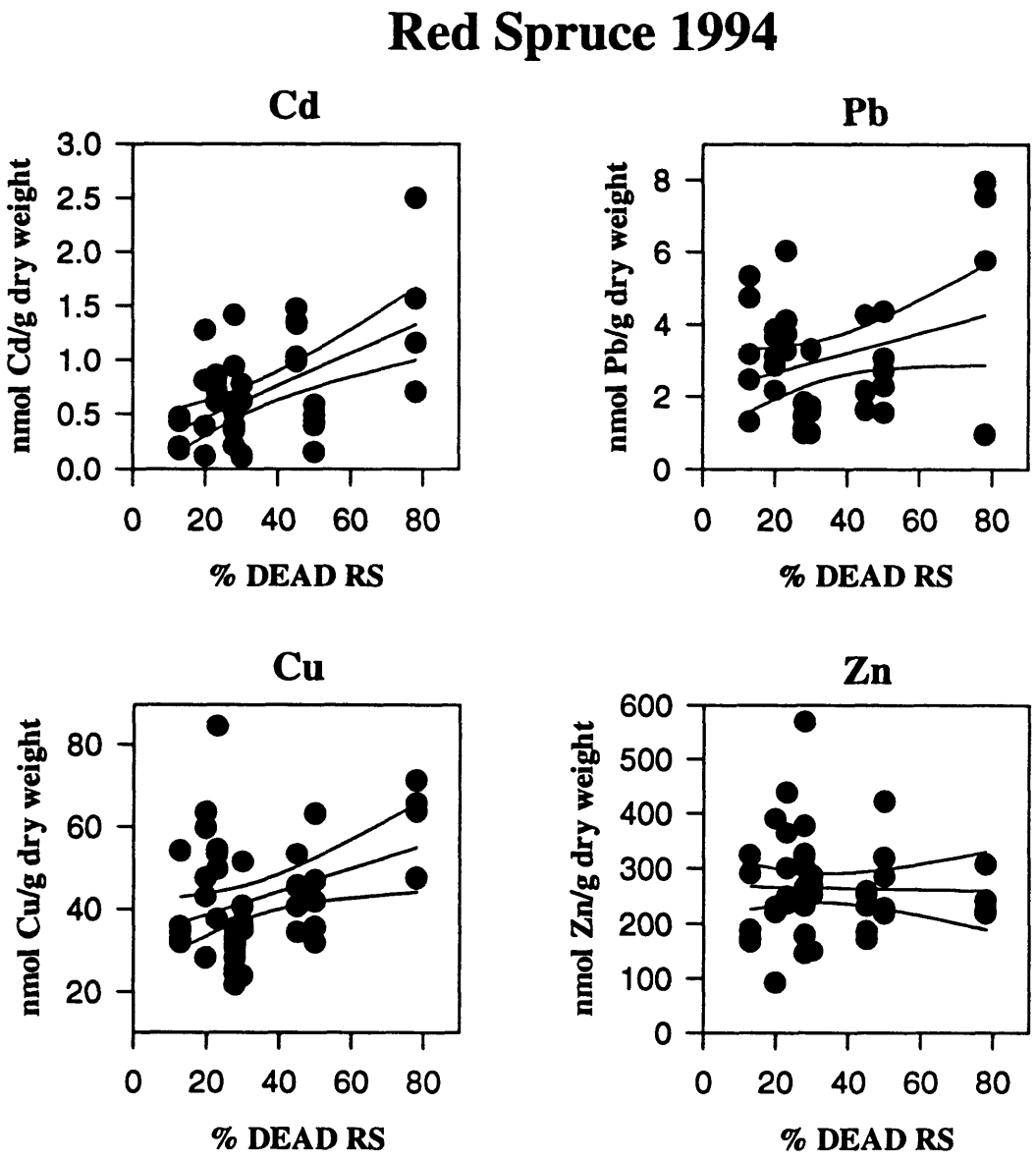

Figure 2.12: Metal concentrations normalized to dry weight, in foliage taken in 1994 from red spruce trees at 1000 m on nine different mountains in the Northeast, compared to percent standing dead red spruce in the stand.

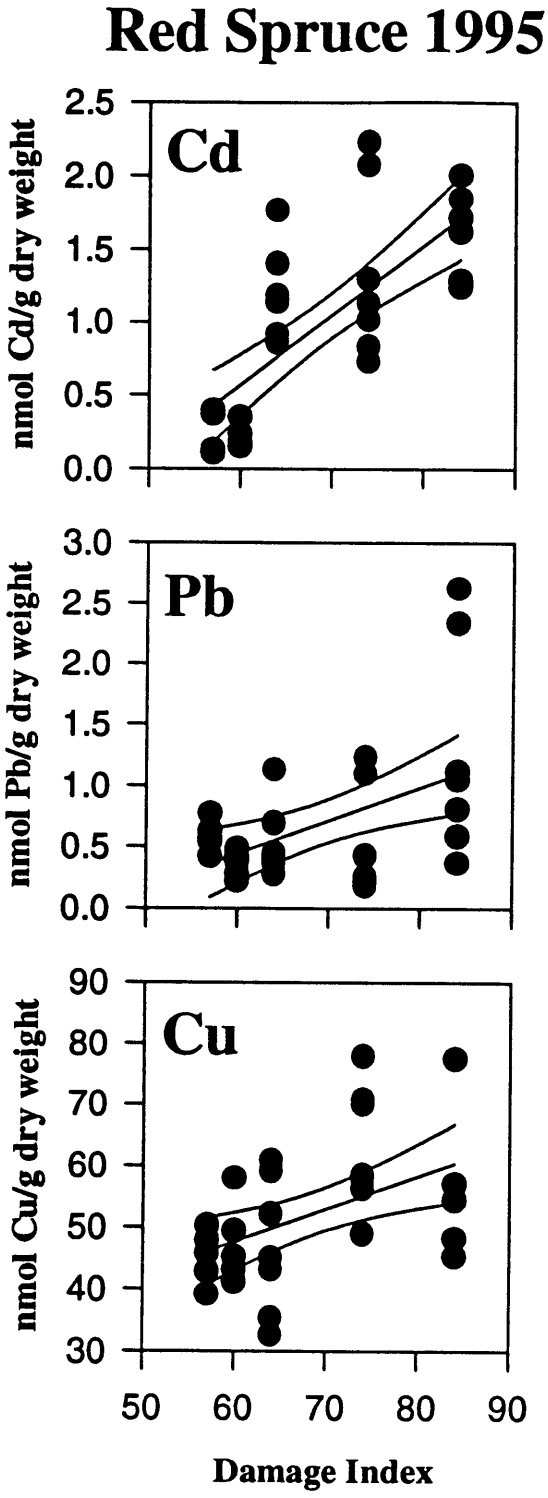

Figure 2.13: Metal concentrations normalized to dry weight, in foliage taken in 1995 from red spruce trees at 1000 m on five different mountains in the Northeast, compared to red spruce damage index in the stand.

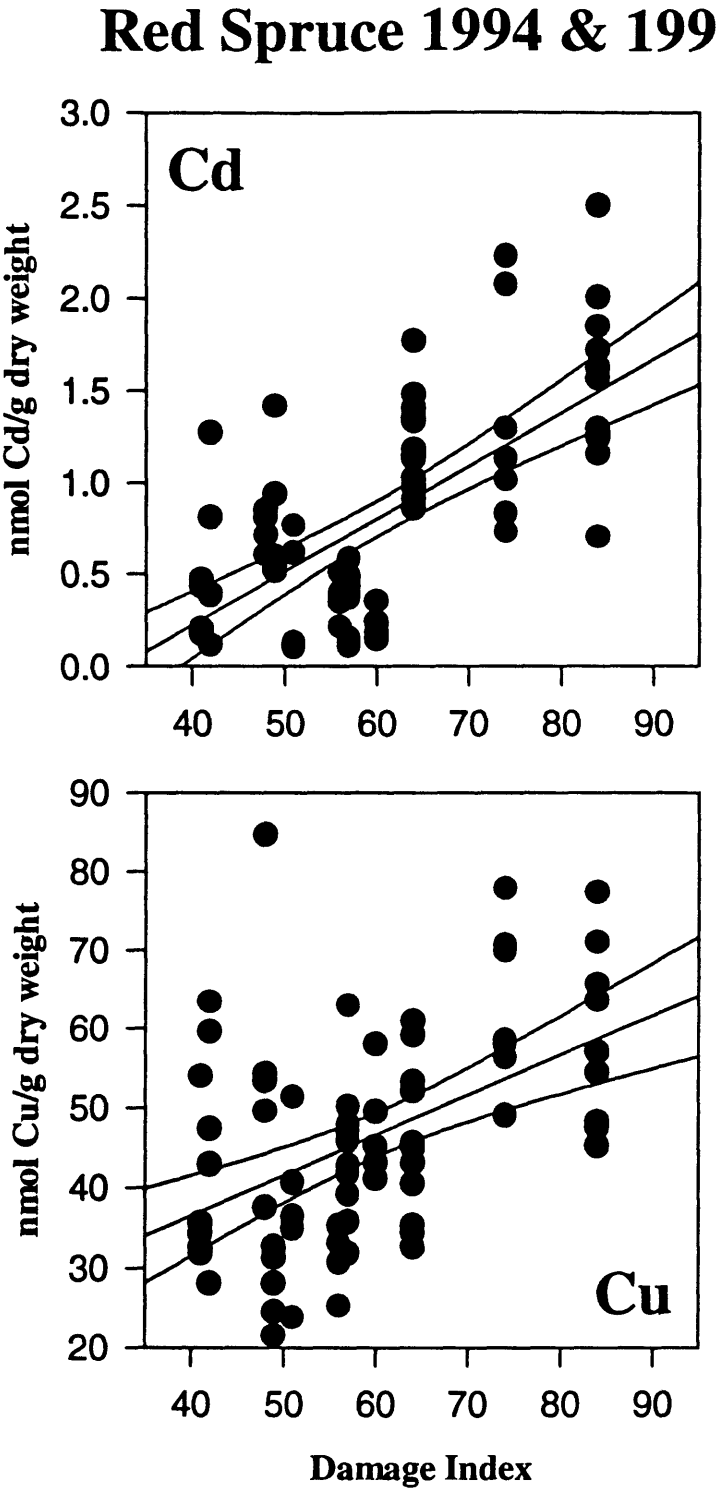

Figure 2.14: Metal concentrations normalized to dry weight, in foliage taken in 1994 and 1995 from red spruce trees at 1000 m on eleven different mountains in the Northeast, compared to red spruce damage index in the stand.

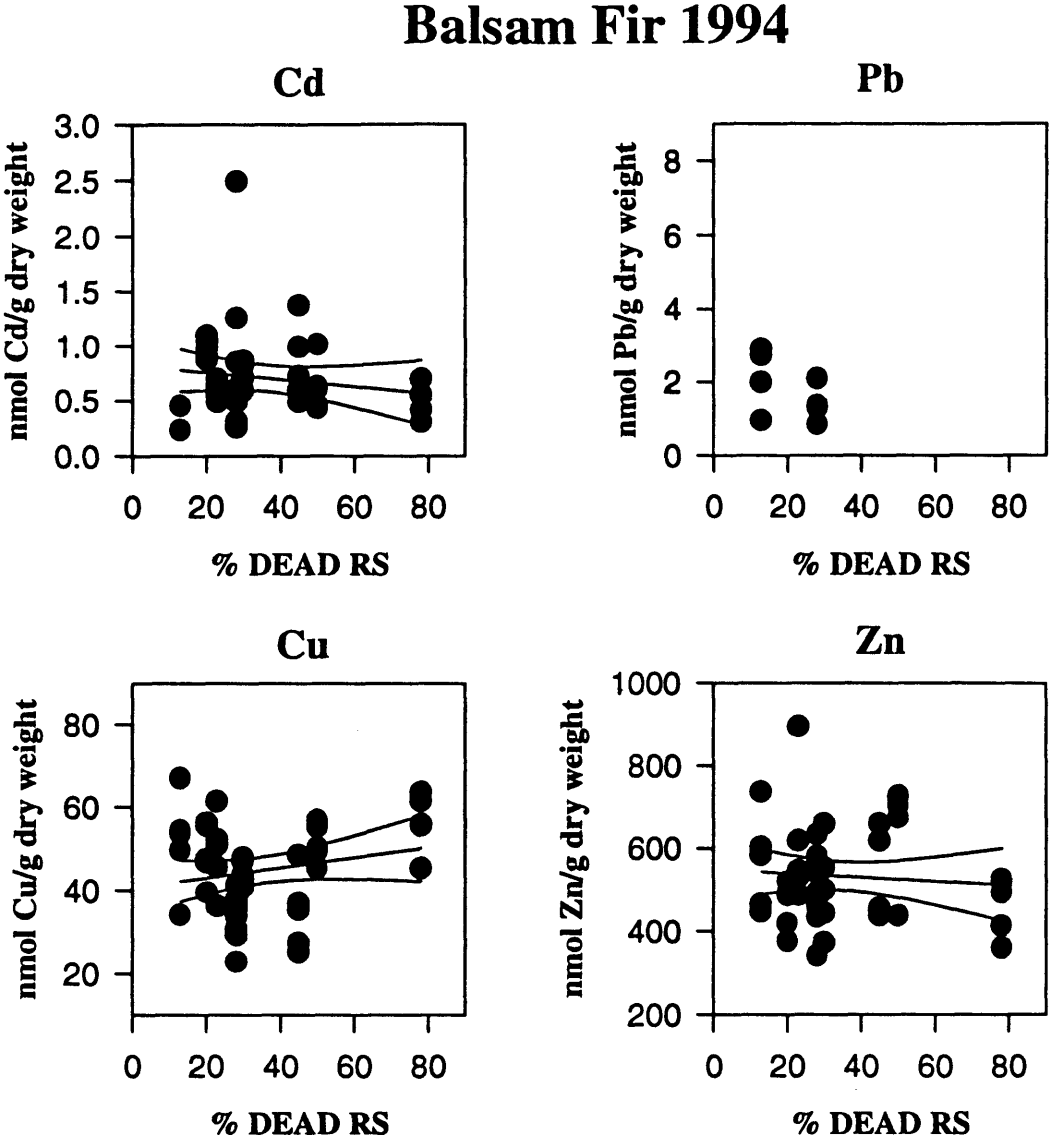

Figure 2.15: Metal concentrations normalized to dry weight, in foliage taken in 1994 from balsam fir trees at 1000 m on nine different mountains in the Northeast, compared to percent standing dead red spruce in the stand.

Discussion

It is apparent from previous research that concentrations of metals in foliage and soils correspond roughly to observed patterns of forest decline across the region. Smith and Siccama (1981) found

higher levels of Pb in needles of red spruce, the species in decline, than in balsam fir, a species which is not (Friedland 1989, Battles et al. 1992, Craig and Friedland 1991), and Johnson et al.

P. rubens	Cd (std.dev.)	Pb (std.dev.)	Cu (std.dev.)	Zn (std.dev.)
Elevation (m)	nmol/g dry wt.	nmol/g dry wt.	nmol/g dry wt.	nmol/g dry wt.
1994				
1000	0.41 (0.16)	2.78 (1.04)	43.9 (12.1)	294 (82)
900	0.70 (0.47)	4.44 (1.38)	55.7 (14.1)	352 (74)
700	0.34 (0.22)	4.50 (1.90)	52.6 (5.5)	285 (98)
1995				
1000	0.23 (0.14)	0.59 (0.13)	45.2 (4.3)	
900	0.63 (0.25)	0.71 (0.63)	52.1 (6.0)	
700	0.55 (0.40)	0.54 (0.35)	48.8 (4.7)	
A. balsamea	Cd (std.dev.)	Pb (std.dev.)	Cu (std.dev.)	Zn (std.dev.)
Elevation (m)	nmol/g dry wt.	nmol/g dry wt.	nmol/g dry wt.	nmol/g dry wt.
1994				
1000	0.62 (0.23)	N/D	51.3 (4.6)	651 (121)
900	0.09 (0.03)	1.89 (2.00)	53.3 (6.7)	616 (78)
700	0.95 (0.63)	2.59 (0.40)	77.5 (9.0)	797 (131)

Table 2.1: Metal concentrations normalized to dry weight, in foliage taken in 1994 and 1995 from red spruce and balsam fir trees at different elevations on Whiteface Mountain, NY.

(1982) measured increasing concentrations of lead with increasing elevation, as well as higher lead levels in the Green Mountains than in the White Mountains, corresponding to patterns of red spruce decline (Craig and Friedland 1991, Battles et al. 1992). Zinc, copper, nickel, and cadmium also exhibit similar distributions across the different mountain ranges in the Northeast (Friedland et al. 1986, Herrick and Friedland 1990). According to laboratory results extrapolated to field conditions, however, metal concentrations measured were for the most part considered too low to

have any effect (McLaughlin 1985). Several authors included metals among the possible culprits in red spruce decline, but generally placed them very low on the list when ranking potential causes by "strength of evidence" (Smith 1991, Hinrichsen 1987).

Our results from three years of sampling in the mountains of the Northeast are consistent, and point to one conclusion; metals probably play a role in red spruce decline. The use of phytochelatins as a direct measure of metal stress in the trees provides a physiological link between metal concentrations and red spruce health. Not only do metal concentrations correlate with red spruce damage, but the actual intracellular production of metal-detoxifying proteins also matches the same interspecies and geographical distributions.

First, concentrations of phytochelatins normalized to protein are higher in red spruce than in balsam fir. Phytochelatin concentrations in balsam fir also exhibit no trend with respect to elevation, geographical distribution, or damage indices. Thus, metals have no measurable effect on balsam fir trees in the mountains of the Northeast. At this point in our research we cannot say for certain what may be protecting balsam fir from metal toxicity. We did find, however, that metal concentrations measured in balsam fir needles are relatively constant in all forest stands [Figure 2.15]. In addition, levels of Cd in damaged stands are higher in red spruce [Figures 2.12] than in balsam fir, while levels of Zn, an essential trace element, are lower. This suggests that balsam fir may be able to selectively take up Zn without allowing toxic metals to enter, and that increased exposure to Cd in red spruce may be the result of non-specific metal uptake by zinc-deficient trees. McLaughlin et al. (1993) have suggested that calcium or magnesium deficiency

may be a factor in red spruce decline. Either of these deficiencies could also result in increased metal stress. With low concentrations of Ca and Mg available in the soil, non-specific Ca- or Mg-uptake pathways may transport higher levels of toxic metals into the tree. Boyce et al. (1991) also found that the needles of red spruce are more easily wetted than balsam fir needles. This may allow greater retention and uptake of metals from cloud droplets deposited on red spruce needles, thus leading to a greater pollutant insult in relation to balsam fir.

Second, concentrations of phytochelatins exhibit a strong correlation with elevation in 1994 [Figure 2.7]. It was found that the 1993 data produced only a weak correlation during mid-July [Figure 2.6], and the 1995 data showed consistently higher phytochelatin levels throughout most of the month at mid-elevation [Figure 2.8]. The lack of an elevational gradient in metal concentrations both in 1994 and 1995 [Table 2.1] suggests that it is not metal deposition that is changing. Thus, some other factor related to elevation controls metal uptake or bioavailability. One possibility that could account for this anomalous behavior is that metal uptake may have been depressed by some factor during the summers of 1993 and 1995. Palmer drought severity indices (PDSI), tabulated monthly by the National Climatic Data Center, a branch of NOAA, for the Whiteface Mountain area, classify July, 1993 as an "incipient drought" and July, 1995 as a "moderate drought." July, 1994 was classified as "incipient wet." However, rainfall data measured less than 100 ft. from the 1000 m site on Whiteface Mountain show that precipitation during the month of July, 1995 was much higher (17.8 cm) than in 1994 (9.3 cm), and rainfall in 1993 (7.88 cm) was only slightly lower [data courtesy of E.K. Miller and A.J. Friedland, Dartmouth College]. The PDSI is based on the principle of a balance between moisture supply

and demand, and it is possible that the few downpour events that made up much of the precipitation in July 1995 provided only temporary relief. Drier conditions in general may have decreased cloud formation at high elevations, thus decreasing metal dissolution and the subsequent transport of metal ions across the cuticle and into foliar cells (Tyree et al. 1990, Tyree et al. 1991, Hauser et al. 1993). It is also possible that the drier conditions may have resulted in the closing of the stomata. Since metals may be able to enter through the stomata directly (Buchauer 1973), this may have effectively decreased metal uptake by the foliage. Metal transport from the root zone would also have been curtailed by decreased transpiration rates.

Finally, a direct comparison between phytochelatin concentrations and red spruce damage at 1000 m across the region produced a very strong correlation ($P < 0.001$). Geographic distribution patterns of phytochelatins, with the highest values found in the Green Mountains and the lowest ones measured in the White Mountains, are also consistent with previous observations. As noted in the results, these measurements were made using only red spruce trees with little or no external damage (i.e. damage class 1). Thus, increasing metal stress precedes damage to individual trees. We also found that phytochelatin concentrations in red spruce from different damage classes were not significantly different. Therefore, all new red spruce foliage in the stand exhibits the same degree of metal stress initially. It follows that increasing phytochelatin concentrations are not simply a result of red spruce damage, rather they indicate that all trees in damaged areas have been subjected to increased metal stress. In addition, metal measurements in red spruce foliage hint that cadmium may be the metal causing problems. The ratio of phytochelatin-sulfhydryl binding groups to total cadmium in the foliage is approximately 1:1, close to the 2:1 ratio found

by Loeffler et al. (1989) for cell suspensions of *Silene cucubalus*. Since rinsing with ascorbic acid did not affect this measurement, it seems likely that most of the cadmium is inside the cell, either bound to phytochelatins or stored in the vacuole. However, it is difficult to conclude that this is the case without further experiments.

A consensus is now emerging that multiple causative factors are involved in forest decline, probably in conjunction with freezing injury (Friedland et al. 1984, Johnson et al. 1992, Sheppard 1994). Metal toxicity is likely to act synergistically with a number of pollutants to decrease the overall health and vigor of red spruce at high elevation. For example, oxidant damage, recently shown to be a contributing factor in the reduced growth of loblolly pine in the southeastern U.S. (McLaughlin and Downing 1995), has also been implicated in red spruce decline in northeastern forests (Madamanchi et al. 1991, Vann et al. 1992, Taylor et al. 1994). Ormrod (1977) documented synergistic effects between cadmium and ozone in crop plants. Doulis et al. (1993) also found that an increased antioxidant response in red spruce trees, which may be caused by ozone exposure (Hausladen et al. 1990), may decrease winter hardiness. It is known that depletion of glutathione, a component of plant antioxidant defense systems, from the production of phytochelatins results in an increased sensitivity to oxidative damage in plants (de Vos et al. 1992). By this mechanism ozone and other oxidants might act synergistically with metals to decrease the health of red spruce by competing for the same resources required for detoxification of these pollutants. Christie and Costa (1984) found that Cd, Pb, and Hg can form very stable complexes with glutathione, interfering with the conversion of oxidized to reduced forms, and decreasing available antioxidant pools. However, using glutathione measurements from Doulis et

al. (1993), we find that phytochelatin production in red spruce would deplete only about one percent of the available glutathione pool. It is unknown whether this is true for all sites, or if under certain conditions this synergism may still be important.

There is also evidence that metals and oxidants will act synergistically with acid precipitation to increase the potential damage to trees at high elevation. Photochemistry in aqueous cloud droplets produces a series of reactions that may increase the availability and concentration of metals and oxygen radical species (Faust 1994). Low pH in cloud droplets dissolves airborne particles, creating high concentrations of metals in solution. These in turn can act as catalysts in chain reactions in the light which produce oxygen radicals in the presence of organic compounds. Berresheim and Jaeschke (1985) also showed that metals such as copper can act as catalysts in the conversion of SO_2 to sulfuric acid in atmospheric droplets. Thus aqueous phase reactions in cloud and rainwater may create high concentrations of oxidants, acids, and metals, which are then deposited to foliar surfaces in bioavailable forms (Thornton et al. 1994).

It is difficult to interpret from phytochelatin concentrations the amount of damage that is a result of their production. In the stressful living environment found at high elevations in northern climates, it is probable that any demand on energy or cellular materials, other than for growth or reproduction, would make survival more difficult. The losses of energy (in the form of ATP), carbon, nitrogen, and sulfur due to phytochelatin production in red spruce are likely inconsequential in relation to total cellular levels. As stated above, total glutathione concentrations are also unlikely to be significantly affected by their use in building phytochelatin

molecules. Further work is required to elucidate the possible strain that this level of phytochelatin production may represent.

It may be that direct metal toxicity, signaled by phytochelatin levels in red spruce, is having a greater physiological impact than the drain it causes on energy and nutrients allocated for phytochelatin production. Because Cd, the most likely metal culprit, strongly binds to sulfhydryl groups, it is able to interfere in normal protein function by forming disulfide bridges, oxidizing free sulfhydryl groups, and replacing metal cofactors (such as Zn, Ni, Cu, and Co) in protein reaction centers. For example, Vangronsveld and Clijsters (1994) list nitrate reductase as "...one of the most affected enzymes by metals." As shown by Sheppard (1994), decreased nitrogen assimilation may exacerbate sulfate-induced damage in red spruce, which require excess N for the formation of reduced-sulfur compounds. In the presence of low N levels, as may be caused by Cd poisoning of nitrate reductase, sulfate toxicity may lead to increased winter damage. Measurements of activities for proteins that are particularly susceptible to Cd toxicity need to be examined in field experiments in the future. Cadmium may also cause increased cell membrane permeability (Vangronsveld and Clijsters 1994), which may add to the impact caused by winter damage. Again, field measurements are necessary to determine if this is an important mechanism in high elevation forests. Experiments utilizing environmentally relevant concentrations of metals over longer time-scales are lacking in the literature. Experimental design should include the examination of possible synergistic effects as well.

Another possible mechanism for Cd toxicity is related to carbon deficiency, a condition which may

exist in red spruce at high elevations (McLaughlin and Kohut 1992). As stated by Vangronsveld and Clijsters (1994), "photosynthesis probably is one of the most metal-sensitive processes of plant metabolism." These authors document numerous studies that show Cd may interfere with the synthesis of chlorophyll, the electron transport chain, and photophosphorylation. Our measurements show lower Zn concentrations in red spruce [Figure 2.12] than in balsam fir [Figure 2.15], which may lead to Cd substitution in metalloenzymes – of which there are many involved in photosynthesis – resulting in inactive complexes and reduced carbon assimilation. Carbon deficiency might also provide an explanation for the results shown in Figure 2.11. Increased damage to individual trees is likely to increase metal intrusion into foliar cells through membrane disruption. However, as damage class increased phytochelatin levels remained about the same. Thus, red spruce trees may be unable to respond to the increased metal insult because of a lack of carbon available for allocation to detoxification processes, an idea stated by Sheppard (1994).

In conclusion, it seems likely that forest decline in the northeastern United States is the result of synergistic effects involving toxic metals, other air pollutants, and natural environmental factors. Elevated concentrations of phytochelatins provide prima facie evidence that trace metal stress is contributing to red spruce decline. Thus, abatement of atmospheric metal pollution should probably be considered as one of the elements of a policy designed to reverse forest decline in this region. Further, phytochelatin measurements in other forested regions of the world exhibiting dieback may allow us to determine whether the role of metals in forest decline is a general phenomenon or limited to the northeastern United States.

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Chapter 3

Metal Stress Near Sudbury, Ontario, Canada: Current Atmospheric Deposition Versus Historic Soil Contamination

Introduction

Sudbury, Ontario, is located approximately 60 km northeast of Lake Huron on the Canadian Shield geologic formation in Central Ontario. In the late 1800's commercial metal ore deposits were discovered there which, with the building of the Canadian Pacific Railroad in 1883, were intensively mined to feed the explosive industrial growth occurring in North America and Europe. These mines have produced enormous amounts of copper and nickel, but iron, cobalt, gold, silver, and selenium have also been extracted. [This and other background material below concerning the history of Sudbury, Ontario, are excerpted from Courtin (1994), and Freedman (1995).]

The smelting methods used originally to reduce the sulfide-laden ore were highly inefficient and very slow. Open-bed roasting, used until it was prohibited in the late 1920's, consisted of filling large pits with layers of metal ore and wood taken from the surrounding forests, and igniting the pile. These usually burned for months, creating ground-level smoke with high concentrations of sulfur dioxide (SO_2) and metals. Damage to the surrounding vegetation from these pollutants was severe, creating a barren wasteland in the vicinity (3-8 km radius) of the pits (Amiro and Courtin 1981).

Following the ban on open-bed smelting, emission stacks of various heights were put into operation at the smelters to disperse and dilute toxic gases and aerosols over a larger area. However, severe fumigation conditions continued to occur because of local meteorological instabilities forming near the ground (Dreisinger and McGovern 1970). In 1972, along with other

improvements to reduce emissions, a 381 m "superstack" was completed at INCO's (International Nickel Company Limited) Copper Cliff plant in an attempt to improve ground-level air quality in the Sudbury area. The tremendous height of this stack was designed to emit the pollutant plume high enough to avoid any near-ground inversions. In addition, this was also an altitude where instability would help disperse the plume above, but also where a stable layer below would keep the plume from reaching the ground, thus lofting conditions would dominate. However, this has not been altogether successful since unstable looping conditions still occur frequently in the summer months, creating episodes where the plume impinges the ground directly only 3-10 km downwind (Chan and Lusis 1985).

At the same time that the "superstack" was brought on-line, INCO ceased operations at its Coniston smelter, 8 km east of Sudbury, and incorporated all production at its Copper Cliff facility, 8 km west of Sudbury. Falconbridge Nickel Mines Limited operates the only other smelter in the area, in the town of Falconbridge, 16 km northeast of Sudbury. However, the Falconbridge smelter emits an insignificant amount of metal particles in comparison to INCO's Copper Cliff facility [e.g. 11 tons Cu/yr. versus 245 tons/yr., respectively, averaged from 1973 to 1981], and will be disregarded in further discussions and calculations (Chan and Lusis 1985). Although there are several emission stacks at the Copper Cliff smelter, the "superstack" is the primary venting stack and subsequently the most important source of metal pollution in the Sudbury area at present.

At the peak of production, from 1965 to 1970, these smelters emitted more than 4% of the

world's anthropogenic SO_2, making Sudbury the largest single source at that time (Freedman 1995). Along with SO_2, large amounts of metal particles were released into the surrounding area. The era of open-bed smelting and the subsequent switch over to relatively short stacks with insufficient dispersal capabilities created a distinct pattern of metal concentrations in the surrounding soils. Due to the nature of these early smelting methods prior to 1972, the highest concentrations of metals, measured in litter and surface soils in the 1970's, occurred immediately adjacent to the smelting facilities, and concentrations decreased rapidly with distance from the stack (Freedman and Hutchinson 1980).

Sudbury is an ideal site for the investigation of metal pollutant dispersal and toxic response in vegetation. Phytochelatin measurements are well suited to the task of mapping bioavailable metal concentrations in the Sudbury area, as well as indicating the magnitude of metal stress exhibited in the surrounding flora, for two reasons. First, both Cu and Ni, the primary smelting targets, and several others co-emitted during the smelting process (Cd, As, Pb, Zn and Ag), may induce the production of phytochelatins when taken up by plants in excess of what is required for normal cell metabolism and growth. Second, the recolonization of previously denuded land around Copper Cliff provides continuously available vegetation for sampling along transects radiating from the "superstack."

Methods

A transect was established for sampling following Highway 144 in a roughly northwest direction

away from the "superstack" [Figure 3.1]. This direction was chosen by Bagatto and Shorthouse (1991) because (1) it was perpendicular to aerial deposition isopleths, (2) easily accessible, and (3) avoided the Sudbury urban area. Six sites were located between 1 and 59 km, and named according to nearby landmarks [base (1 km), Rayside (15 km), Dowling (23 km), Windy Lake (32 km), Cartier (44 km), and Halfway Lake (59 km)]. One other "control" site was located at Harp Lake (170 km southeast of Sudbury) in 1993, and at Killarney Provincial Park (65 km southwest of Sudbury) in 1994 and 1995. Both "control" sites should be out of the zone of influence for emissions from Sudbury. All sites were at least 0.5 km from the highway to minimize the influence of automobile exhaust.

In May of 1993, foliage samples were taken by hand from the north and south sides of three trees at every site. However, repeat sampling in October did not include the base and Rayside sites. *Betula papyrifera* (paper birch) trees were sampled at all locations in May and October, with the exception of the Harp Lake site where *Populus alba* (white poplar) was sampled in May. All samples were placed in separate cryovials, immersed immediately in liquid nitrogen contained in a portable dewar, and sent to MIT for analysis.

Sampling was repeated in 1994 at the same stations along the northwest transect, but the "control" site was relocated as described above. This year three trees each of three different species – *B. papyrifera*, *Abies balsamea* (balsam fir), and *Populus tremuloides* (quaking aspen) – were sampled at each site in order to examine the possibility of interspecies differences in response to emissions. One sample for each tree was pooled from north and south aspects in

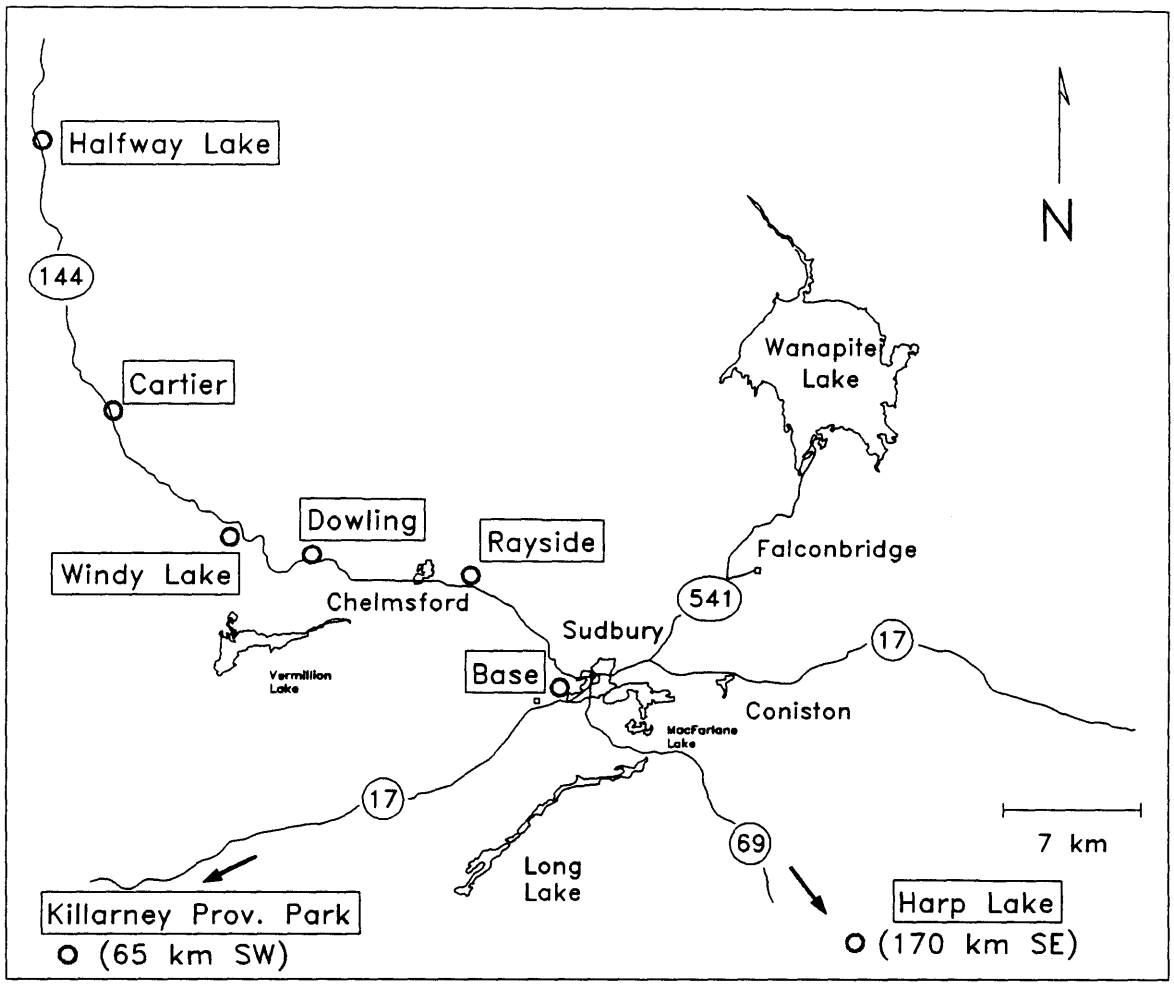

Figure 3.1: Map of sampling locations in the Sudbury, Ontario, area.

May, June, and August, and stored and shipped as in 1993.

Three potted two-year-old *B. papyrifera* saplings, taken from nursery stock, were placed at each of these same sites on June 27, 1994, and again on June 22, 1995. Samples were collected during placement and again on August 10, 1994, and August 11, 1995. Saplings were placed on the ground's surface at the sites such that there was greater than 95% light transfer (using a ceptometer) at the point of placement, and greater than 90% light transfer in a 15 ft radius. This was necessary to insure similar exposure conditions for potted trees at all sites. Trees were subject to atmospheric deposition and precipitation, but not the influence of contaminated soils or groundwater. In 1994 foliage samples were taken as described above for trees growing *in situ* in 1993. In 1995 two samples per tree and three samples per tree were collected on June 22 and August 11, respectively.

In 1995 crude filters for atmospheric metals were added to the potted trees. These consisted of approximately two inches of packed commercial peat moss placed on top of the soil in the pots and held in place by a screen. A side drain was also included on each pot so that excess water would be allowed to run off. Samples of the peat moss used in 1995 were collected in August and placed in cryovials in liquid nitrogen for shipping and storage, then dried in an oven for three days at 80°C. Metals were extracted from approximately 0.1 g peat moss by shaking overnight in 5 ml 0.12 N HCl (Mallinckrodt AR Select). Extracts were centrifuged at 27,000 rpm (approximately 1080g) for 30 min, filtered through 0.2 μm syringe filters (Acrodisk), and stored in the refrigerator. These extracts were analyzed for Cu and Ni using a Perkin-Elmer 4100ZL

graphite furnace atomic absorption spectrophotometer. Handling of samples during analysis produced insignificant contamination for both metals as attested to by method blanks (~5 ppb Cu and undetectable Ni). Concentrations of Cu and Ni in peat controls (i.e. prior to placement in the pots at Sudbury) were 12.7 ± 1.8 and 11.3 ± 0.5 nmol/g dry peat respectively.

Grinding, homogenization, extraction, and analysis methods for phytochelatins and total acid-soluble protein of these foliage samples are described in Chapter 2 and Appendix A. Standardization to protein was carried out for all samples, and wet weight measurements were added in 1994 and 1995.

Results

For trees in the Sudbury area there are two potential sources of metal pollution. Early smelting practices deposited high concentrations of metals adjacent to the roasting pits and early smelter facilities. However, current smelting practices at the INCO facility in Copper Cliff emit metal pollutants high above the ground, thus shifting metal deposition farther away. Our results in 1993 and 1994 for trees growing *in situ* support the hypothesis that current metal emissions have a greater impact. In the Spring of 1993, phytochelatin concentrations normalized to protein in *B. papyrifera* peaked ($P < 0.05$) at 32 km away from the "superstack" [Figure 3.2]. Near the end of the growing season in October of that year, while leaves were still green, phytochelatin levels decreased to a consistently low concentration (~2.6 nmol phytochelatin (n=2)/g protein) throughout the transect. The following year, all three species sampled produced similar results.

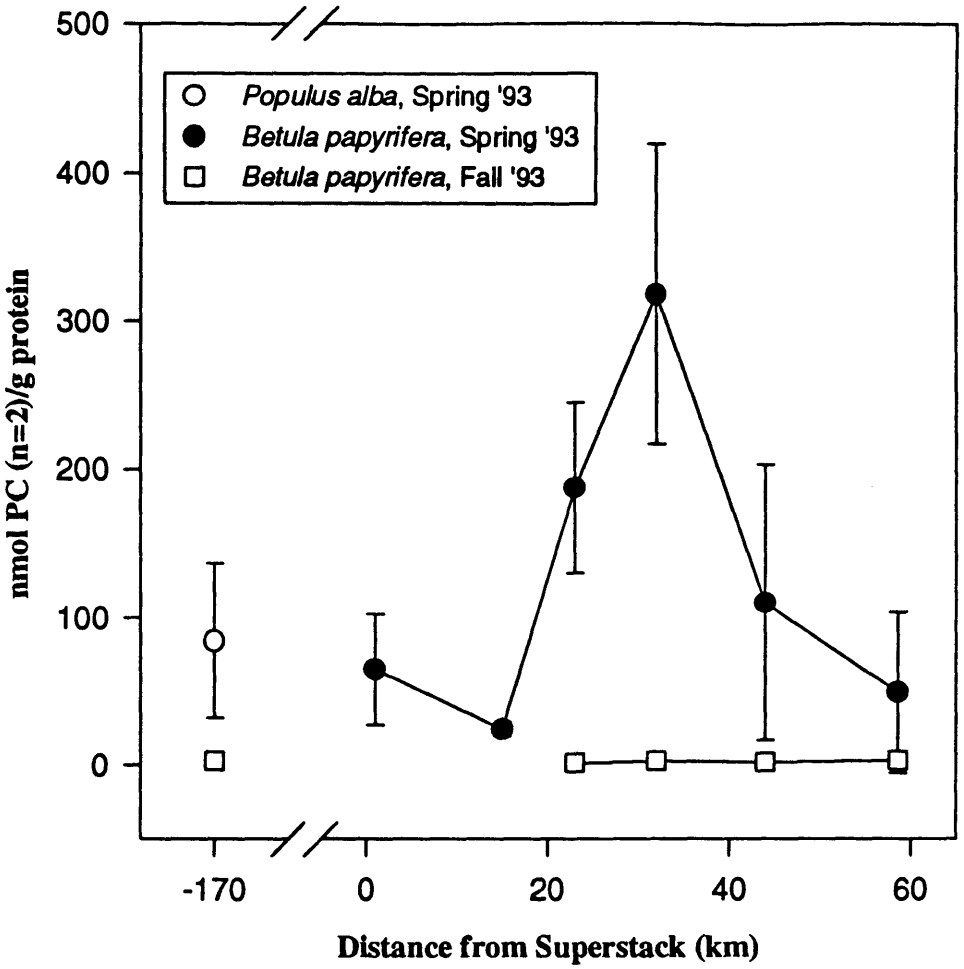

Figure 3.2: Phytochelatin concentrations normalized to protein in trees growing *in situ* along a NW(+)/SE(-) transect away from Sudbury, Ontario, Canada, in 1993.

In May 1994, phytochelatin concentrations normalized to protein peaked in *B. papyrifera* at the 23 and 32 km sites ($P < 0.05$) [Figure 3.3]; in *A. balsamea* at the 32 km site ($P < 0.001$) [Figure 3.4]; and in *P. tremuloides* at the 15, 23, and 32 km sites ($P < 0.001$) [Figure 3.5]. This year, elevated phytochelatin levels were also detected in May at the 59 km site for all species. From May to June concentrations generally decreased throughout the transect for all species [Table 3.1], with the exception of *A. balsamea* at the 59 km site, which still showed high phytochelatin

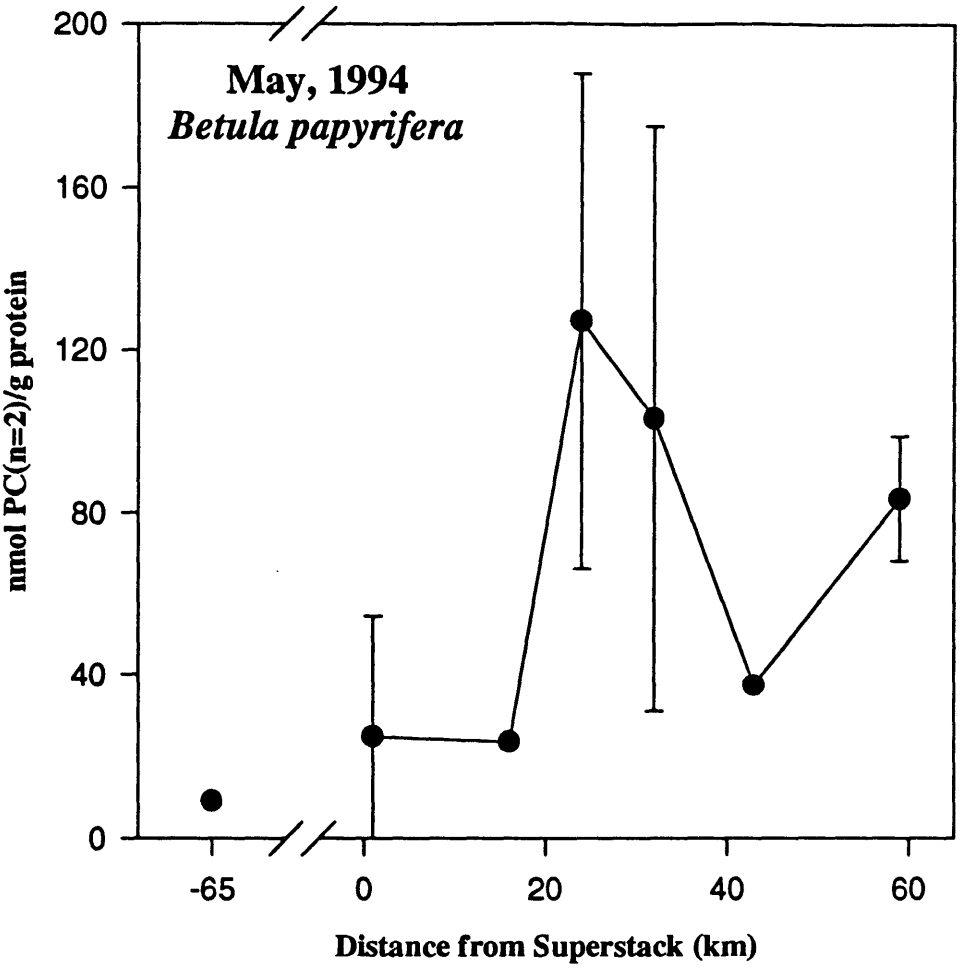

Figure 3.3: Phytochelatin concentrations normalized to protein in paper birch trees growing *in situ* along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1994.

levels. Phytochelatin levels remained low both in 1993 and 1994 for all species sampled at the "control" sites, Harp Lake and Killarney Provincial Park. Normalizing to wet weight measurements in 1994 produced similar results [Table 3.1]. It became apparent after analysis that foliar browning in August 1994 samples interfered with normalizations to wet weight and protein as well as phytochelatin analysis. It is unknown what may have brought on early senescence that year, but it was necessary to discard these results for August. In any case, it is clear from the

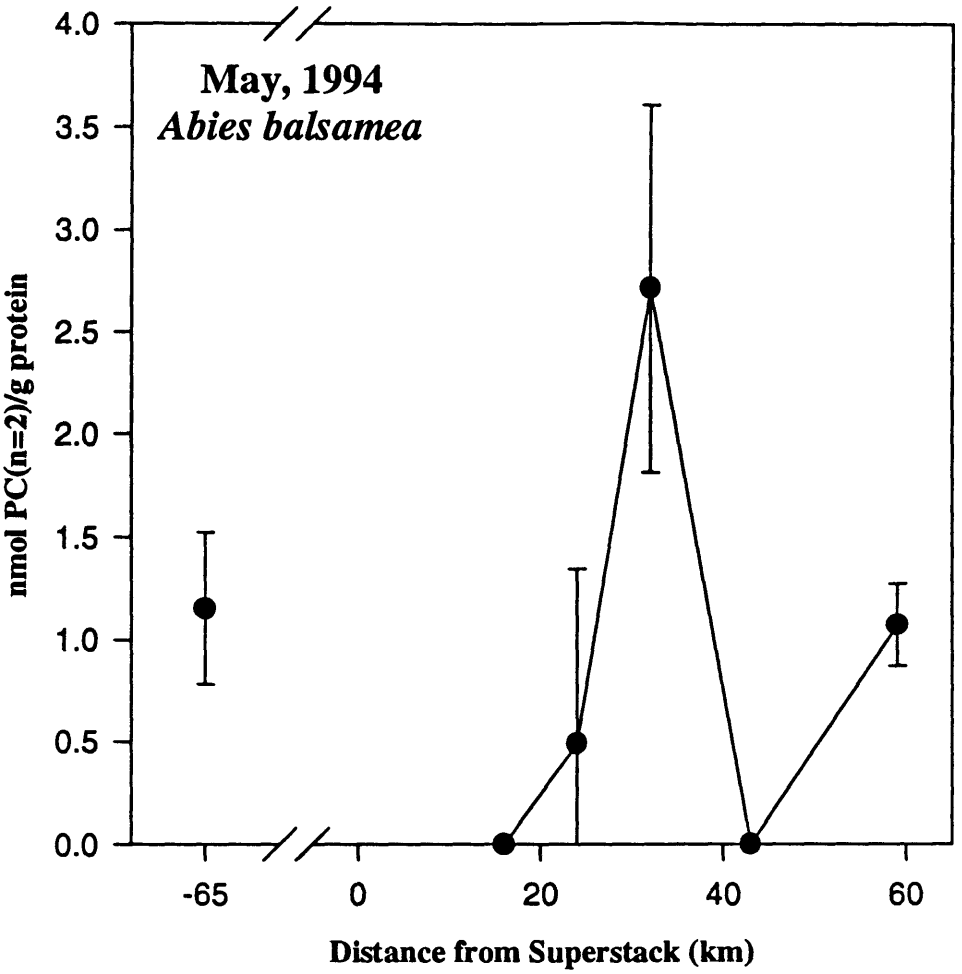

Figure 3.4: Phytochelatin concentrations normalized to protein in balsam fir trees growing *in situ* along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1994.

results above that higher phytochelatin concentrations are produced away from the "superstack" in all species sampled, suggesting that current atmospheric deposition, not soil contamination, is the more important source of bioavailable metals.

In order to exclude the possibility that changes in soil complexation characteristics or adaptation pressures might be responsible for an anomalous result in trees growing *in situ*, a potted tree

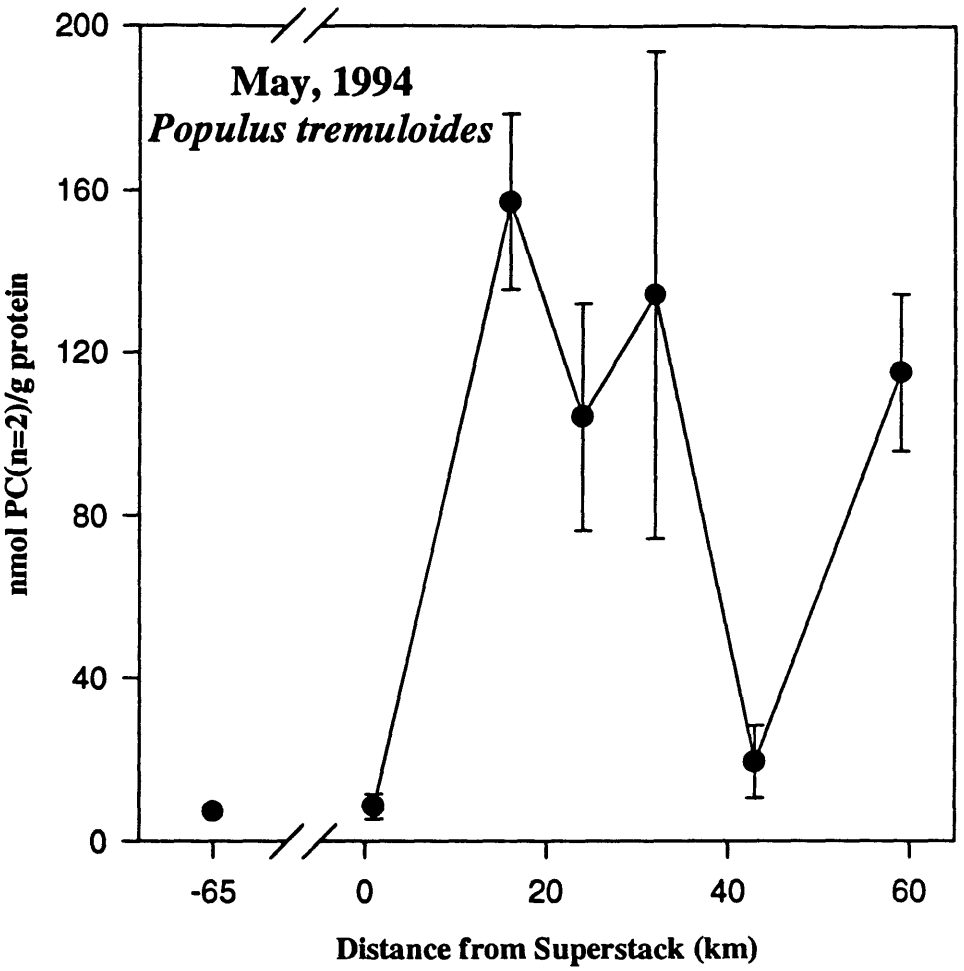

Figure 3.5: Phytochelatin concentrations normalized to protein in quaking aspen trees growing *in situ* along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1994.

experiment was carried out in 1994. This experiment was designed to exclude the influence of historical soil contamination and to investigate separately the role of current atmospheric metals deposition on the phytochelatin response measured in foliage. After a month and a half, phytochelatin concentrations in the foliage of potted trees had increased at all sites, but the largest values occurred at 44 km away from the stack [Figures 3.6a and 3.6b]. Normalization to both protein ($P = 0.12$) [Figure 3.6a] and wet weight ($P = 0.06$) [Figure 3.6b] again produced similar

B. papyrifera					
May-94	distance (km)	mol PC(n=2)/g wet wt.	std.dev.	mol PC(n=2)/g protein	std.dev.
Killarney Park	-65	1.78E-09	9.58E-11	9.04E-09	1.18E-09
Base	1	6.20E-09	3.12E-09	2.49E-08	2.96E-08
Rayside	16	5.88E-09	3.61E-09	2.36E-08	9.13E-10
Dowling	24	2.00E-09	1.36E-10	1.27E-07	6.08E-08
Windy Lake	32	4.19E-09	1.69E-09	1.03E-07	7.20E-08
Cartier	43	6.66E-10	1.02E-10	3.74E-08	6.92E-10
Halfway Lake	59	1.50E-09	1.66E-09	8.34E-08	1.54E-08
Jun-94					
Killarney Park	-65	4.64E-10	1.71E-10	3.00E-09	7.75E-10
Base	1	4.98E-10	1.09E-10	2.97E-09	8.96E-10
Rayside	16	1.52E-09	2.01E-09	1.78E-08	2.66E-08
Dowling	24	3.86E-10	1.62E-10	1.73E-09	7.01E-10
Windy Lake	32	3.25E-10	3.13E-11	1.52E-09	2.75E-10
Cartier	43	5.91E-10	3.12E-11	8.44E-09	2.52E-09
Halfway Lake	59	4.31E-10	1.04E-10	1.68E-09	5.17E-10
A. balsamea					
May-94	distance (km)	mol PC(n=2)/g wet wt.	std.dev.	mol PC(n=2)/g protein	std.dev.
Killarney Park	-65	1.77E-10	6.43E-11	1.15E-09	3.73E-10
Base	1	N/M	N/M	N/M	N/M
Rayside	16	N/D	N/D	N/D	N/D
Dowling	24	5.72E-11	9.91E-11	4.91E-10	8.51E-10
Windy Lake	32	5.29E-10	2.15E-10	2.71E-09	8.98E-10
Cartier	43	N/D	N/D	N/D	N/D
Halfway Lake	59	1.55E-10	2.03E-11	1.07E-09	2.00E-10
Jun-94					
Killarney Park	-65	1.41E-10	1.88E-10	2.93E-09	4.10E-09
Base	1	N/M	N/M	N/M	N/M
Rayside	16	1.24E-08	3.37E-09	2.31E-07	1.23E-07
Dowling	24	N/D	N/D	N/D	N/D
Windy Lake	32	N/D	N/D	N/D	N/D
Cartier	43	N/D	N/D	N/D	N/D
Halfway Lake	59	4.64E-10	1.52E-10	3.32E-09	1.02E-09
P. tremuloides					
May-94	distance (km)	mol PC(n=2)/g wet wt.	std.dev.	mol PC(n=2)/g protein	std.dev.
Killarney Park	-65	4.86E-10	7.46E-11	7.20E-09	1.02E-09
Base	1	5.39E-10	1.93E-10	8.33E-09	3.02E-09
Rayside	16	5.49E-09	5.86E-10	1.57E-07	2.17E-08
Dowling	24	3.85E-09	6.99E-10	1.04E-07	2.77E-08
Windy Lake	32	5.47E-09	2.09E-09	1.34E-07	5.97E-08
Cartier	43	8.79E-10	4.11E-10	1.93E-08	8.84E-09
Halfway Lake	59	3.48E-09	6.41E-10	1.15E-07	1.92E-08
Jun-94					
Killarney Park	-65	7.40E-10	5.75E-09	2.32E-10	3.97E-10
Base	1	2.27E-09	1.03E-08	2.34E-09	1.08E-08
Rayside	16	1.67E-09	1.41E-08	8.35E-10	9.90E-09
Dowling	24	1.49E-09	9.53E-09	3.06E-10	1.63E-09
Windy Lake	32	1.25E-09	1.18E-08	1.78E-10	1.91E-09
Cartier	43	3.93E-09	4.12E-08	1.47E-09	2.62E-08
Halfway Lake	59	6.29E-10	6.25E-09	2.05E-10	3.26E-09

Table 3.1: Phytochelatin concentrations normalized to protein and wet weight in trees growing *in situ* along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in May and June, 1994.

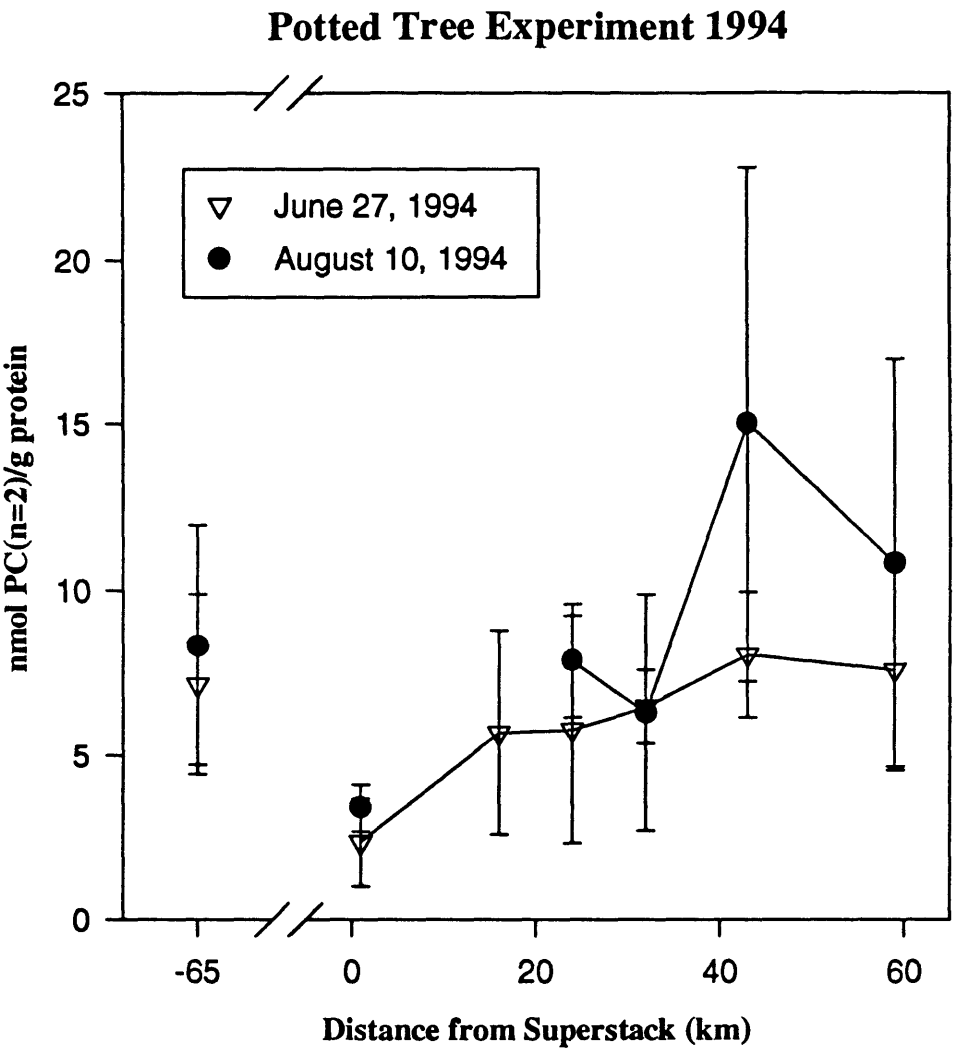

Figure 3.6a: Phytochelatin concentrations normalized to protein in potted paper birch trees placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1994.

results, although the difference was only highly significant for the latter. These data show the importance of atmospheric deposition in the Sudbury area. The highest phytochelatin response was again seen far away from the emissions stack, without the possible influence of differential adaptation or metal-binding characteristics of the soils.

We have established that atmospherically deposited metals are responsible for high phytochelatin

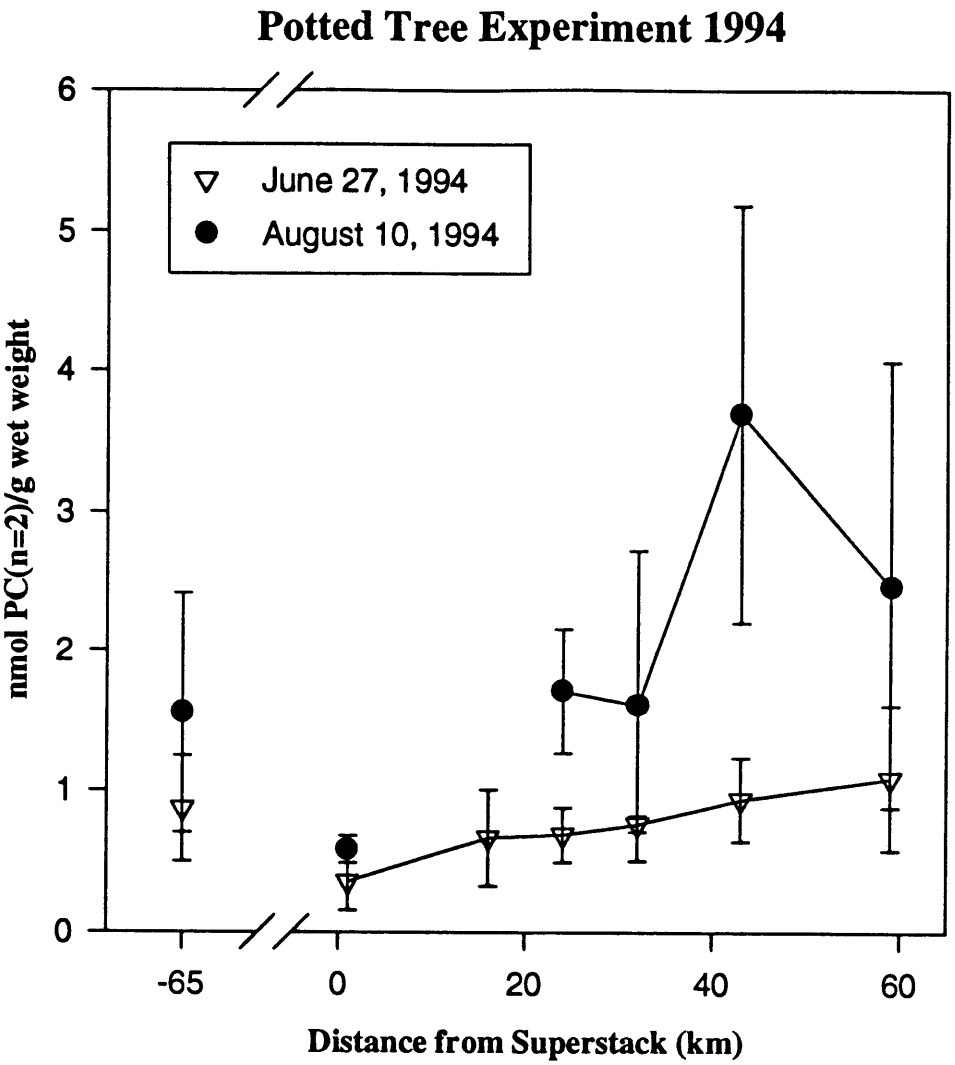

Figure 3.6b: Phytochelatin concentrations normalized to wet weight in potted paper birch trees placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1994.

concentrations in trees in the Sudbury area, but the question remains as to which pathway is responsible for metal intrusion. In the 1994 potted tree experiment, metals deposited from the atmosphere could have infiltrated the soil in the pots and been transported from roots to shoots, rather than entering directly through the leaves. Thus, the peat moss filters were added to the pots in 1995 to trap metals in precipitation or ambient air and keep them from contaminating the potted soil. Peat moss has a high affinity for metal ions, and should be able to effectively prevent

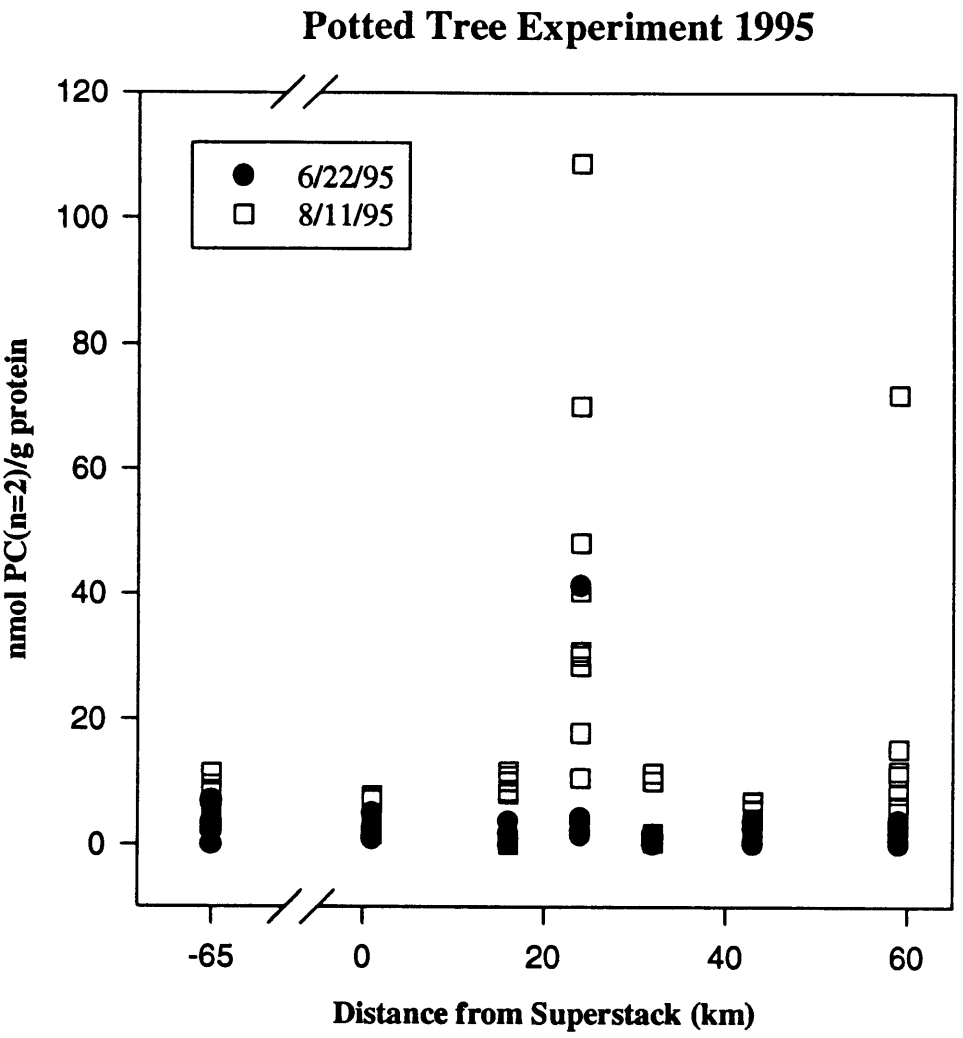

Figure 3.7a: Phytochelatin concentrations normalized to protein in potted paper birch trees with peat moss filters placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1995.

metal infiltration while allowing passage of water into the soil. Thus direct foliar uptake would be the only available pathway in this experiment for atmospheric metal pollution to enter the foliage. Again, phytochelatin concentrations increased at all sites after a month and a half, but the highest values by far were measured at 23 km from the stack [Figures 3.7a and 3.7b], and differed significantly from June levels normalized to both protein [Figure 3.7a] and wet weight [Figure

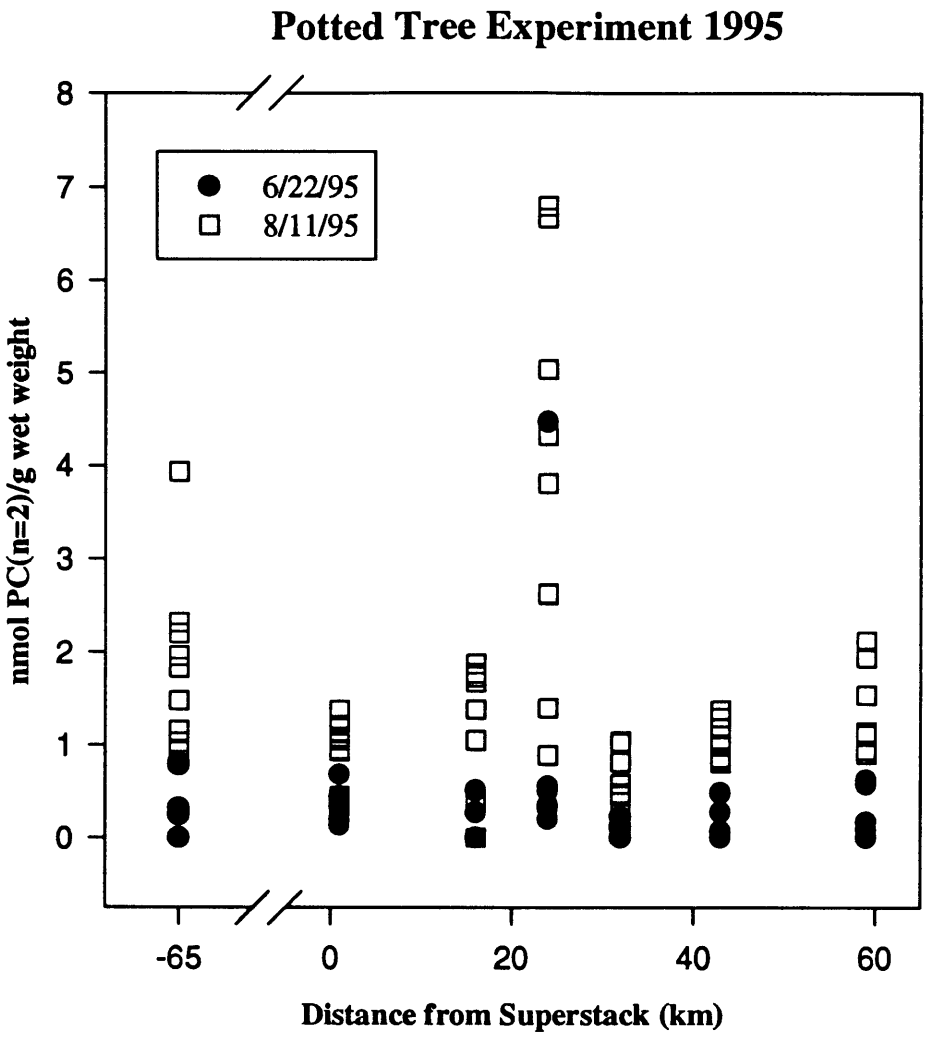

Figure 3.7b: Phytochelatin concentrations normalized to wet weight in potted paper birch trees with peat moss filters placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1995.

3.7b] ($P < 0.001$ for both). In addition, phytochelatin concentrations were even higher this year than in the 1994 potted trees [Figures 3.6a and 3.6b]. Therefore, the phytochelatin levels produced in 1995 by direct foliar uptake alone may account for the entire stress response measured in the trees, indicating that uptake and transport of metals by the roots is insignificant.

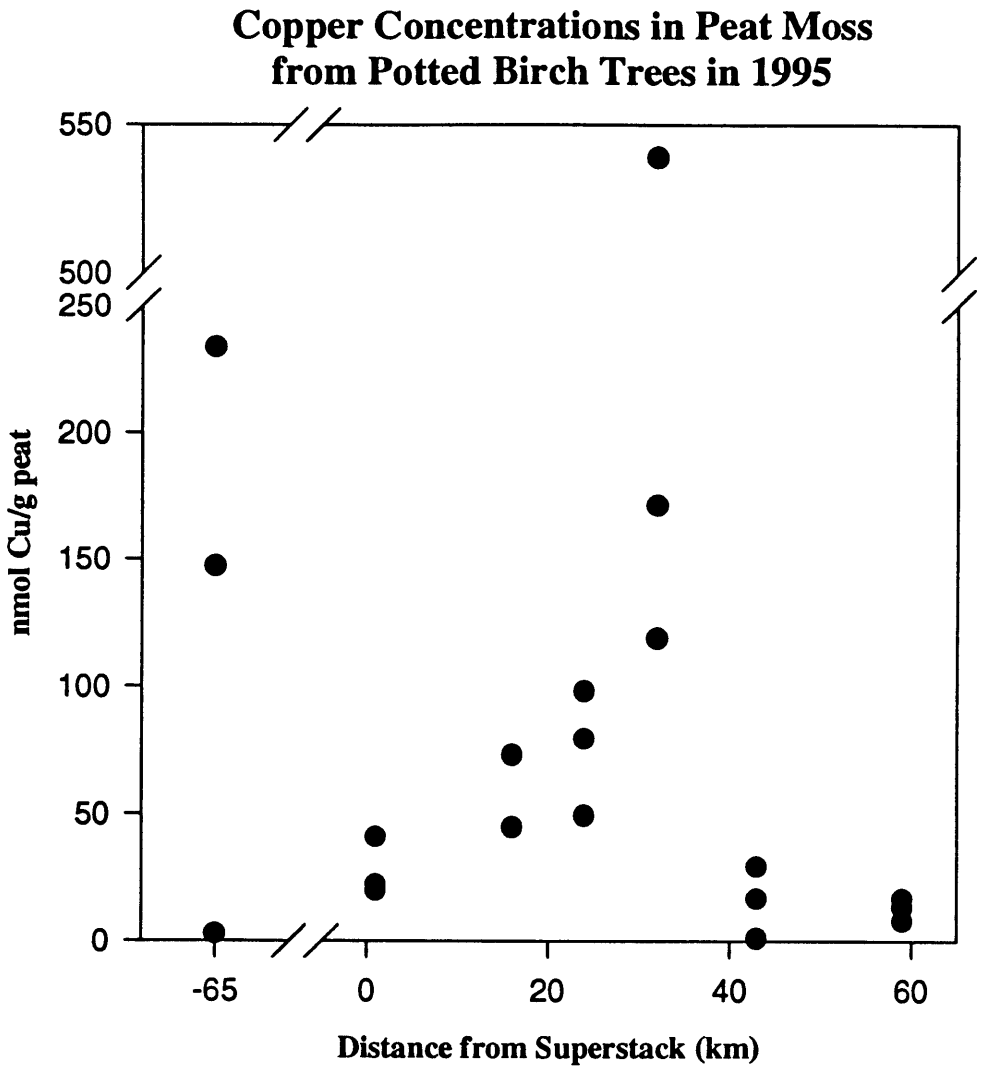

Figure 3.8: Copper concentrations from peat moss filters in the potted paper birch trees placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1995.

Since atmospheric deposition in 1995 produced the most significant metal stress response in trees 23 km away from the "superstack", bioavailable metal concentrations should be reflected by these measurements. Copper and nickel, with the two highest concentrations of the phytochelatin-inducing metals emitted by the Copper Cliff facility, were chosen as representative of bioavailable metal deposition in the area. Extracts from the peat moss used as filters in 1995 along the

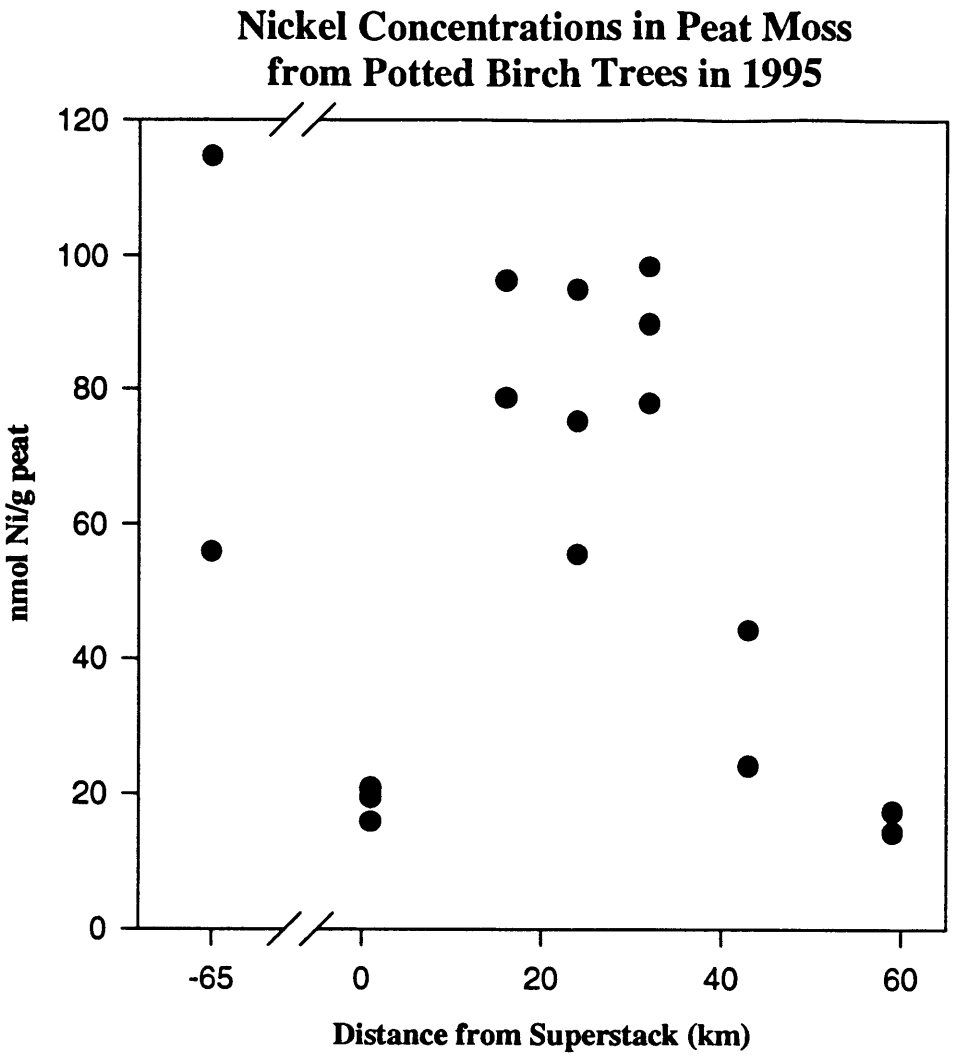

Figure 3.9: Nickel concentrations from peat moss filters in the potted paper birch trees placed along a NW(+)/SW(-) transect away from Sudbury, Ontario, Canada, in 1995.

northwest transect showed significantly ($P < 0.05$) higher concentrations of Cu at the 32 km site [Figure 3.8]. Ni concentrations were significantly higher ($P < 0.001$) at the 15, 23, and 32 km sites [Figure 3.9]. Thus, we have shown that both metal deposition and phytochelatin production exhibit maxima far away from the emissions stack at Copper Cliff. Concentrations of both Cu and Ni were also high in Killarney Provincial Park, the "control" site 65 km southwest of Sudbury,

even though phytochelatin levels were found to be low there [Figures 3.7a and 3.7b]. This fact, coupled with obvious discrepancies between patterns in phytochelatin levels and Cu and Ni concentrations along the northwest transect, suggests that either (1) phytochelatin production responds to short-term metal fluxes not indicated by total metal accumulation in the peat, (2) total metal levels do not correlate well with bioavailable metal levels, or (3) Cu and Ni are not the metals responsible for the stress response seen in the foliage.

Discussion

Our measurements, conducted over three years in the Sudbury area, provide very strong evidence that current emissions practices have resulted in high bioavailable metal concentrations and an elevated metal stress response in trees far away from the "superstack" near Sudbury. Three species of trees, growing *in situ* and sampled during two different years, showed almost identical patterns of phytochelatin concentrations along a northwesterly transect [Figure 3.1], with the highest levels between 15 and 32 km away [Figures 3.2, 3.3, and 3.4]. Trees placed along the same transect two consecutive years – in pots which isolated them from metal contamination in the native soils – also responded to local metal pollution in the same manner as trees rooted in the ground. The highest concentrations in these trees were found at the 44 and 23 km sites [Figures 3.6a, 3.6b, 3.7a, and 3.7b]. A comparison between trees in pots with and without peat moss filters revealed that direct foliar uptake may dominate over subsurface routes [Figures 3.6a and 3.7a]. In addition, concentrations of Cu and Ni, two primary smelter pollutants measured in peat moss filters used in the potted tree experiment, exhibited a pattern of deposition which also

peaked from 15 to 32 km away from the "superstack" [Figures 3.8 and 3.9]. Thus, all our data point to a downwind maximum in metal exposure to vegetation, corresponding to a much greater influence of current atmospheric emissions compared to historical contamination in the soils.

The location of the peak concentrations of phytochelatins fluctuates over time, and elevated Cu and Ni deposition do not necessarily correspond to either elevated phytochelatin levels or to each other. Discrepancies are also seen in the responses of different species to the same ambient conditions. However, the differences listed here may be expected to result from several environmental, chemical, and physical factors, which are discussed below.

Interspecies differences can be expected for two reasons: (1) differential sensitivity to metal pollution and (2) differential exposure to metals due to morphological characteristics. In support of the latter, McMahon and Denison (1979) have compiled extensive lists of measured deposition velocities to different plant surfaces for particles and gases. They found that differences between tree species can be almost an order of magnitude for dry deposition. However, wet deposition of metals may be more important in the Sudbury area (Chan et al. 1984), and morphological differences may have an even greater impact on water retention. Size, shape, and cuticle properties are very important in determining the wettability of foliage (Boyce et al. 1991). Wettability, in turn, influences metal exposure (McCune and Boyce 1992). As seen in our 1994 data, *B. papyrifera* [Figure 3.2] and *P. tremuloides* [Figure 3.4] – with relatively large surface areas, serrated edges, and thinner cuticles – show higher phytochelatin concentrations over a larger area than *A. balsamea* [Figure 3.4] – which has small needles, smooth edges, and thick

cuticles. Differences in phytochelatin production may also be due, in part, to microclimatological effects on metal deposition created by the trees themselves. Thus, it is not at all unexpected that different species may produce slightly different phytochelatin profiles when exposed to similar ambient conditions.

Even with these differences, all three species produced overlapping peaks in 1994. However, all of these were sampled at the same time. The location where peak phytochelatin levels are observed does not always remain the same from one sampling period to the next, as seen in Figures 3.2, 3.3, 3.6, and 3.7. However, since it was found that atmospheric metal deposition is controlling the phytochelatin response, changes in wind speed, atmospheric stability, and precipitation will all affect the pattern of phytochelatin production in the trees. Because phytochelatins may have a rapid turnover rate (de Knecht et al. 1995), small-scale fluctuations in these climatic factors may result in variable peak locations, even if average conditions remain the same.

Neither macro- nor microclimatological factors nor differences in surface morphologies, however, should have been responsible for the differences seen in patterns of Cu and Ni deposition since sampling time scales were identical and the same peat moss was used for all measurements. The differences between these two metals are likely a result of the dependence of wet deposition on particle size. It is evident for both Ni and Cu deposition that the 15, 23, and 32 km sites all show elevated values as compared to the base of the stack and the two furthest sites along the northwest transect [Figures 3.8 and 3.9]. For copper, however, the 32 km site dominates the

profile. Metals emitted from the INCO 'superstack" may exhibit different mass median particle diameters and modal diameter distributions as shown by Chan et al. (1983). In their study, particles containing Cu or Ni showed the same mass median diameters (> 9 μm) most of the time. However, Cu frequently showed a significant small-diameter mode (0.7 to 1.1 μm) when Ni did not. This difference may result in contrasting deposition patterns due to changes in the relative influence of various physical removal mechanisms as shown by Wayne (1991). Thus, discrepancies between Cu and Ni deposition may be the result of overlapping deposition patterns for the two different sizes of Cu-containing particles. The Cu particles around 1 μm in diameter are very ineffectively scavenged by rain (McMahon and Denison 1979). Thus, these particles are able to travel farther downwind before being removed, most likely by growth due to coagulation or heterogeneous SO_2 oxidation reactions (Wayne 1991). In addition, these particles – which would still be much smaller than 9 μm – have large surface area to volume ratios, making them more easily dissolved by our extraction methods. This may result in the increased Cu deposition seen in our data for the 32 km site.

This does not explain, however, why high concentrations of Cu and Ni were found in Killarney Provincial Park, or why deposition patterns for these metals do not match phytochelatin concentrations. Several possible explanations exist. First, rapid rates of phytochelatin production and destruction (de Knecht et al. 1995) may cause phytochelatin patterns to indicate short-term metal deposition and uptake, rather than long-term integrated metal patterns. Second, total metal concentrations may not be proportional to bioavailable metal levels. Finally, Cu and Ni may not be responsible for inducing the phytochelatin response measured in the Sudbury area. Because

these two metals are only toxic to plants at very high concentrations, co-emitted metals such as Cd and Pb, which are much more toxic (Bala and Setia 1990), may have a greater influence on phytochelatin production. Chan et al. (1984) found that 23% of Cd and 33% of lead emitted by the "superstack" is scavenged within 40 km, almost all by wet deposition. In addition, particles containing Cd and Pb are primarily in the sub-micron size range (Chan et al. 1983), and may be readily dissolved in precipitation. Thus, any of these three mechanisms, or some combination of them, could be responsible for discrepancies among the concentrations of Cu, Ni, and phytochelatins.

While it is unknown what the source of elevated Cu and Ni concentrations in Killarney Provincial Park may be, metal deposition northwest of Sudbury is almost certainly due to emissions from the INCO Copper Cliff smelting facility. Because wet deposition processes are more important than dry ones – up to a six-fold difference for Cd and Pb (Chan et al. 1984) – dry air modeling may not be helpful. This is especially true for the Sudbury area since the smelter plume is emitted at an altitude where lofting conditions predominate. While it is true that unstable, looping conditions occasionally cause fumigation episodes 3-10 km from the stack (Chan and Lusis 1985), current operating protocols minimize this impact by halting operations during unstable conditions (Potvin and Negusanti 1995). In addition, this alone would not account for the high concentrations of phytochelatins produced up to 59 km away [Figures 3.3, 3.4, and 3.5]. Without further data on particle coagulation rates or heterogeneous chemical reactions (resulting in SO_2 oxidation to SO_4^{2-} on particle surfaces) under current operational controls, calculations for comparison to observed metal deposition patterns are difficult. However, our Cu and Ni deposition measurements are

supported by two field studies. First, Gatz (1975) observed greater washout ratios (i.e. greater wet scavenging of particles) with increasing distance from pollutant sources over the Great Lakes. Second, Gundermann and Hutchinson (1995) recently found decreasing metal concentrations and increasing pH values in the surface soils close to the old Coniston smelter. At the same time, they found that metal concentrations are increasing and pH decreasing in surface soils greater between 20 and 60 km away. Although these measurements were made along a transect running south and southeast of Coniston, this area is currently subject to emissions from the Copper Cliff facility (Costescu 1974). Thus, not only do observed changes in scavenging ratios with distance support our measurements, but actual values of current metal concentrations in soils along a transect similar to ours (i.e. perpendicular to the dominant wind direction) also show corresponding results.

Patterns of phytochelatin concentrations and metal deposition, measured from 1993 to 1995, point to current atmospheric emissions as the dominant influence on metal stress in the Sudbury area. Although done in an attempt to improve local conditions, changes made in the design and operation of the Copper Cliff facility have resulted in continuing metal stress conditions farther from the stack. Monitoring design often does not take into account the possibility that greater dispersal may improve local air quality at the expense of increased damage downwind. In addition, metal concentrations – which are often used as the primary indicator of metal exposure – alone reveal little about relative impacts on vegetation. Therefore, phytochelatin measurements may be an ideal method for monitoring air quality changes both here and elsewhere, and repeated sampling may help in documenting long-term trends in metal pollution on local, regional, and

global scales.

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Chapter 4

Aberjona Watershed, Woburn, Massachusetts: Phytochelatin Concentrations as an Indicator of Metal Contamination in Groundwater

Introduction

Groundwater contamination is a growing problem throughout the world. In the northeastern United States, past industrial practices have produced hazardous waste materials which have been indiscriminately left behind in open lagoons, dumpsites, and landfills. These contaminants have leached into the groundwater over time, spreading pollution sometimes over very large areas, fouling drinking water sources, and poisoning vegetation in the process. However, because there are so many of these sites throughout the Northeast and limited economic resources available to characterize them, it is desirable to utilize inexpensive methods to measure the extent of contamination at each site and to monitor the progress of remediation efforts.

Biomonitoring – i.e. using organisms to monitor pollution levels and toxicity (Streit and Stumm 1993) – may be the most appropriate tool for groundwater investigations at sites where there may be direct contact between plant roots and polluted water. Aquatic plants can be used to monitor groundwater discharging to a stream, and trees – with long tap roots – can be used for monitoring away from the stream bed in shallow aquifers. Since metals are not subject to loss through biodegradation, unlike many organic compounds, biomonitoring has traditionally focused on measuring metal concentrations in the local biota (Streit and Stumm 1993). However, metal concentrations alone may be misleading, both because metals sorbed on plant surfaces may interfere with internal metal measurements and because some "hyper-accumulator" species may take up high concentrations of metals even when groundwater concentrations are low.

As shown in the two previous chapters, measurements of phytochelatins in plants may be used to track the mobility and bioavailability of metal contaminants in the field through time. Unlike metal concentrations, phytochelatin levels signal a metal stress-induced response since they are produced by plants specifically to detoxify free metal ions in the cytoplasm. Thus, phytochelatin concentrations in the foliage of plants able to access groundwater might perhaps be used to monitor the extent and magnitude of metal stress due to subsurface contamination in a field site. However, this is only true if the impact from atmospheric deposition is relatively minor in comparison. Thus, a third field site, where subsurface metal pollution exceeds atmospheric sources, is needed.

The Aberjona River watershed, 10 miles north of Boston, Massachusetts [Figure 4.1], provides an opportunity for examining the potential of phytochelatin measurements as biomonitors for metal stress due to groundwater contamination. This watershed is burdened with two Superfund sites – the "Industri-Plex" site and the "Wells G&H" site – and numerous other sites "confirmed" as containing hazardous materials by the Massachusetts Department of Environmental Protection. Tanning, hide and leather rendering, leather finishing, and chemical manufacturing were historically important industries in this area (Durant et al. 1990). The processes used by these industries resulted in the production of large amounts of waste with high concentrations of metals. Chromium, arsenic, copper, lead, and zinc contaminated wastes were dumped into open lagoons or buried in hide piles on site. These wastes were sometimes dumped directly into the Aberjona River and its tributaries. In addition, over forty waste disposal sites have been listed in the watershed, most of which were not designed to contain leaching, leaks, or fires (GeoTrans, Inc.

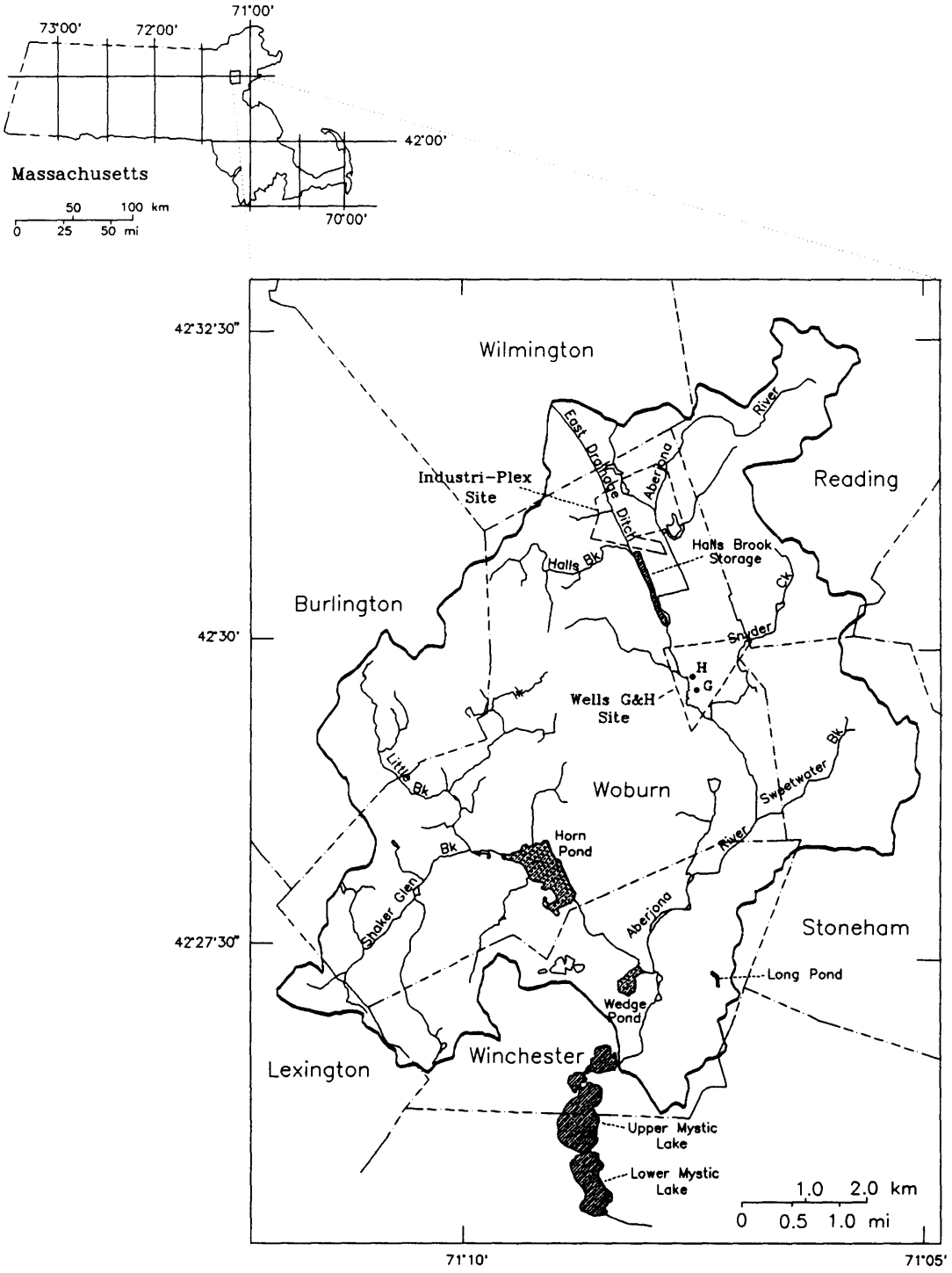

Figure 4.1: Locations of the Aberjona watershed, Industri-Plex site, and Wells G&H site.

and RETEC 1994). Over the years metals have leached into groundwater and surface water, contaminating the shallow aquifer under the watershed, the Aberjona River and some of its tributaries, as well as the Mystic Lakes into which they feed. Of the pollutants detected at elevated concentrations in the watershed, arsenic, lead, zinc, and copper, as well as co-contaminants such as cadmium and nickel, are known to induce the production of phytochelatins in plants (Grill et al. 1987). Trees are available for sampling in most areas since the Aberjona watershed is largely forested throughout, and tree roots easily reach the water table, which is close to the surface in most areas (Roux Associates, Inc. et al. 1991, GeoTrans, Inc. 1987). In addition, potential sources of atmospheric metal pollution (e.g. automobiles and residential oil or gas heating) are spread throughout the watershed, which should produce a relatively homogeneous deposition pattern. Therefore, if phytochelatin production induced by groundwater contamination is greater than the general urban background signal, then monitoring of subsurface metal pollution using phytochelatin concentrations should be possible in the Aberjona watershed.

Methods

As in our other studies, foliage samples collected were placed immediately in liquid nitrogen and brought back to the lab for analysis. Grinding and analysis methods are discussed in Chapter 2 and in Appendix A. Normalization to protein was done for 1992 and 1994 data, and normalization to wet weight was calculated for 1993 and 1994. Initially, in order to investigate whether there was a measurable difference between phytochelatin concentrations attributable to subsurface metal pollution and the general urban background air pollution, samples in 1992 were

collected by hand from the same three species – *Populus tremuloides* (quaking aspen), *Betula populifolia* (gray birch), and *Rubus allegheniensis* (blackberry) – in two different areas: (1) just down-gradient from the Industriplex site, near the Halls Brook Storage Area (HBSA), and (2) in the relatively pristine Middlesex Fells Reservation about three miles south.

A more extensive survey of the watershed was carried out in 1993. Samples were collected from 27 different locations throughout the watershed [Figure 4.2] on October 7 and 14, 1993. Sampling sites were located along the entire length of the Aberjona River, as well as all tributaries feeding into it from both the Aberjona River sub-basin and the Horn Pond sub-basin. Two samples were taken by hand from each of three trees per site and analyzed separately. The tree species sampled – in order of sampling frequency – were identified as *Acer platanoides* (Norway maple), *Rhamnus frangula* (glossy buckthorn), *B. populifolia*, *Viburnum dentatum* (arrow-wood), *Acer negundo* (box elder), *Acer rubrum* (red maple), and *P. tremuloides*.

In 1994 sampling was intensified in the immediate vicinity of the Industri-Plex and Wells G&H investigation areas [Figure 4.3]. In an attempt to compare published groundwater data, collected from monitoring wells during the investigations of contaminant migration at these sites, with phytochelatin concentrations in trees, sampling sites were located near past or current well locations. Near the Industri-Plex site, all wells ('OW' series) had been removed and well locations were approximated from detailed maps (Roux Associates, Inc. et al. 1991). Unfortunately, it was not possible to collect samples within the Industri-Plex site itself, since the designated area was completely cleared of vegetation in 1992. All wells used in this study near the Wells G&H site ('S'

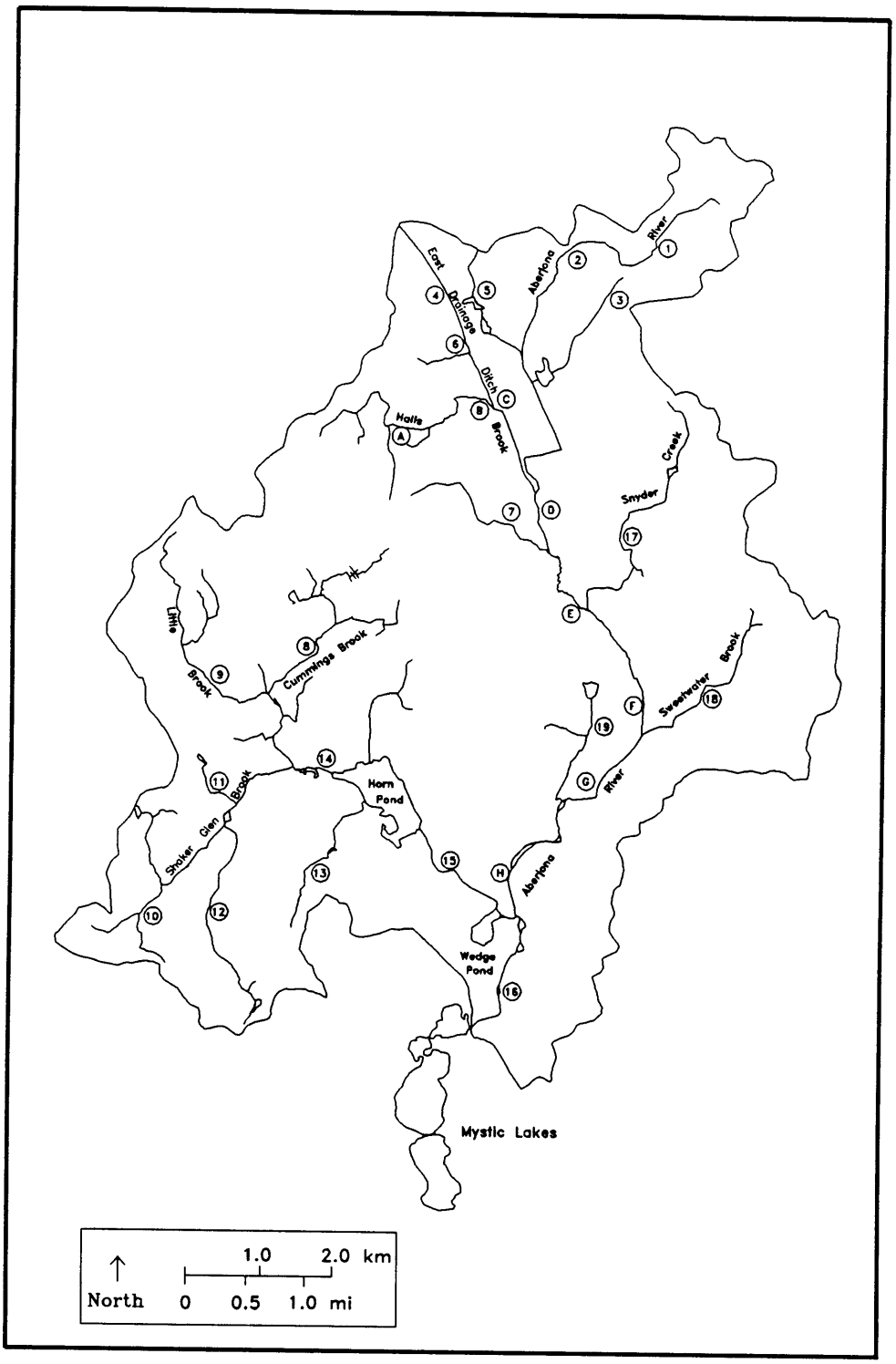

Figure 4.2: Map of locations sampled in 1993 throughout the Aberjona watershed.

Figure 4.3: Well locations near the Industri-Plex and Wells G&H sites.

series wells and BUG1) were still in place (GeoTrans, Inc. 1987). Three *R. frangula* trees were located near all but one site (only two were found near S47), and single samples were taken from each tree using a pole-pruner. All sampling took place on September 12, 1994.

Results

It was first necessary to find whether the metal stress induced by general urban background air pollution would exceed groundwater contamination effects in the Aberjona watershed. As seen in Sudbury, Ontario, atmospheric sources may dominate even when total metal levels in the soil are very high [Chapter 3]. Our data from 1992 show that concentrations of phytochelatins (normalized to protein) in *B. populifolia* and *R. allegheniensis* in the most contaminated area near the Industri-Plex site, were higher than those measured in the same species in the Middlesex Fells Reservation [Figure 4.4]. However, some species may not be as sensitive to metal pollution in the groundwater, as seen for *P. tremuloides*, which did not show a significant difference between the two sites. The lack of replicates for more than one species makes these results suspect. However, none of the species measured showed higher concentrations in the Middlesex Fells Reservation. In addition, measurements made in 1993 normalized to wet weight, using several species with repeated sampling in different areas, also showed low levels of phytochelatins in remote corners of the watershed itself as compared to the polluted areas along the Aberjona River [Figure 4.5 and Table 4.1]. Thus, background urban air pollution contributes less to the phytochelatin response seen in the foliage of these plants than metal contamination in the groundwater.

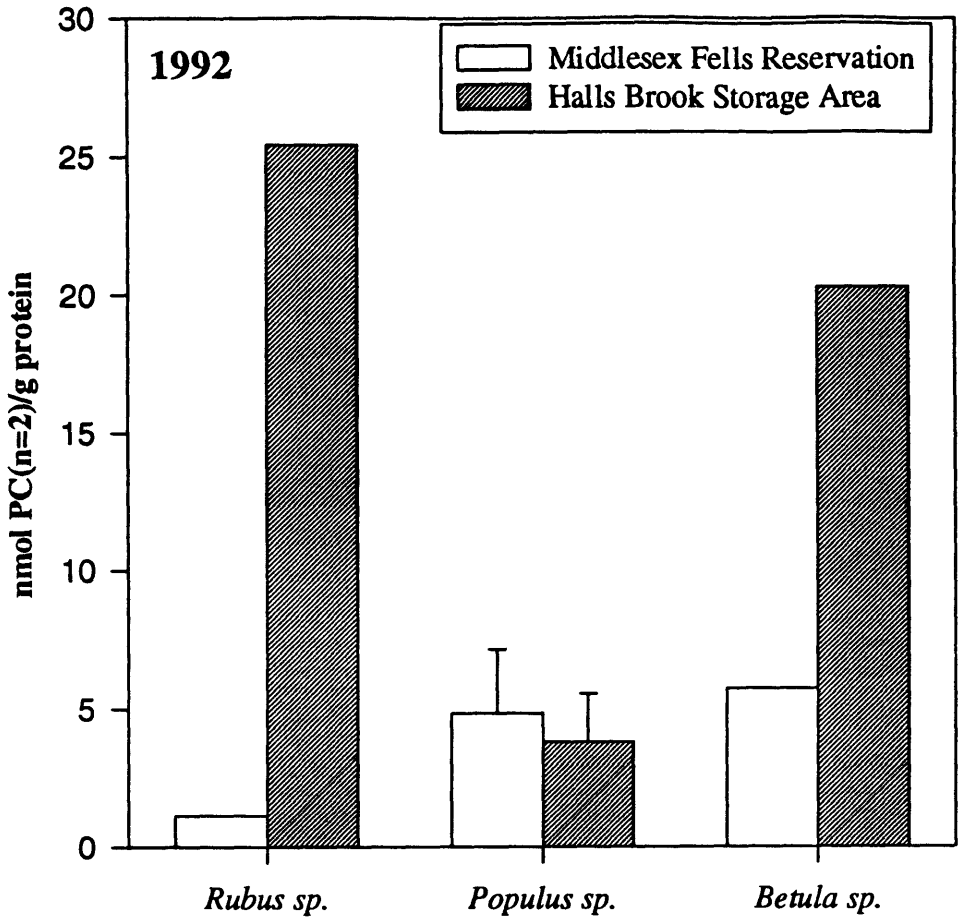

Figure 4.4: Phytochelatin concentrations normalized to protein in three plant species located near the Industri-Plex site and the Middlesex Fells Reservation.

The next step was to look at the overall pattern of phytochelatin concentrations throughout the watershed in order to locate those areas showing elevated metal stress. This was used to correlate known sources of metal contamination with increased phytochelatin production. In 1993, concentrations of phytochelatins normalized to wet weight, in species with overlapping distributions (i.e. *R. frangula*, *A. platanoides*, and *B. populifolia*), show high mean concentrations near the East Drainage Ditch (site 4), Landfill Creek (site 6), Halls Brook (sites A and B), the north end of HBSA (site C), and downstream along the Aberjona River (sites D and E) [Figure

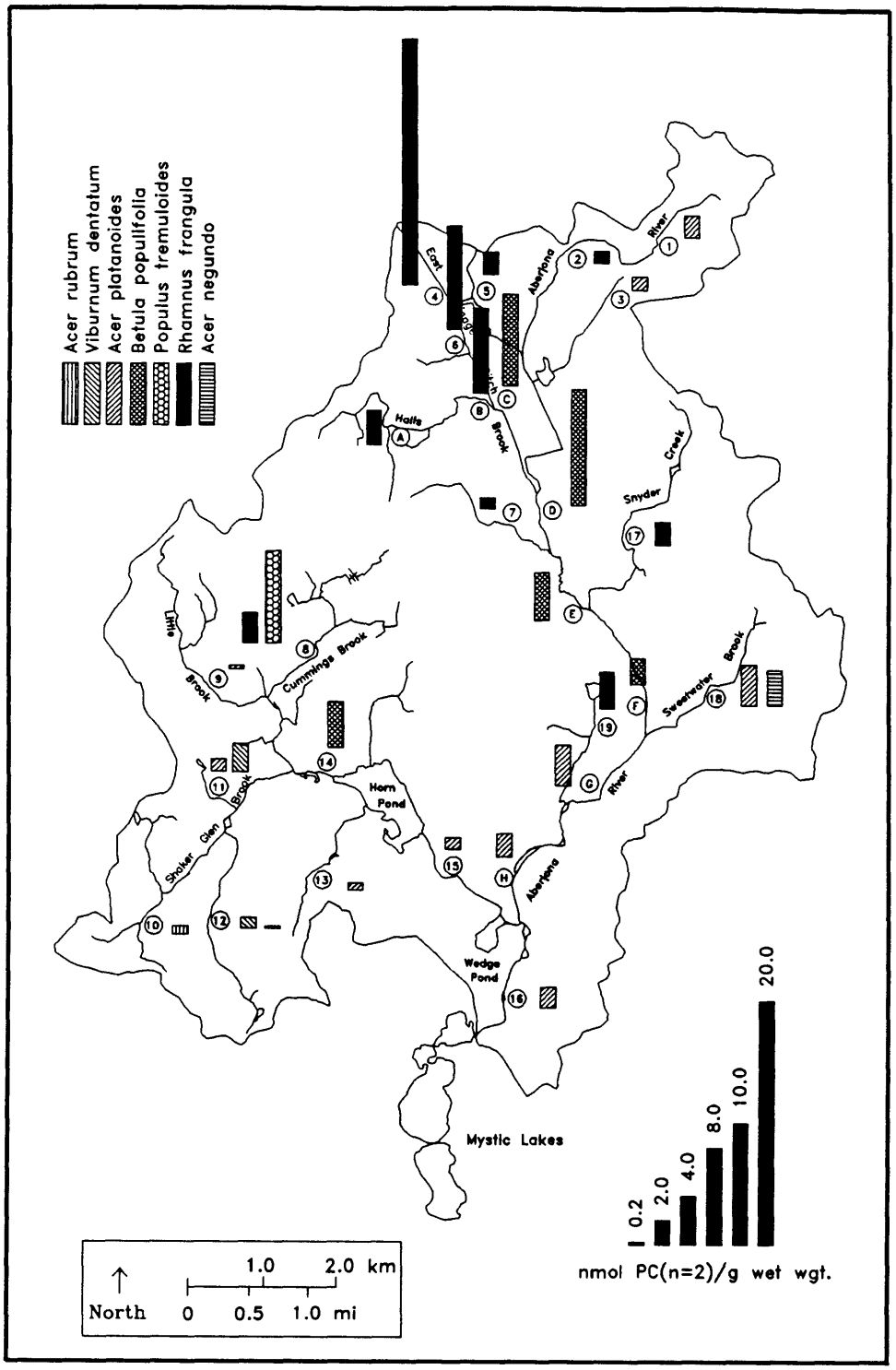

Figure 4.5: Mean phytochelatin concentrations normalized to wet weight in foliage samples collected throughout the Aberjona watershed in 1993.

station	species	mol PC(n=2)/g wet wt.	standard deviation
1	*A. platanoides*	1.84E-09	1.27E-09
2	*R. frangula*	9.56E-10	1.34E-09
3	*A. platanoides*	1.14E-09	3.85E-10
4	*R. frangula*	1.98E-08	1.40E-08
5	*R. frangula*	1.75E-09	1.39E-09
6	*R. frangula*	8.67E-09	7.88E-09
7	*R. frangula*	8.84E-10	8.33E-10
8	*R. frangula*	2.40E-09	8.14E-10
8	*P. tremuloides*	7.45E-09	7.14E-10
9	*A. platanoides*	3.34E-10	1.57E-10
10	*A. rubrum*	7.26E-10	1.29E-10
11	*A. platanoides*	1.02E-09	2.46E-12
11	*V. dentatum*	2.32E-09	7.86E-10
12	*R. frangula*	2.44E-10	3.79E-11
12	*V. dentatum*	9.10E-10	3.29E-10
13	*A. platanoides*	6.11E-10	4.48E-10
14	*B. populifolia*	3.69E-09	6.07E-10
15	*A. platanoides*	9.13E-10	1.19E-09
16	*A. platanoides*	1.71E-09	1.33E-09
17	*R. frangula*	1.86E-09	1.35E-09
18	*A. platanoides*	3.35E-09	6.87E-10
18	*A. negundo*	2.89E-09	4.00E-10
19	*R. frangula*	3.03E-09	1.11E-09
A	*R. frangula*	2.82E-09	2.35E-09
B	*R. frangula*	6.99E-09	2.73E-09
C	*B. populifolia*	7.54E-09	2.59E-09
D	*B. populifolia*	9.43E-09	8.47E-09
E	*B. populifolia*	3.96E-09	1.65E-09
F	*B. populifolia*	2.12E-09	6.61E-10
G	*A. platanoides*	3.28E-09	1.21E-09
H	*A. platanoides*	1.94E-09	5.18E-10

Table 4.1: Phytochelatin concentrations normalized to wet weight in trees located throughout the Aberjona River watershed.

4.5 and Table 4.1]. These concentrations can be compared to values in the same species outside this corridor, which are low throughout most of the Horn Pond sub-basin and along the other

Aberjona River tributaries. This pattern is consistent with known sources of metal contamination in the watershed.

Having shown that the highest concentrations of phytochelatins were found in the vicinity of the Industri-Plex and Wells G&H sites, we then narrowed the sampling area to better describe the extent of groundwater contamination in these areas. This was done for comparison with recorded groundwater metal concentrations, and to try to elucidate on a smaller scale the actual locations of current metal pollution sources in these areas. In 1994, high concentrations of phytochelatins normalized to wet weight were again found near "Arsenic Springs" at the north end of the HBSA (OW-18) and near the East Drainage Ditch (OW-1) [Figure 4.6 and Table 4.2]. Higher concentrations were found on the east bank of the Aberjona River than on the west bank from the Industri-Plex down past Wells G&H. Phytochelatin levels gradually decreased from "Arsenic Springs" southward, but again reached high values to the east of Well G (S39) and Well H (S40). Slightly lower, but still high, phytochelatin concentrations were measured south of this to the edge of our sampling just below Salem Street (S-11). The only site within the Wells G&H wetland itself (S88) also showed high concentrations. Normalization to protein produced similar results [Table 4.2]. As reflected by phytochelatin measurements, high levels of bioavailable metals are likely to exist in these same areas, and possible sources of contamination can be inferred from this information.

Discussion

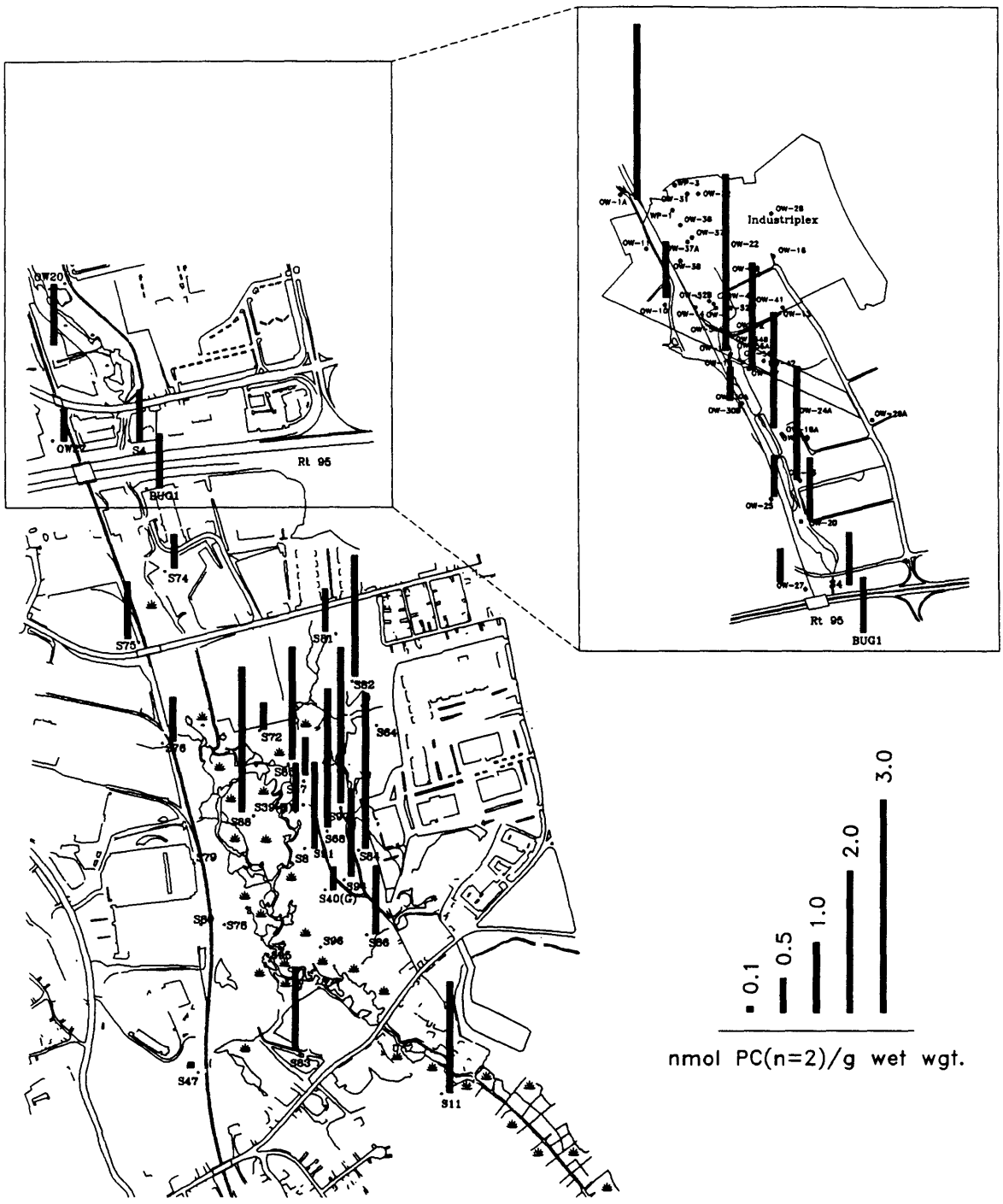

Figure 4.6: Mean phytochelatin concentrations normalized to wet weight in foliage samples collected near the Industri-Plex and Wells G&H Sites in 1994.

well #	mol PC(n=2)/g protein	standard deviation	mol PC(n=2)/g wet weight	standard deviation
OW1A	1.83E-08	1.96E-08	2.43E-09	2.72E-09
OW27A	5.39E-09	2.33E-09	4.65E-10	2.26E-10
OW25A	7.29E-09	3.91E-09	5.83E-10	3.30E-10
OW30A	4.07E-09	5.41E-09	4.85E-10	6.72E-10
OW10	7.43E-09	3.52E-09	7.96E-10	2.30E-10
OW17	1.35E-08	9.46E-09	1.51E-09	1.06E-09
OW18A	2.70E-08	1.18E-08	2.48E-09	9.06E-10
OW19A	1.32E-08	7.72E-09	1.63E-09	8.70E-10
OW33A	1.27E-08	7.44E-09	1.60E-09	9.79E-10
OW20	8.48E-09	4.54E-09	8.71E-10	3.93E-10
S75	5.79E-09	4.83E-09	8.02E-10	6.53E-10
S81	5.89E-09	3.99E-09	6.01E-10	4.15E-10
S82	2.43E-08	7.52E-09	1.69E-09	5.63E-10
S76	5.43E-09	2.43E-09	6.34E-10	2.60E-10
S47	1.35E-09	1.91E-09	1.00E-10	1.42E-10
S11	1.82E-08	1.20E-08	1.57E-09	1.34E-09
S83	1.08E-08	1.25E-08	1.17E-09	1.40E-09
S88	1.93E-08	1.75E-08	2.04E-09	1.75E-09
S8	1.63E-08	9.91E-09	1.23E-09	8.06E-10
S40	3.75E-09	2.91E-09	3.33E-10	2.55E-10
S84	2.78E-08	2.89E-08	2.19E-09	2.58E-09
S86	1.22E-08	4.00E-09	9.65E-10	4.05E-10
S4	1.22E-08	3.67E-09	7.37E-10	2.64E-10
BUG1	8.88E-09	5.85E-09	7.73E-10	5.21E-10
S74	6.85E-09	2.98E-09	4.73E-10	2.18E-10
S72	4.37E-09	4.36E-09	3.86E-10	4.11E-10
S94	1.14E-08	1.81E-08	1.23E-09	1.97E-09
S90	3.39E-08	2.80E-08	2.19E-09	1.84E-09
S85	2.13E-08	1.85E-08	1.59E-09	1.36E-09
S87	7.07E-09	1.05E-09	5.35E-10	1.09E-10
S39	7.41E-09	3.28E-09	6.77E-10	3.21E-10
S68	2.28E-08	1.71E-08	1.95E-09	1.46E-09

Table 4.2: Mean phytochelatin concentrations normalized to protein and wet weight in glossy buckthorn trees located near monitoring wells for the Industri-Plex and Wells G&H Superfund Sites in the Aberjona watershed.

The Aberjona watershed is subject to groundwater metal contamination, which exceeds the general urban background air pollution in inducing a metal stress response in plants. Elevated phytochelatin concentrations, indicating increased metal stress, were found in trees growing along

the East Drainage Ditch, Landfill Creek, and the HBSA near the Industri-Plex Superfund site, and downstream past the Wells G&H Superfund site. Phytochelatin levels were low in trees along the remaining length of the Aberjona River, and all the tributaries in both the sub-basins of the watershed. High density sampling in trees growing away from the stream itself revealed greater metal stress again near the East Drainage Ditch and the north end of HBSA, along with the area immediately to the east of Well G and Well H. Over the entire length of the waterway, from HBSA to below the Wells G&H site, phytochelatin concentrations were higher on the east side of the river than the west side. The possible sources of metal pollution in these areas are discussed below.

In the north, the locations of sites showing high concentrations of phytochelatins in 1993 and 1994 correspond primarily to the largest suspected sources of metals contamination in the watershed; the hide-piles, waste lagoons, and settling basins at the Industri-Plex site. This site was used by five different chemical manufacturers since 1883 (Aurilio 1992), which produced compounds containing Sn, Cu, Pb, As, and Zn (Cherry et al. 1989). "Arsenic springs", the discharge area at the north end of HBSA fed by groundwater flowing southwest from the Industri-Plex site, was shown by Aurilio (1992) to have up to 1100 nM As. Measurements of groundwater in 1990 and 1991 from shallow sampling wells located near "arsenic springs" (OW-18 and OW-18A) also produced the highest concentrations of Cd and Zn found anywhere in the northern part of the watershed, as well as the highest levels of As found off the Industri-Plex site itself (Roux Associates, Inc. et al. 1991). Cadmium is an impurity in zinc ores used for tanning processes, but it may also have been used specifically for making dyes and pigments at the

Industri-Plex site (Knox 1991). Data collected by Knox (1991) showed that concentrations of As, Cd, Cu, and Zn in stream sediments from HBSA, Halls Brook, and the East Drainage Ditch were much higher than levels downstream, prior to reaching the Wells G&H wetland. Knox found significant inter-correlations between these four metals, suggesting that the majority of metal contamination comes from the same sources.

Several caveats need to be mentioned when comparing published metals data with our findings. First, records of metal concentrations may exhibit apparent discrepancies due to differences in sampling methods, movement of the contaminant plume over time, or human error. Groundwater from wells in the Aberjona watershed was collected by several different consulting companies using different flow rates and sampling apparatus, and analyzed by different laboratories using methods with varying detection limits. In addition, sampling was spread out over several years, and multiple samples from the same wells over time are few. Therefore, it is difficult to amass a set of metal concentrations from the recorded data that includes a large number of wells sampled during the same time period. Adding to the problem of arriving at a clear picture of groundwater metal contamination is the fact that, even including only shallow wells, the depth of the aquifer screened for sampling varies, and larger screened intervals may dilute high surface metal concentrations with "clean" water from deeper in the aquifer. The reverse may also be true. Finally, concentrations of metals in groundwater samples may not accurately reflect bioavailable metal levels. Even "dissolved" metal measurements are done using simple filtration to separate this fraction from "total" levels. This may erroneously include colloidal metal, which is not directly available to plants for uptake, in the "dissolved" fraction. However, given these

restrictions, it is still useful to examine similarities and differences between various metal pollutant concentrations and phytochelatin levels in the Aberjona watershed. [see Tables 4.3 and 4.4 for metal measurements with associated disclaimers]

Concentrations of phytochelatins and dissolved As, Cd, Cu, and Zn for the Industri-Plex investigation area are shown in Figure 4.7 and Figures 4.8 to 4.11 respectively. Metal levels, excerpted from Roux Associates, Inc. et al. (1991), were measured during 1990, and include only those wells screened to within at least 20 ft of the surface. As can be seen in these plots, Cd and Zn maximums are found to generally correlate with phytochelatin maximums. Arsenic concentrations also show a corresponding distribution outside the Industri-Plex site itself. Unfortunately, phytochelatin levels could not be measured within the site boundary because it was cleared of vegetation. Copper, on the other hand, was found to be highest near OW-10, where phytochelatin levels were relatively low. Comparing phytochelatin values directly with metal concentrations found in the nearest wells produces few data points for comparison, however, weak correlations are found between Cd ($r^2 = 0.44$) and Zn ($r^2 = 0.39$) and phytochelatin concentrations in this area, whereas Cu and As show no correlation with phytochelatin levels [Figure 4.12]. In any case, the pattern of phytochelatin levels seen in this portion of the watershed again points to the Industri-Plex site as a primary source of metal contamination in this area. Other sources may be responsible for elevated phytochelatin levels and sediment metal concentrations (Knox 1991) seen in the East Drainage Ditch, Landfill Creek, and Halls Brook. Possible sources are listed by Cherry et al. (1989) and include a chemical manufacturer (Olin Chemical Group) and dye maker (New England Pigments and Resins) among others.

Mar-90	As(mg/L)	Cd(mg/L)	Cu(mg/L)	Zn(mg/L)
OW-1A	0.002U	0.005U	0.0115A	0.0728R
OW-6	N/M	0.005U	0.006U	0.0232R
OW-10	0.002U	0.0088A	0.321A	3.18R
OW-11	0.162A	0.005U	0.006U	0.913R
OW-12	0.422A	0.005U	0.006U	0.0463R
OW-13	0.0204A/0.0206A	0.005U/0.005U	0.006U/0.006U	0.0389R/0.0483R
OW-14	0.0075A	0.0055A	0.0176A	2.11R
OW-15	0.002U	0.005U	0.006U	0.0266R
OW-16	2.86A	0.005U	0.006U	0.0979R
OW-17	0.164A	0.005U	0.006U	0.0785R
OW-18	0.002U	0.0252A	0.122A	8.00R
OW-18A	0.002U	0.0205A	0.0351A	7.22R
OW-19A	0.0177A	0.005U	0.006U	0.219R
OW-21	0.002U/0.002U	0.005U/0.005U	0.006U/0.006U	0.0319R/0.121R
OW-22	0.0044A	0.005U	0.006U	0.0457R
OW-28	0.002U	0.005U	0.006U	0.0287R
Jun-90				
OW-23	0.002U	0.005U	0.0075J	0.0648J
OW-24A	0.002U	0.005U	0.0076J	0.0509J
OW-25A	0.002U	0.005U	0.006U	0.0259J
OW-26A	0.0171A	0.005U	0.0121J	0.0505J
OW-29	0.002U/0.002U	0.005U/0.005U	0.0099J/0.006U	0.0729J/0.0376J
OW-30A	0.0414J/0.047A	0.005U/0.005U	0.006U/0.006U	0.1J/0.128J
OW-31	0.518A	0.005U	0.006U	0.0272J
OW-32	0.023A	0.005U	0.006U	0.0264J
Aug-90				
OW-1A	0.002U	N/M	N/M	0.0254J
OW-6	0.002U	N/M	N/M	0.012J
OW-7	0.002U	N/M	N/M	0.0207J
OW-10	0.002U	N/M	N/M	0.231J
OW-11	0.198J	N/M	N/M	1.6J
OW-12	0.0364J	N/M	N/M	0.009J
OW-13	0.0435J	N/M	N/M	0.0076J
OW-14	0.0041J	N/M	N/M	1.61J
OW-15	0.002U	N/M	N/M	0.0172J
OW-16	2.4J	N/M	N/M	0.006U
OW-17	0.083J	N/M	N/M	0.0632J
OW-18	0.0031J	N/M	N/M	8.99J
OW-18A	0.002U	N/M	N/M	4.13J
OW-19A	0.0224J	N/M	N/M	0.166J
OW-21	0.0028J/0.004J	N/M	N/M	0.0157J/0.0378J
OW-22	0.002U	N/M	N/M	0.025J
OW-28	0.002U	N/M	N/M	0.0151J

Table 4.3: "Dissolved" metal concentrations in groundwater from monitoring wells near the Industri-Plex site screened to within 20 ft of the surface (data from Roux Associates, Inc. et al. 1991). R = rejected, A = quantitative, U = not detected at indicated detection limit, J = below method detection limit, reported value is estimated, N/M = not measured.

Oct-90				
OW-10	0.003UJ	0.0159A	0.852U	7.25A
OW-11	0.0984A	0.0017U	0.0045UJ	0.942A
OW-12	0.556A	0.0034U	0.059U	0.0323U
OW-13	0.0252A	0.0017U	0.0045UJ	0.008U
OW-16	2.3A	0.0061J	0.0555U	0.0311U
OW-17	0.164A	0.0033A	0.188U	0.037U
OW-18	0.003U	0.0267A	0.137U	7.3A
OW-18A	0.003U	0.0073U	0.0268U	2.15A
OW-19A	0.0354A	0.0017U	0.0045UJ	0.12A
OW-21	0.003UJ	0.0017UJ	0.0411U	0.0251U
OW-22	0.003U	0.0017U	0.0357U	0.0611U
OW-24A	0.003U	0.0017U	0.0045UJ	0.0174U
OW-28	0.003U	0.0017U	0.0172U	0.0164U
OW-30A	0.0273A	0.0017U	0.0045UJ	0.107A
OW-31	0.63A	0.0017UJ	0.0045UJ	0.0098U
OW-32	0.0218A	0.0027A	0.161U	0.0479U
OW-36	0.256A	0.0027J	0.164U	0.042U
OW-37	0.343A	0.0017UJ	0.0927U	0.0267U
OW-38	0.12A/0.132A	0.0018A/0.0017U	0.0732U/0.0416U	0.291A/0.275A
OW-39	0.0293A/0.0303A	0.0017U/0.0017U	0.0045UJ/0.0045UJ	0.0113U/0.0146U
OW-40	0.003U	0.0017U	0.124U	0.132A
OW-41	0.0344A	0.0017U	0.0075U	0.0125U

Table 4.3: continued

Downstream of Industri-Plex lies the Wells G&H Superfund site. This site was originally designated because of organic chemical contamination. However, Knox (1991) found that concentrations of As, Cu, and Zn were just as high in the sediments of the Wells G&H wetland as they were in HBSA, while the stream bed between the two sites had relatively low levels. In addition, recent measurements made by the United States Environmental Protection Agency (1995) show higher concentrations of As, Cd, Cu, Pb, Ni, and Zn in the stream water leaving the Wells G&H wetland than in the water that is entering. However, the contamination at this site is not confined to the Aberjona River and wetlands. Levels of Cd were higher in some groundwater samples away from the stream at the Wells G&H site (GeoTrans, Inc. 1987) than the highest levels measured near the Industri-Plex site (Roux Associates, Inc. et al. 1991). In addition,

S64S	6/25/85	Arsenic, dissolved	3.0U	ug/l
S64S	2/22/91	Arsenic, dissolved	1.0U	ug/l
S68S	4/23/85	Arsenic, dissolved	10.0U	ug/l
S68S	6/26/85	Arsenic, dissolved	3.0U	ug/l
S68S	8/21/91	Arsenic, dissolved	10.0U	ug/l
S72S	6/25/85	Arsenic, dissolved	3.0U	ug/l
S72S	8/21/91	Arsenic, dissolved	10.0U	ug/l
S74S	4/23/85	Arsenic, dissolved	17.0A	ug/l
S74S	8/31/93	Arsenic, dissolved	1.4U	ug/l
S75S	6/27/85	Arsenic, dissolved	16.0J	ug/l
S76S	6/26/85	Arsenic, dissolved	3.0U	ug/l
S78S	6/27/85	Arsenic, dissolved	3.0U	ug/l
S81S	6/26/85	Arsenic, dissolved	31.0A	ug/l
S81S	6/26/85	Arsenic, dissolved	22.0A	ug/l
S81S	6/26/85	Arsenic, dissolved	27.0J	ug/l
S81S	6/26/85	Arsenic, dissolved	38.0J	ug/l
S81S	2/21/91	Arsenic, dissolved	1.1UJ	ug/l
S82	2/22/91	Arsenic, dissolved	1.0U	ug/l
S83SS	8/30/93	Arsenic, dissolved	10.0U	ug/l
S83SS	8/30/93	Arsenic, dissolved	10.0U	ug/l
S84S	6/27/85	Arsenic, dissolved	3.0U	ug/l
S84S	8/20/91	Arsenic, dissolved	10.0U	ug/l
S85S	8/23/91	Arsenic, dissolved	10.0U	ug/l
S85S	9/2/93	Arsenic, dissolved	1.4U	ug/l
S86S	8/26/91	Arsenic, dissolved	10.0U	ug/l
S86S	8/26/91	Arsenic, dissolved	10.0U	ug/l
S87S	8/23/91	Arsenic, dissolved	10.0U	ug/l
S90S	8/22/91	Arsenic, dissolved	10.0U	ug/l
S91S	8/21/91	Arsenic, dissolved	10.0U	ug/l
S91S	9/1/93	Arsenic, dissolved	1.4U	ug/l
UG4-5	8/23/91	Arsenic, dissolved	10.0U	ug/l
S64S	2/22/91	Cadmium, dissolved	3.3U	ug/l
S64S	6/25/85	Cadmium, dissolved	4.0U	ug/l
S68S	6/26/85	Cadmium, dissolved	1.9U	ug/l
S68S	6/26/85	Cadmium, dissolved	4.0UJ	ug/l
S68S	4/23/85	Cadmium, dissolved	60.0UJ	ug/l
S72S	6/25/85	Cadmium, dissolved	5.7A	ug/l
S74S	8/31/93	Cadmium, dissolved	2.8UJ	ug/l
S74S	4/23/85	Cadmium, dissolved	60.0UJ	ug/l
S75S	6/27/85	Cadmium, dissolved	1.9U	ug/l
S76S	6/26/85	Cadmium, dissolved	6.1J	ug/l
S77SS	6/27/85	Cadmium, dissolved	1.9U	ug/l
S78S	6/27/85	Cadmium, dissolved	5.7J	ug/l
S78S	6/27/85	Cadmium, dissolved	6.5A	ug/l
S78S	10/26/87	Cadmium, dissolved	8.1A	ug/l
S81S	2/21/91	Cadmium, dissolved	3.3U	ug/l
S81S	6/26/85	Cadmium, dissolved	5.6J	ug/l
S81S	6/26/85	Cadmium, dissolved	6.0A	ug/l
S81S	6/26/85	Cadmium, dissolved	6.2A	ug/l
S81S	6/26/85	Cadmium, dissolved	12.0J	ug/l
S82	2/22/91	Cadmium, dissolved	3.3U	ug/l
S83SS	8/30/93	Cadmium, dissolved	5.0U	ug/l
S83SS	8/30/93	Cadmium, dissolved	5.0U	ug/l
S84S	6/27/85	Cadmium, dissolved	4.0UJ	ug/l
S85S	9/2/93	Cadmium, dissolved	2.8UJ	ug/l
S91S	9/1/93	Cadmium, dissolved	3.6U	ug/l

Table 4.4: "Dissolved" metal concentrations in groundwater from monitoring wells near the Wells G&H site screened to within 20 ft of the surface (data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994). A = quantitative, U = not detected at indicated detection limit, J = below method detection limit, reported value is estimated, UJ = not detected, indicated detection limit is approximate, B = below CRDL but above IDL.

S64S	2/22/91	Copper, dissolved	5.8U	ug/l
S64S	6/25/85	Copper, dissolved	15.5UJ	ug/l
S68S	6/26/85	Copper, dissolved	4.5UJ	ug/l
S72S	6/25/85	Copper, dissolved	15.5UJ	ug/l
S74S	8/31/93	Copper, dissolved	4.7U	ug/l
S74S	4/23/85	Copper, dissolved	185.0A	ug/l
S76S	6/26/85	Copper, dissolved	34.0J	ug/l
S81S	2/21/91	Copper, dissolved	5.8U	ug/l
S81S	6/26/85	Copper, dissolved	172.0J	ug/l
S81S	6/26/85	Copper, dissolved	250.0J	ug/l
S81S	6/26/85	Copper, dissolved	253.0J	ug/l
S81S	6/26/85	Copper, dissolved	328.0J	ug/l
S82	2/22/91	Copper, dissolved	5.8U	ug/l
S83SS	8/30/93	Copper, dissolved	25.0U	ug/l
S83SS	8/30/93	Copper, dissolved	25.0U	ug/l
S85S	9/2/93	Copper, dissolved	4.7U	ug/l
S91S	9/1/93	Copper, dissolved	4.7U	ug/l
S64S	6/25/85	Zinc, dissolved	147.0A	ug/l
S64S	2/22/91	Zinc, dissolved	8.0U	ug/l
S68S	6/26/85	Zinc, dissolved	128.0A	ug/l
S72S	6/25/85	Zinc, dissolved	54.0A	ug/l
S74S	4/23/85	Zinc, dissolved	215.0A	ug/l
S74S	8/31/93	Zinc, dissolved	2.8U	ug/l
S76S	6/26/85	Zinc, dissolved	55.0A	ug/l
S78S	6/27/85	Zinc, dissolved	42.0A	ug/l
S78S	10/26/87	Zinc, dissolved	44.4A	ug/l
S81S	6/26/85	Zinc, dissolved	326.0A	ug/l
S81S	6/26/85	Zinc, dissolved	276.0A	ug/l
S81S	2/21/91	Zinc, dissolved	20.0U	ug/l
S82	2/22/91	Zinc, dissolved	15.0U	ug/l
S83SS	8/30/93	Zinc, dissolved	20.0U	ug/l
S83SS	8/30/93	Zinc, dissolved	20.0U	ug/l
S84S	6/27/85	Zinc, dissolved	54.0A	ug/l
S85S	9/2/93	Zinc, dissolved	2.8U	ug/l
S91S	9/1/93	Zinc, dissolved	3.8B	ug/l

Table 4.4: continued

maximum concentrations of Pb and As measured since 1991 are high in wells to the northeast of Well G and Well H, some distance from the wetland and river (GeoTrans, Inc. and RETEC 1994).

Phytochelatin concentrations in trees away from the wetland on the east side of the river are similar in magnitude to those found near the Industri-Plex site by "arsenic springs" [Figure 4.13]. In fact, some of the highest levels of phytochelatins at the Wells G&H site are found farthest away from the wetland (S90, S84, and S68). Maximum concentrations of dissolved As, Cu, and Zn

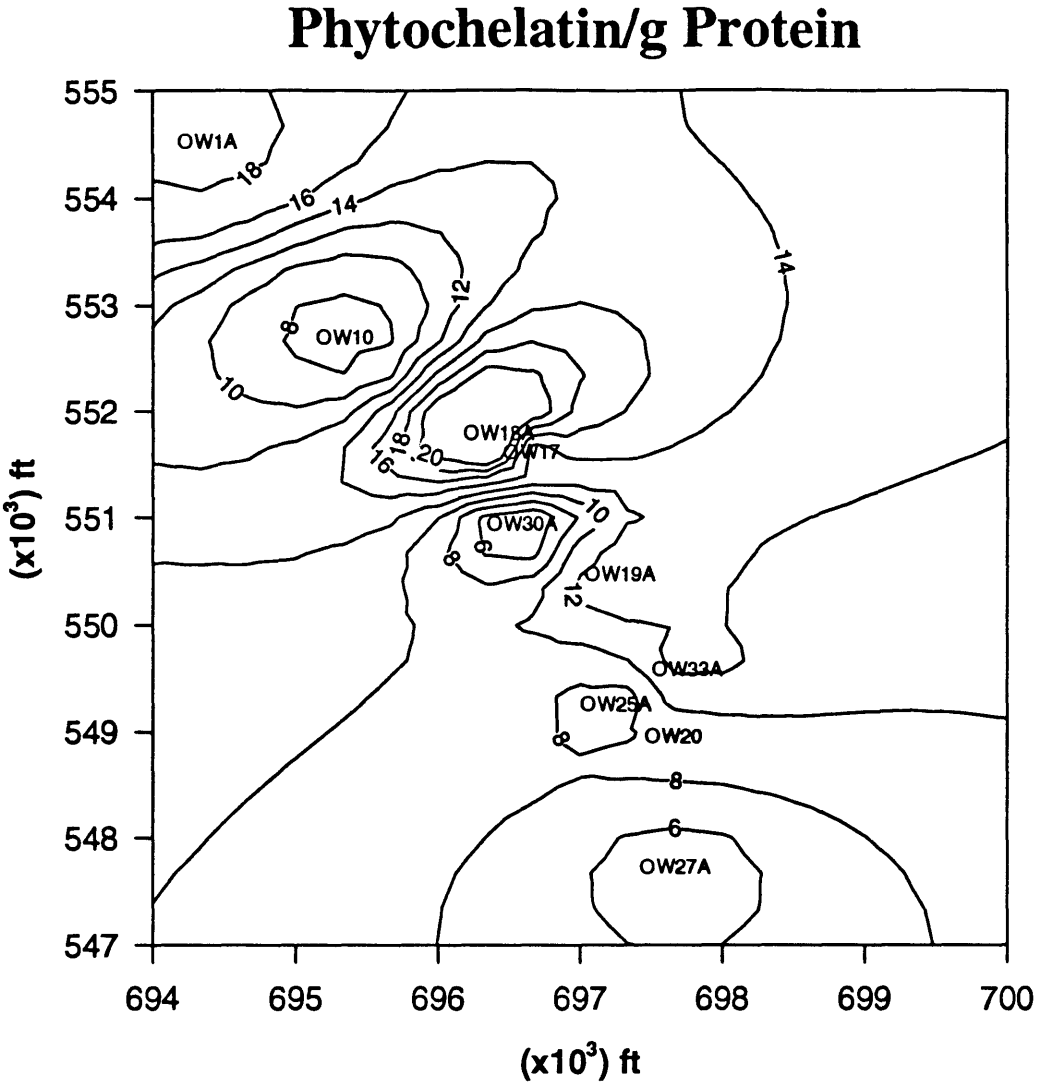

Figure 4.7: Spatial distribution of phytochelatin concentrations normalized to protein (given as nmol PC(n=2)/g protein) in glossy buckthorn trees located near monitoring wells around the Industri-Plex site.

(pooled from data for 1981 to 1993) have been measured in groundwater just to the north of these wells (GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994) [Figures 4.14, 4.15, and 4.17]. Cadmium exhibits high levels both in the same area where maximum phytochelatin levels are found on the east side of the river, and on the west side where phytochelatin levels are low [Figure 4.16]. However, no significant correlations were found between these four metals and

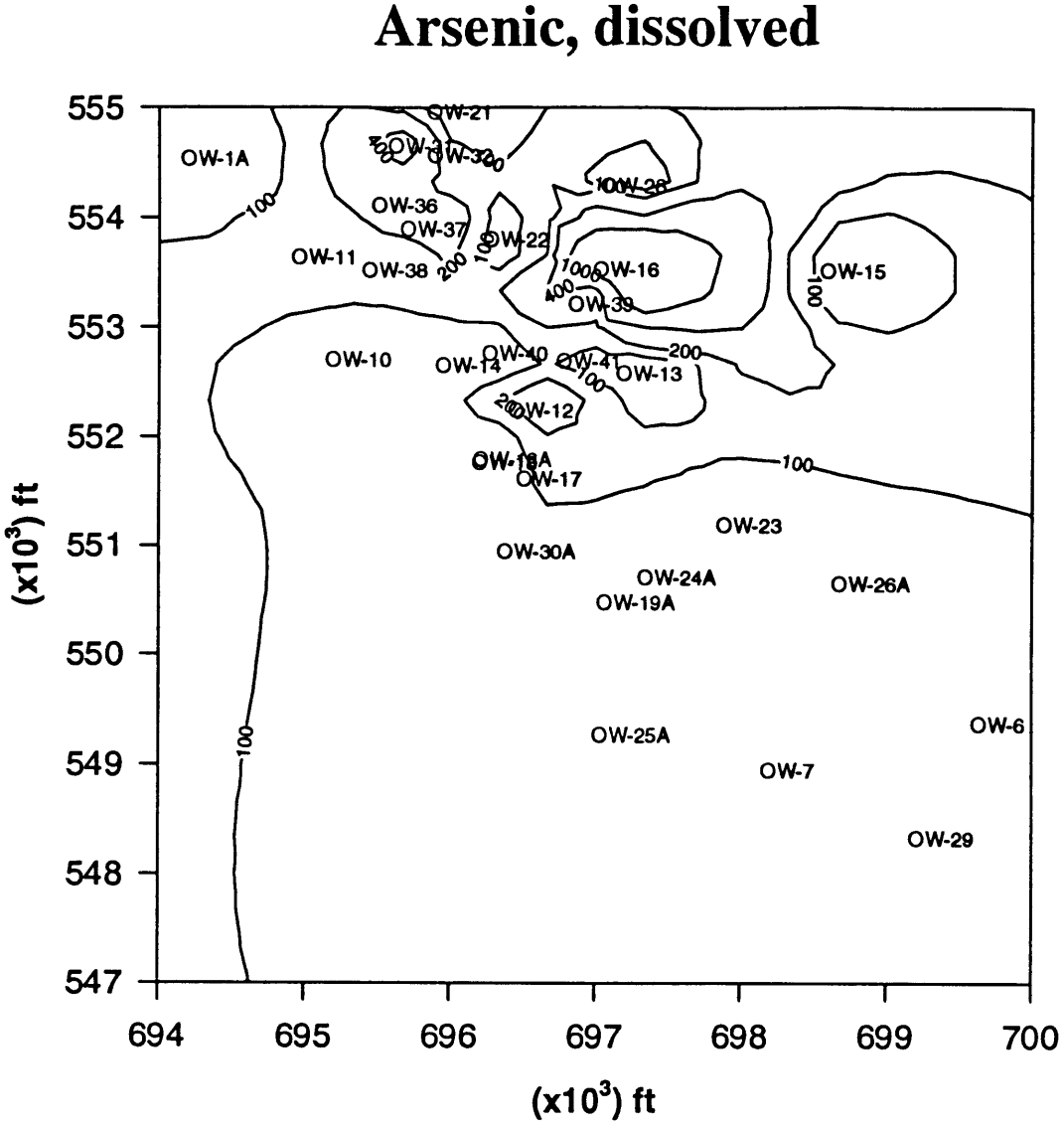

Figure 4.8: Spatial distribution of "dissolved" arsenic concentrations (given in ppb) in groundwater from monitoring wells around the Industri-Plex site (data from Roux Associates, Inc. et al. 1991).

phytochelatins when compared directly, possibly due to a lack of metals data and the time span of different sampling periods that are included [Figure 4.18]. A majority of the recorded metal measurements used in these figures for the Wells G&H area were collected in the early 1980's, and may not be consistent with measurements made a decade later.

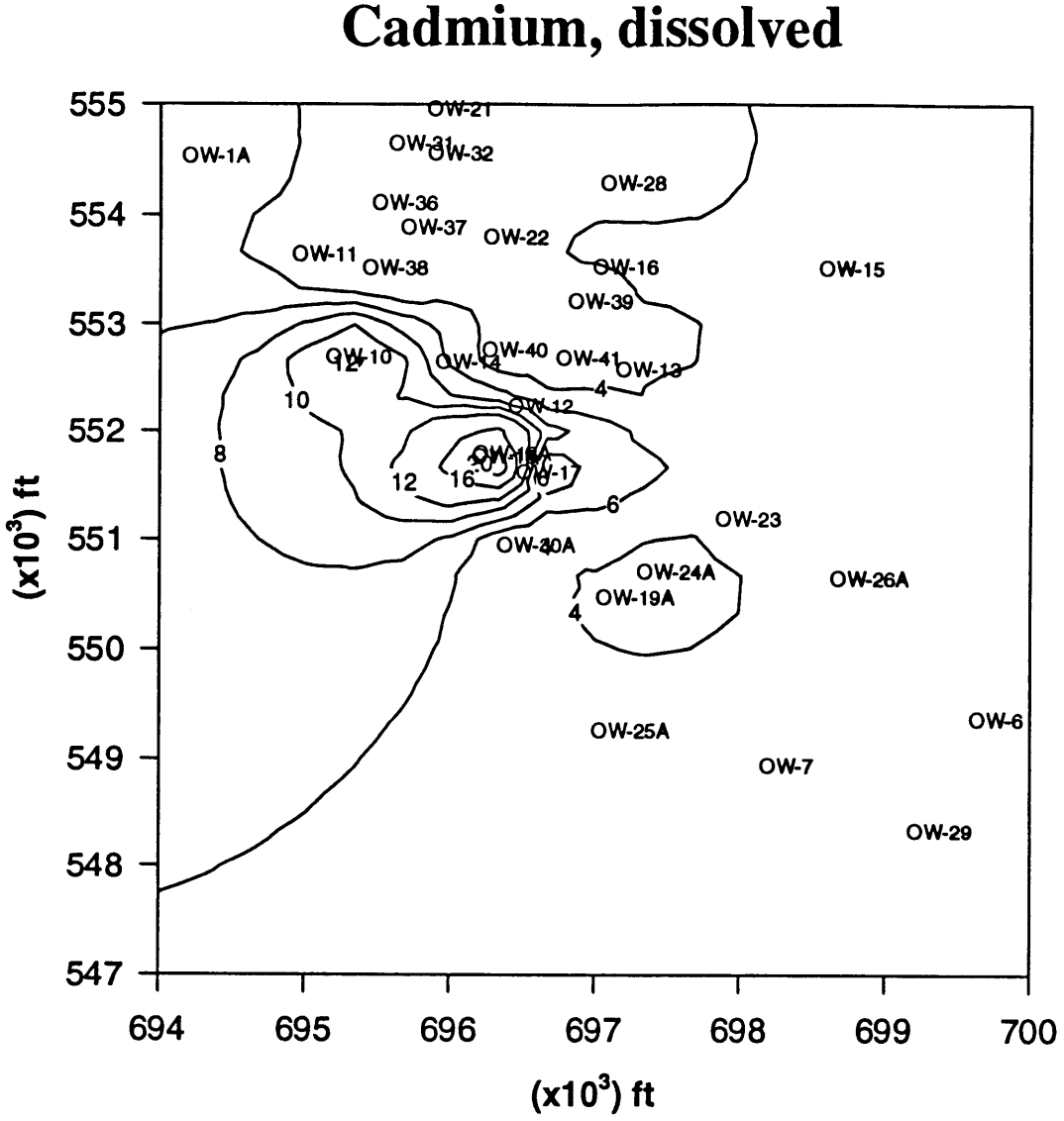

Figure 4.9: Spatial distribution of "dissolved" cadmium concentrations (given in ppb) in groundwater from monitoring wells around the Industri-Plex site (data from Roux Associates, Inc. et al. 1991).

Deciphering the metal pollutant source responsible for the high phytochelatin concentrations observed near Wells G&H is not easy without much further research. As stated by GeoTrans, Inc. and RETEC (1994), the source of the metals may still be the Industri-Plex site to the north. Metal contaminants are transported downstream from the Industri-Plex area, especially during

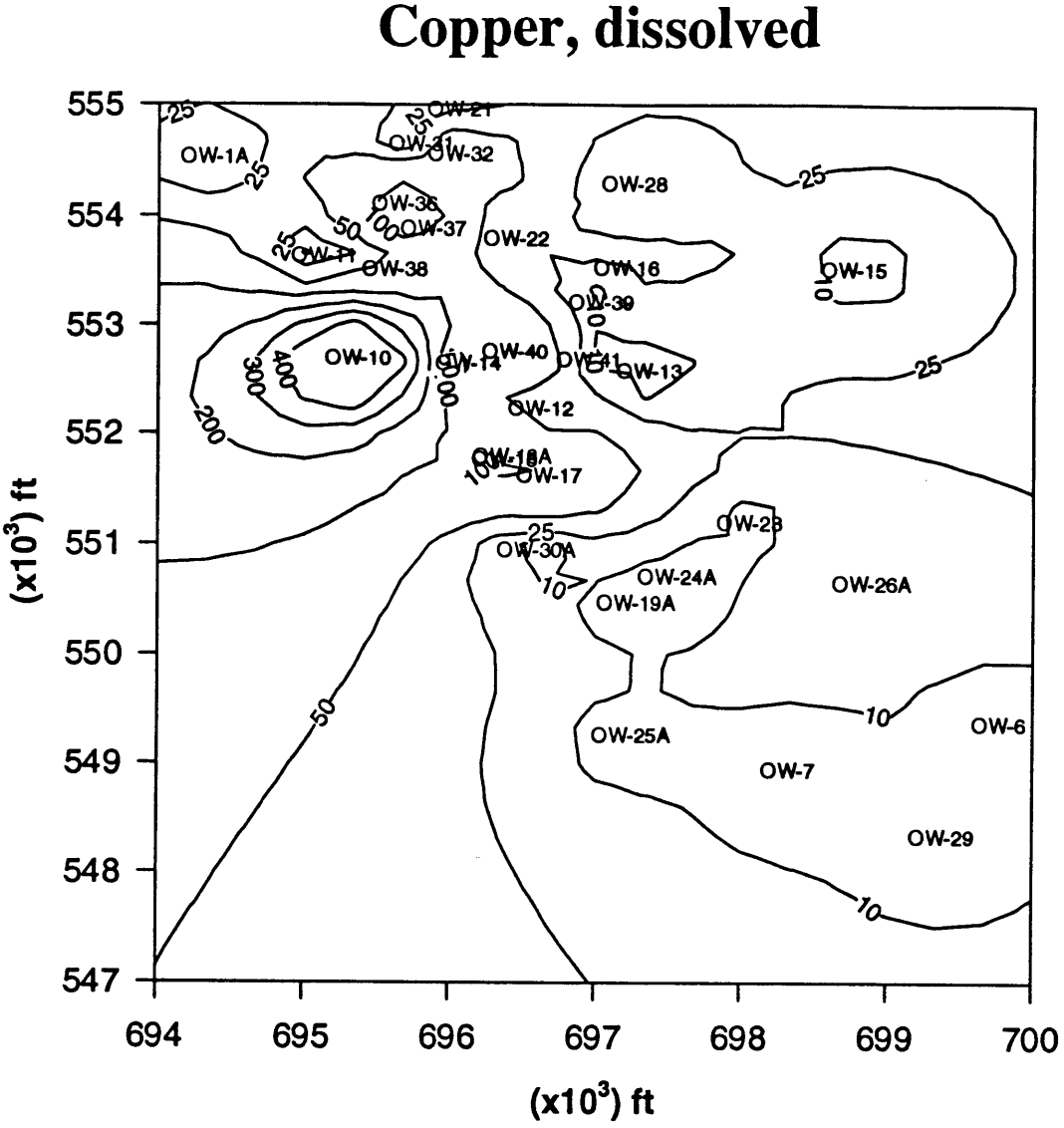

Figure 4.10: Spatial distribution of "dissolved" copper concentrations (given in ppb) in groundwater from monitoring wells around the Industri-Plex site (data from Roux Associates, Inc. et al. 1991).

storm flow (Solo-Gabriele 1995). Deposition of metals in the Wells G&H wetlands is aided by slow velocities and high concentrations of organic matter. Because groundwater feeds the Aberjona River, and not the opposite, under normal conditions contamination does not leave the wetlands. However, periodic flooding may provide a pathway for the movement of metals from

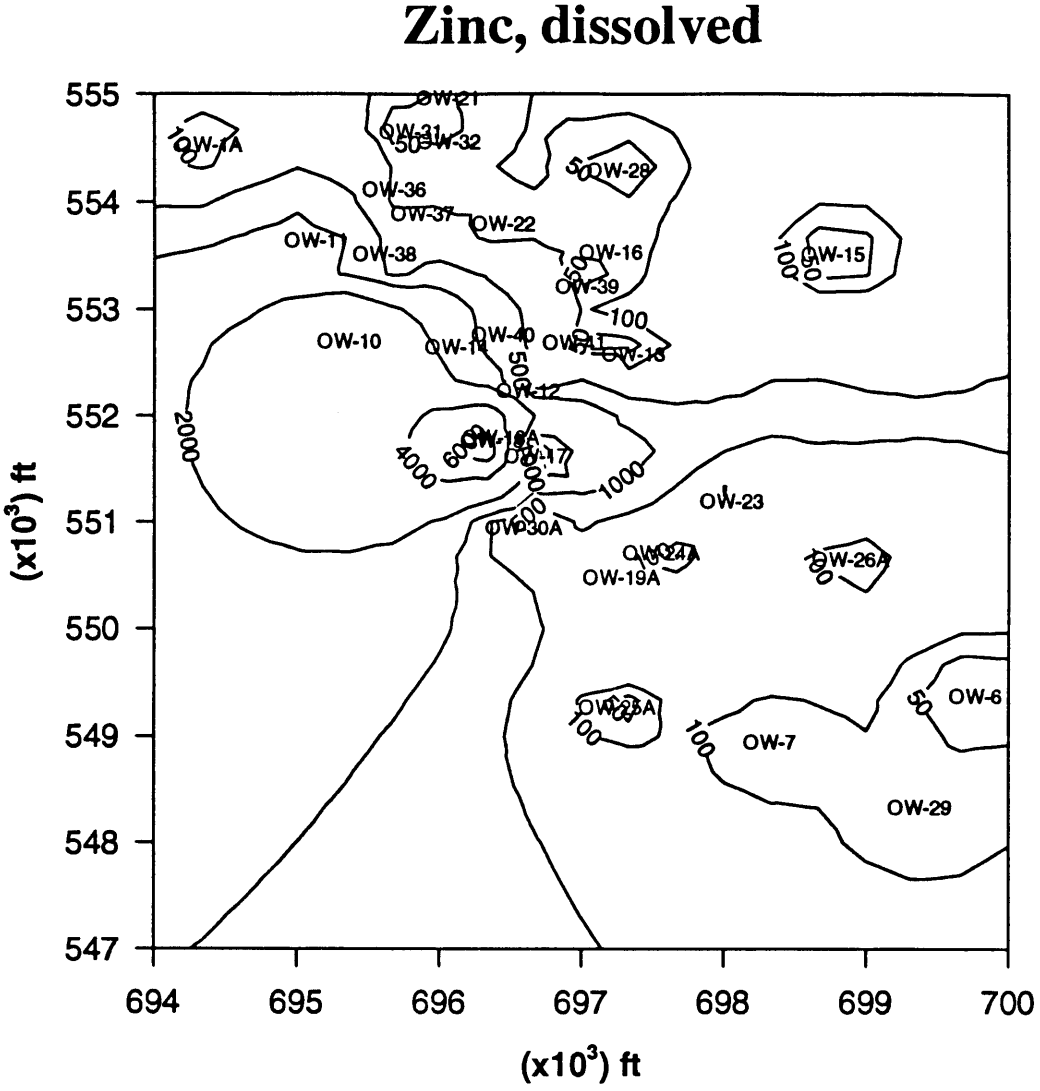

Figure 4.11: Spatial distribution of "dissolved" zinc concentrations (given in ppb) in groundwater from monitoring wells around the Industri-Plex site (data from Roux Associates, Inc. et al. 1991).

the wetland to the surrounding area. Another possible pathway was opened by the pumping of Well G and Well H for drinking water. Drawdown tests conducted by the USGS (1987) showed that pumping caused a reversal of groundwater flow in the area, thus contaminants in the stream were drawn toward the wells to the east. However, metals being pumped directly from the wetland toward the wells would not contaminate the area to the east of Well G and Well H. In

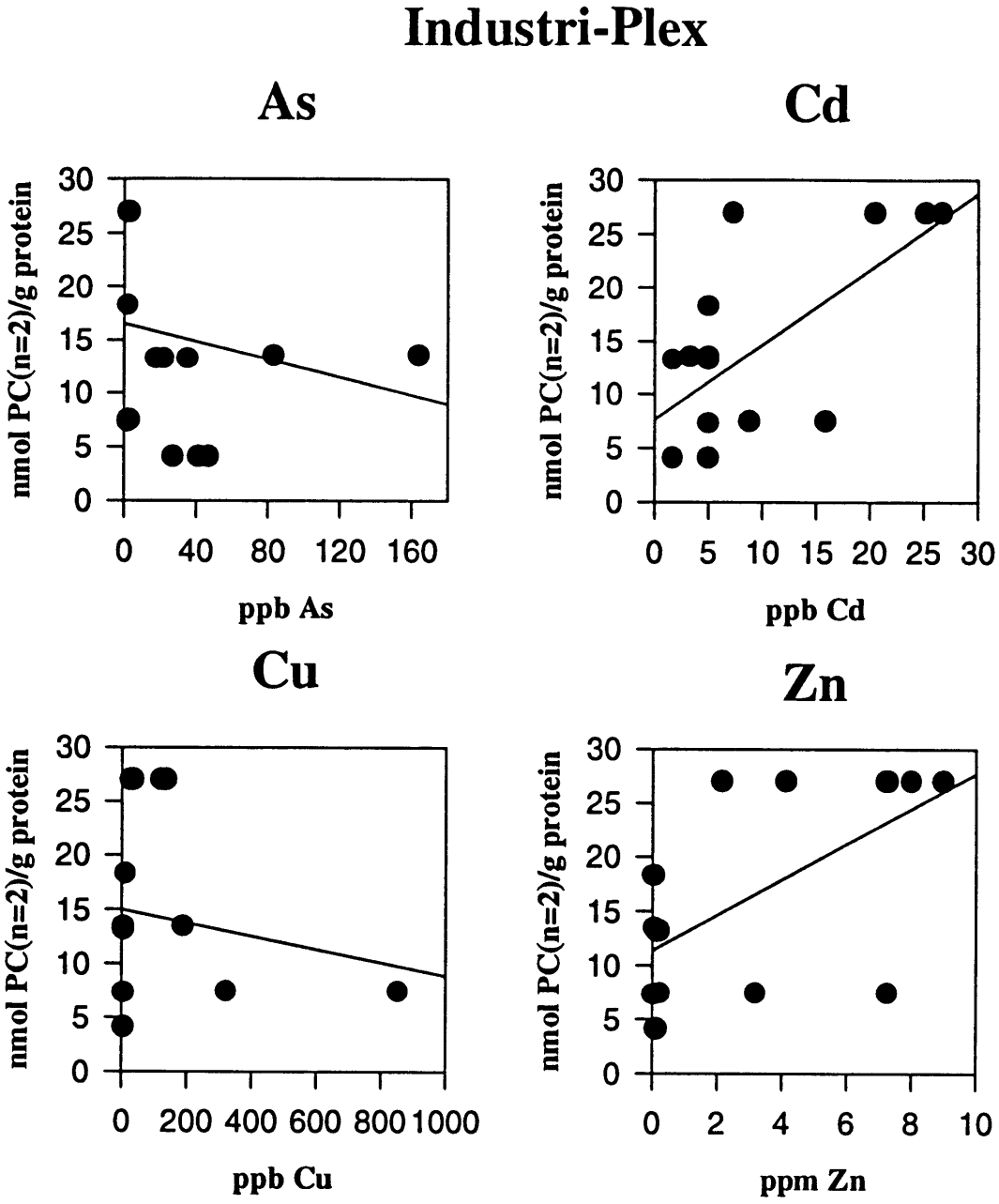

Figure 4.12: Phytochelatin and metals concentrations in glossy buckthorn trees growing near and groundwater from monitoring wells around the Industri-Plex site (metals data from Roux Associates, Inc. et al. 1991).

addition, work done by Zeeb (1996) has shown that the peat below the wetlands may have formed a fairly effective barrier against the movement of water from the stream adjacent to the wells.

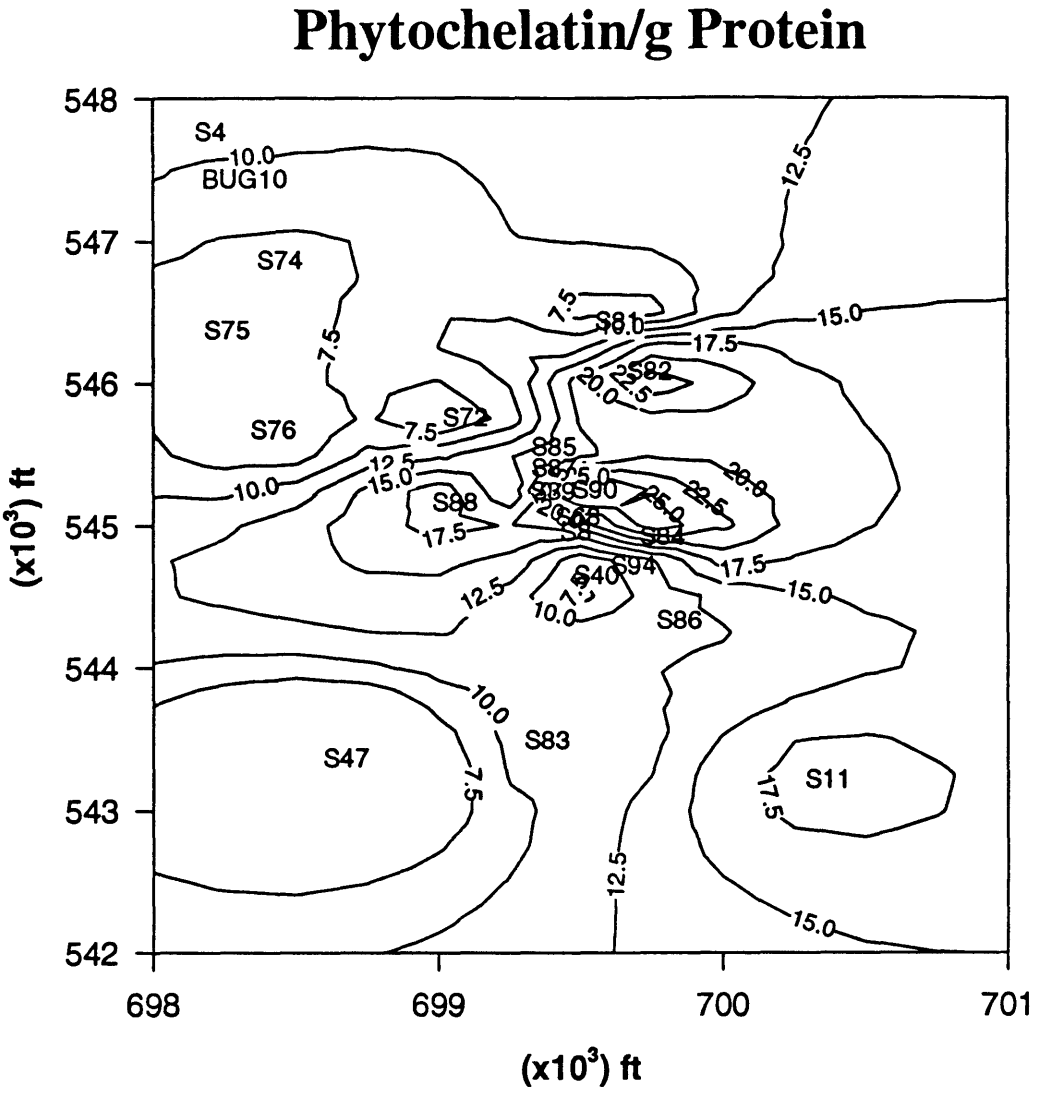

Figure 4.13: Spatial distribution of phytochelatin concentrations normalized to protein (given as nmol PC(n=2)/g protein) in glossy buckthorn trees located near monitoring wells around the Wells G&H site.

Since water was definitely lost from the river during pumping (USGS 1987), it is possible that the avenue for water to leave the river may have been at some distance upstream. This may have caused contaminated stream water from the north to infiltrate the area to the east of Wells G&H. This could also have been accomplished if Well G was pumping alone. However, flooding and pumping have not occurred for some time, and phytochelatin concentrations may respond rapidly

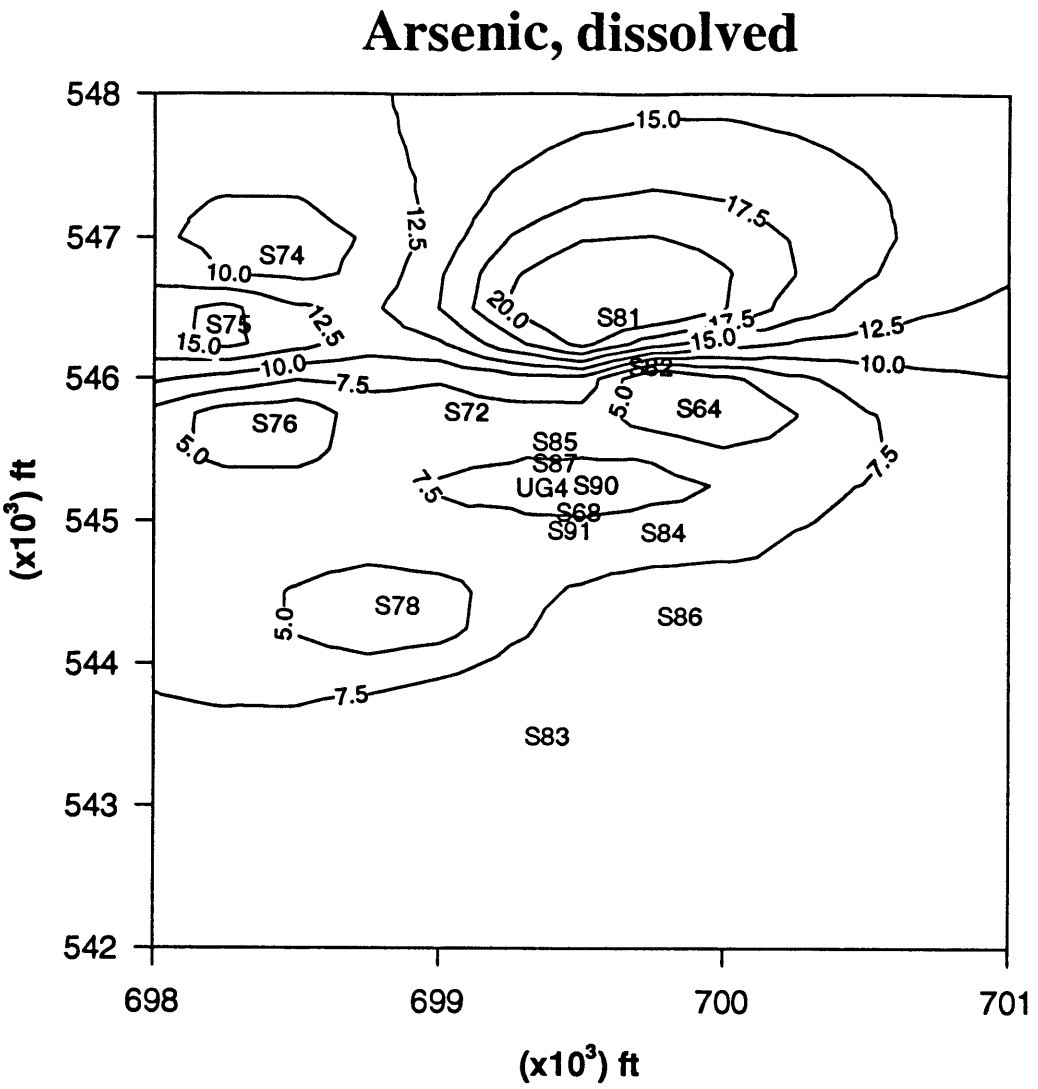

Figure 4.14: Spatial distribution of "dissolved" arsenic concentrations (given in ppb) in groundwater from monitoring wells around the Wells G&H site (data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994).

to changes in metal levels, therefore reflecting current rather than historic contamination. In addition, the highest concentrations of phytochelatins were found farther away from the wetlands, pointing to higher metal levels in that area as well. The wells where the highest values occur are located immediately to the west of a bedrock outcrop (USGS 1987). This barrier may cause decreased flow through the area to the west since groundwater generally moves from east to west

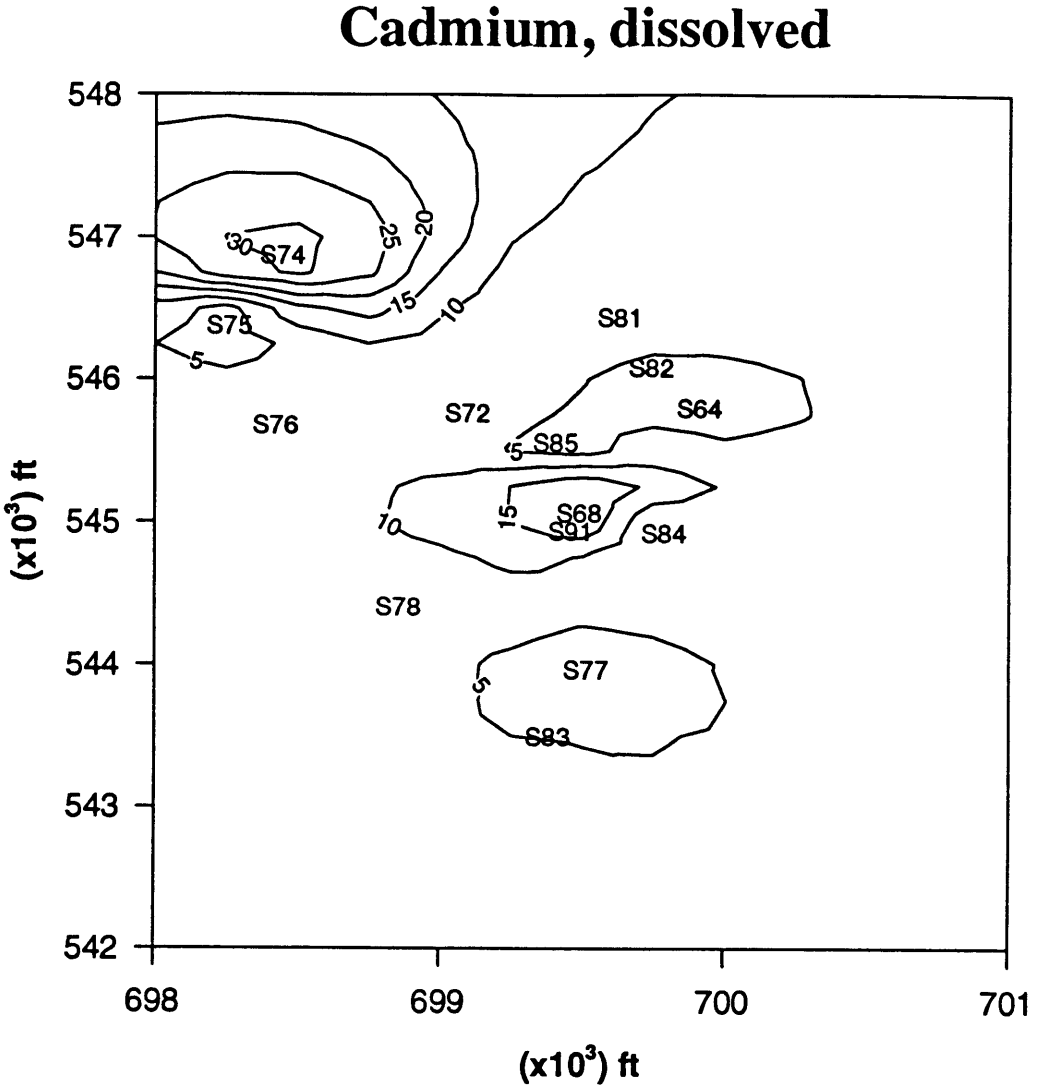

Figure 4.15: Spatial distribution of "dissolved" cadmium concentrations (given in ppb) in groundwater from monitoring wells around the Wells G&H site (data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994).

here. Thus, flushing of metal contaminants brought by past infiltration events may be slower near this barrier, leaving a pocket of pollution behind. The other possibility, given the number of industries that operated in this area (GeoTrans, Inc. and RETEC 1994), is that an unknown source of contamination exists upgradient of the Wells G&H site. In this case high phytochelatin values may indicate the leading edge of a contaminant plume coming from the west or northwest.

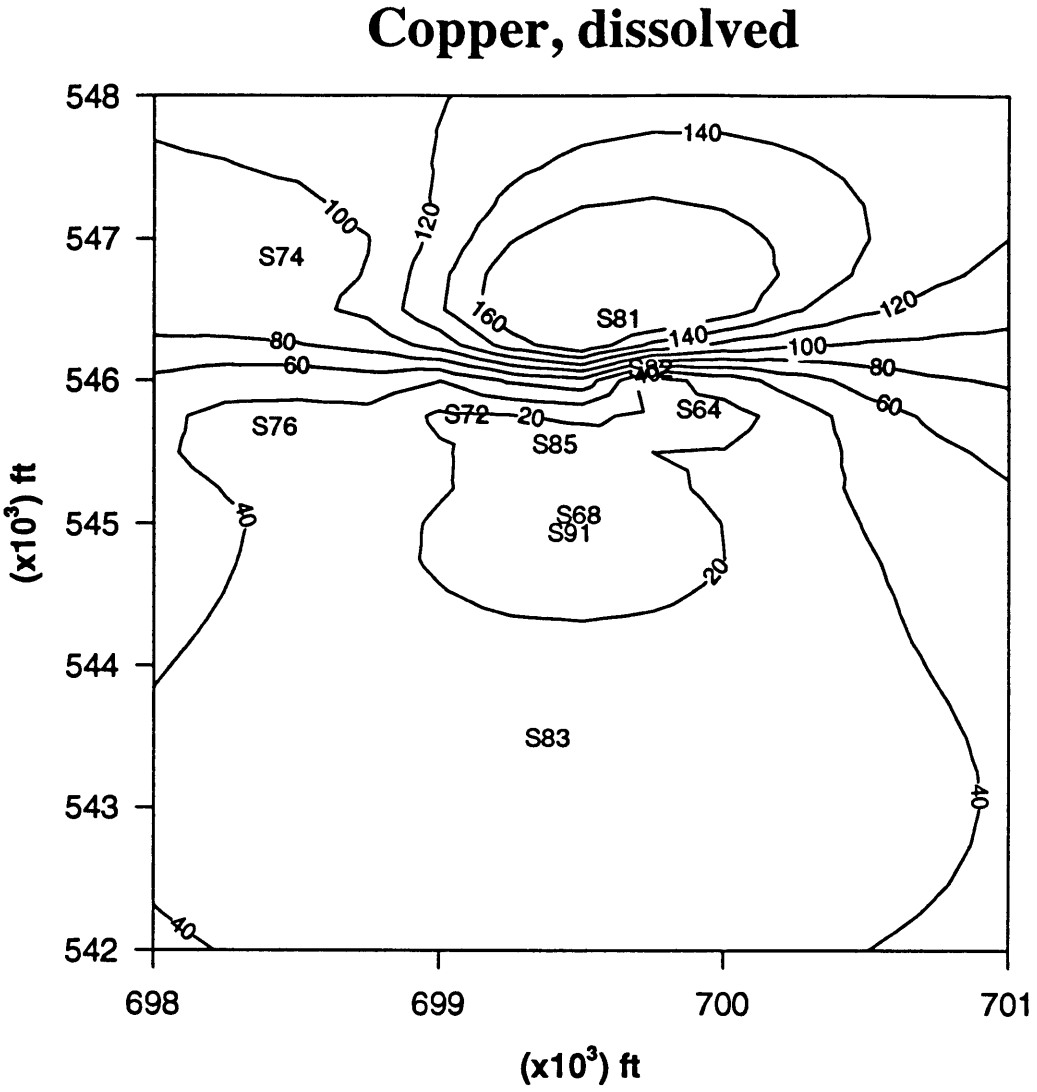

Figure 4.16: Spatial distribution of "dissolved" copper concentrations (given in ppb) in groundwater from monitoring wells around the Wells G&H site (data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994).

Again, further research is needed to pinpoint the source responsible for our findings in the Wells G&H area.

It has been stated by GeoTrans, Inc. and RETEC (1994) that:

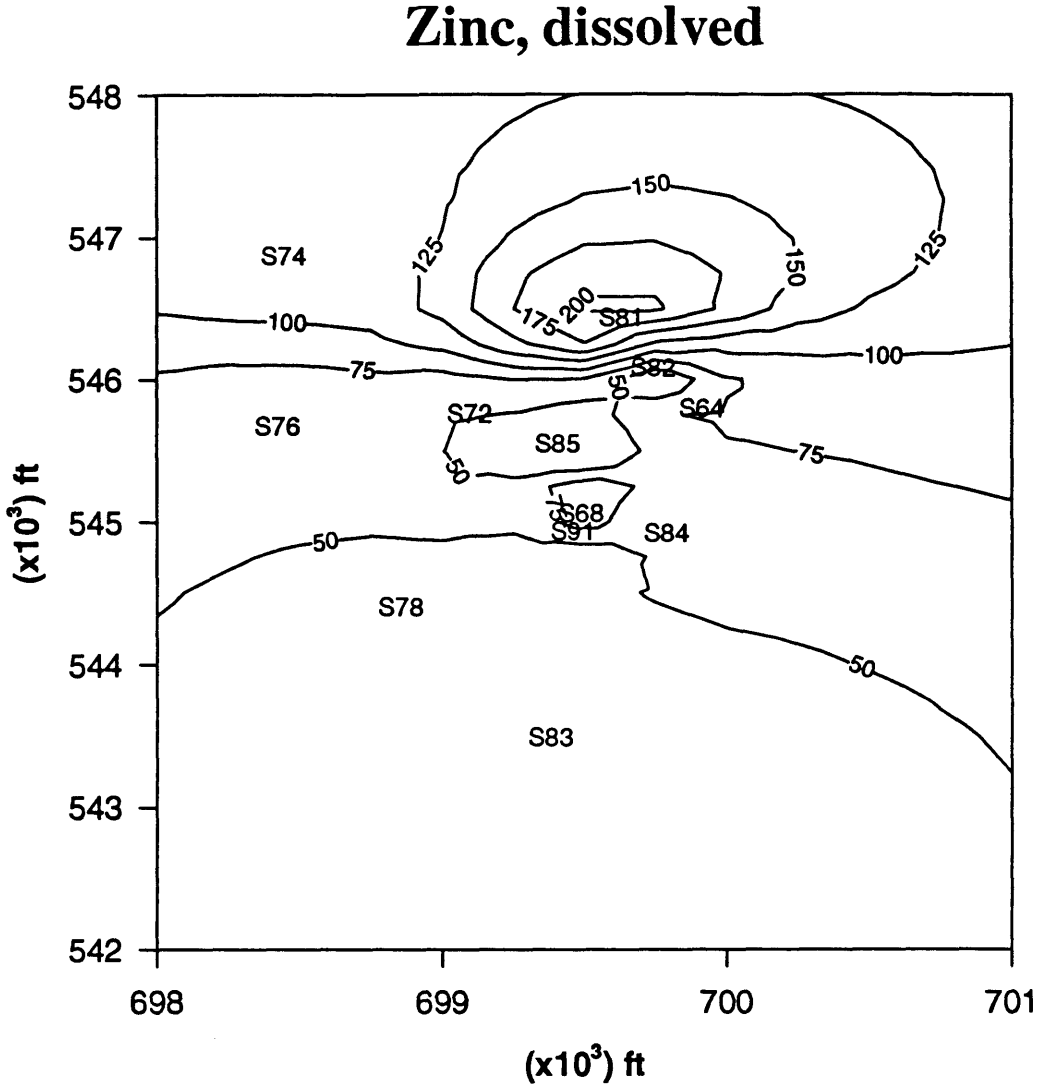

Figure 4.17: Spatial distribution of "dissolved" zinc concentrations (given in ppb) in groundwater from monitoring wells around the Wells G&H site (data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994).

The numerous contamination sources and historic changes in hydrologic conditions within the Central Area have resulted in widespread distribution of the many types of contaminants, such that it is not possible to define or map individual contaminant plumes for any significant distance.

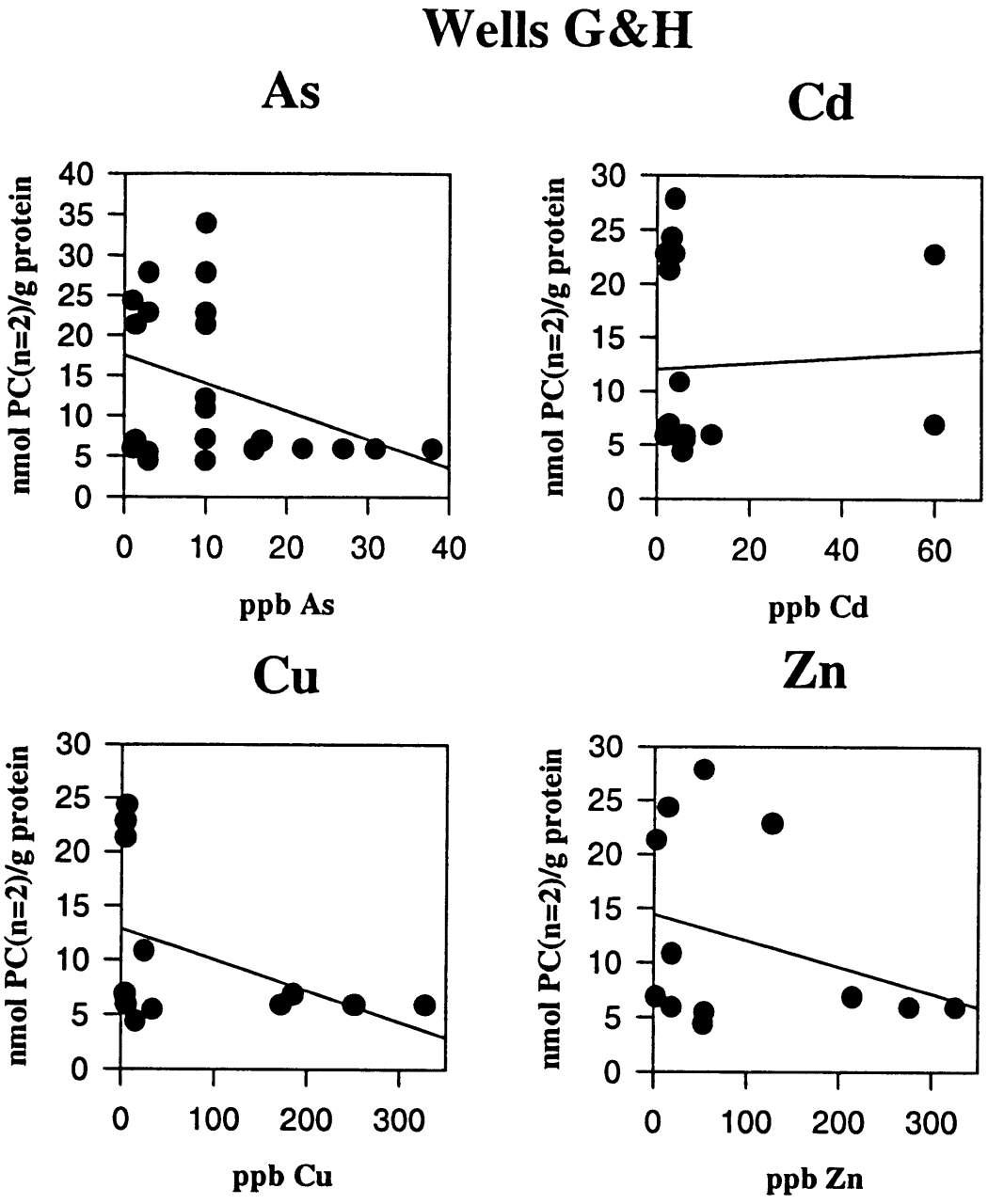

Figure 4.18: Phytochelatin and metals concentrations in glossy buckthorn trees growing near and groundwater from monitoring wells around the Wells G&H site (metals data from GeoTrans, Inc. 1987, GeoTrans, Inc. and RETEC 1994).

Using phytochelatin measurements alone, however, we have defined three distinct zones of metal contamination in the shallow groundwater of the Aberjona watershed: (1) the area extending from

the East Drainage Ditch to the bottom of HBSA in the north, (2) the area in and immediately downstream of the Wells G&H wetlands, and (3) the area to the east of the old drinking water wells (Well G and Well H) and to the west of the bedrock outcrop. The first and second locations have also been determined to be highly contaminated areas by consulting companies using large numbers of expensive wells, but our data defines a third location that was not found by these consultants. Therefore, phytochelatin measurements may be a more sensitive and robust indicator of subsurface metal pollution than metal concentrations collected from monitoring wells. In addition, our sampling technique is ideally suited to sites where access to private land may be a problem, since it is non-destructive and non-intrusive, and samples may be collected from a large area in a short span of time. As long as plants are available and groundwater is accessible to plant roots, this technique is applicable and much faster and easier than alternative methods. We look forward to applying this technique to other field sites where its effectiveness may be tested further.

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Chapter 5

Other Experiments and Future Work

Our results from three different field sites with very different metal pollution conditions show that phytochelatin measurements are effective biomonitors of metal stress for *in situ* studies of metal contamination. Having three different field sites also makes it possible to compare phytochelatin values among them. In addition, samples collected on a regular basis at MIT from 1994 to 1995 provide data from which we can infer a general pattern for background urban metal pollution effects and seasonal changes in phytochelatin levels. Site-to-site comparisons, some other preliminary work, and future research plans are discussed in this chapter.

It is known that metal contamination in the vicinity of Sudbury, Ontario, is extremely high and easily surpasses levels found in either the Aberjona watershed or the Appalachian Mountains. Pollution in the Aberjona watershed, with contributions from both urban air and groundwater contamination, comes in second, leaving the Appalachians with the lowest metal levels. Phytochelatin concentrations in the leaves and needles of trees growing on these sites also exhibit this order. Figure 5.1 and Table 5.1 show that the maximum phytochelatin level measured in Sudbury is almost seven times greater than in the Aberjona watershed, which in turn is almost five times greater than in the Appalachian Mountains. Data in Figure 5.1 include measurements of red spruce in 1995 (Appalachian Mountains), glossy buckthorn in 1994 (Aberjona watershed), and paper birch in 1993 (Sudbury, Ontario), all normalized to protein. Although the n=3 and n=4 chainlengths were detected in foliage from all sites except the Appalachians, this was almost always a small fraction of the total γGlu-Cys units measured, and the n=2 chainlength alone is reported at all sites for consistency. Maximum values from MIT (measured in *Rhododendron sp.*) are similar in magnitude to those found in the Aberjona watershed, but baseline values are higher

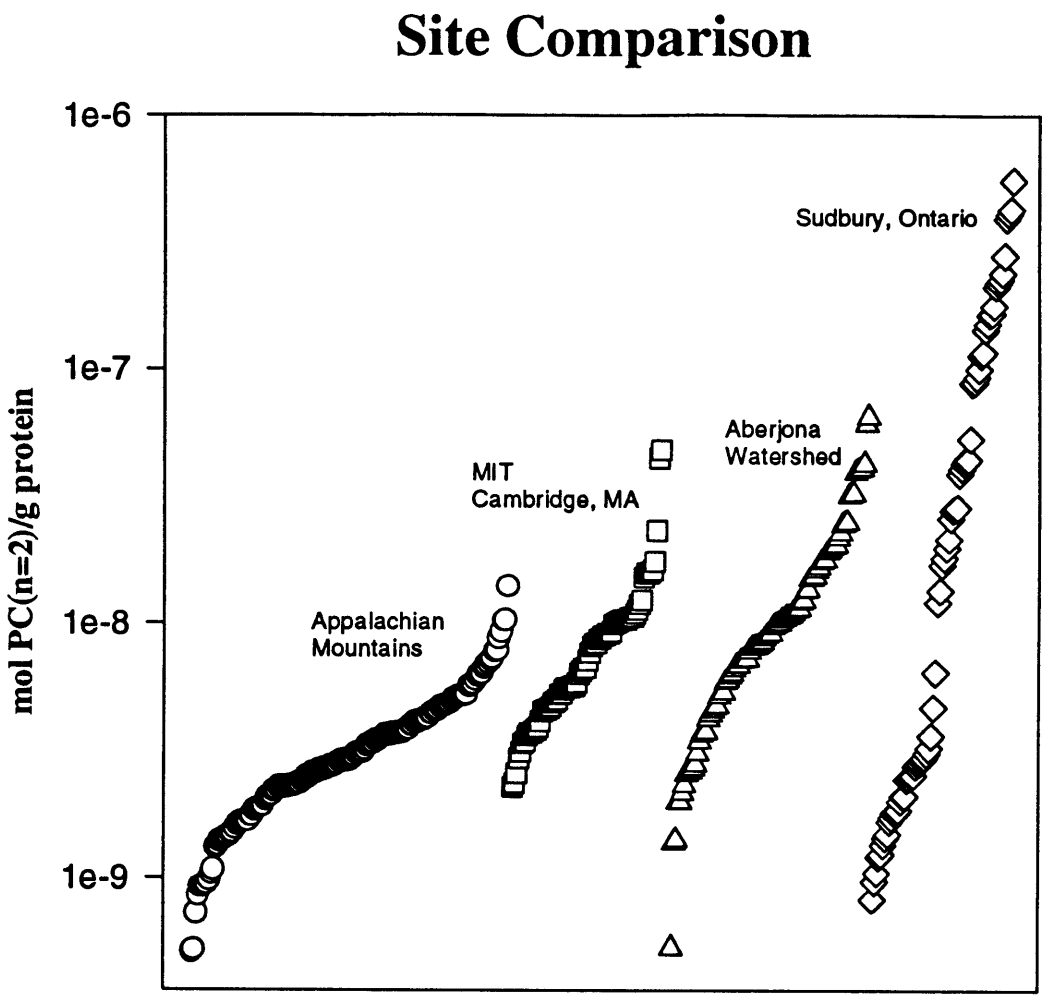

Figure 5.1: Site-to-site comparison of the range of measured phytochelatin concentrations normalized to protein. Each data point is an individual sample.

than at any other site. The location of the tree sampled at MIT is near to a heavy traffic area. This source may provide a relatively high and consistent metal load such that baseline phytochelatin levels are elevated. All other sites reach values below 1 nmol PC(n=2)/g protein, even with maximum values differing by one and a half orders of magnitude. This suggests that if phytochelatins are used for metal homeostasis in plants, as discussed in Chapter 1, constitutive levels are on the order of 1 nmol PC(n=2)/g protein in all trees.

Sampling Site	Minimum Concentrations [nmol PC(n=2)/g protein]	Average Concentrations [nmol PC(n=2)/g protein]	Maximum Concentrations [nmol PC(n=2)/g protein]
Whiteface Mountain, NY	< 0.5	3.3	14.0
MIT, Cambridge, MA	2.3	8.9	48.2
Aberjona Watershed, MA	< 0.5	12.8	65.3
Sudbury, Ontario, Canada	0.8	74.4	426.4

Table 5.1: Minimum, average, and maximum phytochelatin concentrations at all sites.

Numerous questions arise from the research described in the previous chapters. We have already undertaken answering some of these, although further analyses are required. One of the first questions that arises from our research in the northern Appalachians is, to what degree does the production of these levels of phytochelatins stress the trees? This is difficult to quantify in the field, and as imperfect as they may be, initial laboratory studies using red spruce seedlings have been attempted to help derive a dose/response curve for metals. We are attempting to quantify correlations between metal dose, phytochelatin production, and changes in gross physiological parameters – i.e. chlorophyll concentrations and root elongation. To do this quantitatively, Laurel Schaider, an undergraduate working in the Parsons Laboratory at MIT, has developed an initial protocol for growing red spruce seedlings hydroponically in well-characterized aqueous medium. A description of the methods used and initial results are included in Appendix D. Initial results are inconclusive, probably due to germination of seeds in vermiculite and the subsequent carryover of metals which could not be effectively removed without damaging the roots. This points out the importance of using trace metal clean techniques in the laboratory. We have, however, remedied this and hope to analyze the results in the near future.

This attempt to develop a dose/response relationship for metals does not imply that we believe

that metals are the only cause of red spruce decline. On the contrary, we also wish to examine in the future the possible synergistic relationships with other pollutants discussed in Chapter 2. Because the building blocks of phytochelatins, glutathione molecules, are also important in the antioxidant defense systems of plants, we would like to examine glutathione levels in field samples already collected to determine whether phytochelatin production is a significant sink, thus increasing oxidative damage from oxygen radicals produced by air pollutants. This possibility was addressed by De Vos et al. (1992) and Tukendorf and Rauser (1990) who showed that phytochelatin production depleted the available glutathione pool in plants causing increased oxidative damage. In addition, Rennenberg and Brunold (1994) discuss the large number of other stresses that also involve glutathione, including drought, pathogen attack, cold, xenobiotic exposure, excess sulfur, and heat. The relationship between glutathione levels and forest damage has been investigated in Europe (Schmieden et al. 1993), where a correlation has been found between ratios of oxidized to reduced glutathione and ozone levels. It thus seems possible that a drain of the glutathione pool by metal-induced phytochelatin production may have numerous repercussions for tree health, especially at high altitudes where environmental conditions impose added strains.

Another aspect of the relationship between phytochelatin production and glutathione levels that bears investigation is the effect of natural cycles and timing on possible synergisms. Schupp and Rennenberg (1988) found that levels of glutathione vary diurnally, and Esterbauer and Grill (1978) measured seasonal changes in glutathione concentrations in *Picea abies*. Our results from *Rhododendron sp.* growing near MIT – and thus subject to urban air pollution – suggest a short-

term increase in phytochelatin levels during the month of May, the beginning of the growing season in Cambridge [Figure 5.2]. Additional samples were collected during 1994 and 1995, but they have not yet been analyzed. Phytochelatin concentrations in red spruce from the Appalachian Mountains during 1993 also show a distinct peak near the beginning of the growing season [Figure 2.2]. Further measurements of seasonal and diurnal variations in phytochelatin and glutathione levels may possibly show that trees are exceptionally vulnerable to metal poisoning or oxidative damage during this window in time.

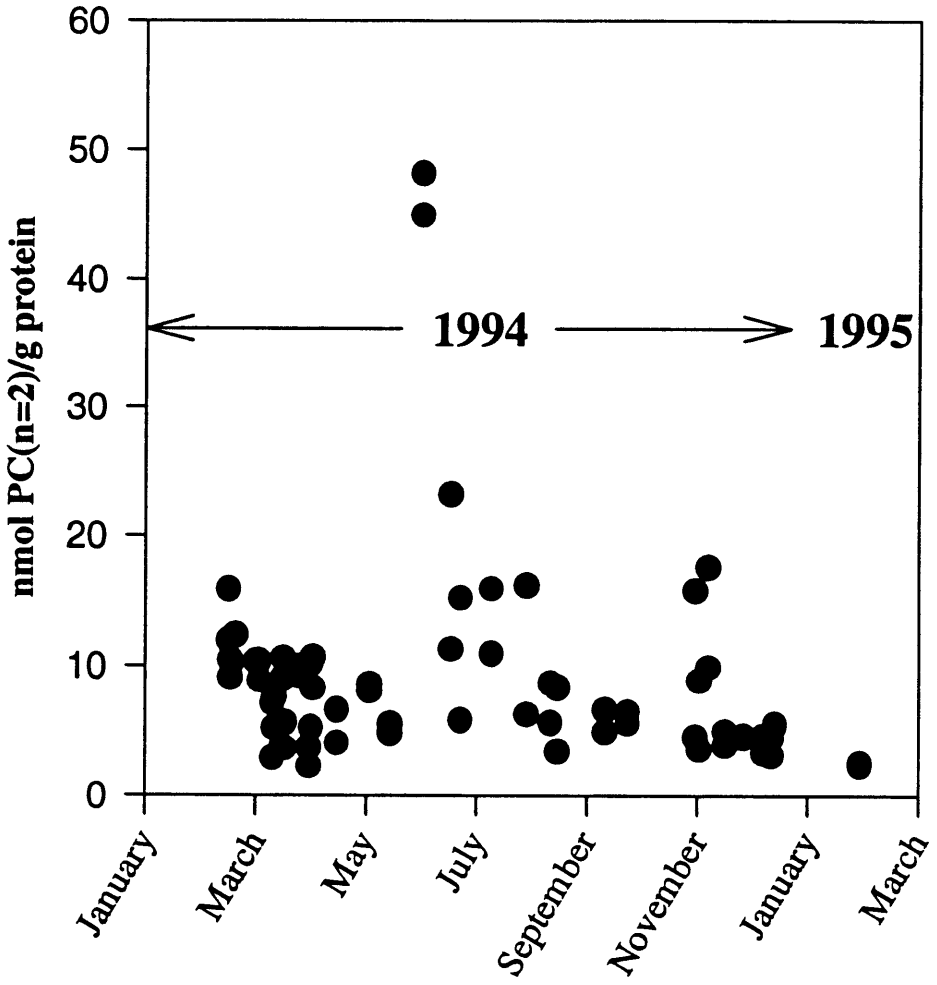

Figure 5.2: Phytochelatin concentrations normalized to protein over one year in a single *Rhododendron sp.* tree located at MIT, in Cambridge, Massachusetts.

In addition, it would be informative to assess whether cloud immersion, precipitation, or fog events create short-term stressful conditions due to relatively high influxes of metals. Results from Sudbury, Ontario, in Chapter 3 show that direct uptake of metals by foliage may be an important pathway for metal uptake. This may also be important for red spruce, growing on soils with high concentrations of metal-complexing organic matter. Thus repetitive applications of metals to the needles by moisture-events may be more stressful than metals accumulated in montane soils. Direct applications of cadmium to red spruce saplings on Mt. Moosilauke in 1994 were inconclusive (data not shown), but further field experiments are planned. We would also like to investigate further the actual chemical and physical processes involved in the direct uptake of metal ions by foliage.

Finally, our results from the northern Appalachians beg a follow-up study comparing red spruce decline in the Northeast to episodes of forest decline documented in the southern Appalachians, Europe, and elsewhere. Concentrations of metals in the montane soils of Europe are at least an order of magnitude greater than in the northern Appalachians (McLaughlin 1985), suggesting that phytochelatins may show an even stronger correlation between metal stress and forest damage there.

Some of the avenues for future research discussed for the Appalachian Mountains are also applicable to the other field sites we have studied, and other specific questions have evolved from our research in Sudbury and the Aberjona watershed. Our data show that aerial deposition of metal pollution is responsible for the metal stress response seen in trees in Sudbury.

Measurements of metals deposited at these sites support this finding. However, we have not been able to show what transport and deposition mechanisms are responsible for this pattern of metal deposition, or why high metal concentrations in the soils are not mobile enough to influence phytochelatin levels in the foliage. In the Aberjona watershed we hope to further elucidate the source of metal contamination responsible for high phytochelatin levels to the east of Wells G&H. In addition, this site offers an ideal area for testing the use of phytochelatin concentrations in aquatic plants as biomonitors of metal stress in waterways.

The answers to the questions listed above are applicable not only to the sites we have already studied, but also to the general characterization of metal contamination and mobility anywhere. Metals are unique among pollutants in that they cannot be destroyed by biotic metabolism. Thus, problems associated with metal contamination will not necessarily go away if left alone. However, since limited resources are available for dealing with cleanup operations, it is necessary to be able to assess the potential impact of metals at a particular site before committing funds to remediation. Phytochelatin measurements offer a low cost method for determining the physiological response of native plants to metal pollution, thus perhaps providing a relevant quantification of the need for cleanup. The use of specific molecular markers, such as phytochelatins, for field studies is a developing area, but it may become an important method for research in the future.

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Appendix A

Phytochelatin Analysis Method

Grinding and Filtration

(1) Add approximately 0.1 g wet weight needles or leaf tissue to 5 ml 0.01 M methanesulfonic acid in a glass test tube.

(2) Grind on ice for 5 min at 15,000 rpm using Brinkmann Instruments' Polytron Homogenizer PT3000 (with or without pulverizing first in liquid nitrogen with a ceramic mortar and pestle).

(3) Decant into 1.5 ml eppendorf tubes and centrifuge at 16,000g for 30 min in the refrigerator.

(4) Filter approximately 1 ml through 0.2 μm Nalge syringe filter and store the remainder in the refrigerator for further analyses.

Sample Preparation for HPLC

(1) Add 960 μl filtered (and diluted) sample, 100.8 μl of buffer solution containing 100 mM sodium borate and 10 mM diethylenetriaminepentaacetic acid (DTPA), and 36 μl of 15 mM dithiothreitol (DTT) [made fresh daily] to a 1.5 ml eppendorf tube. Shake and let react 10 min.

(2) Add 36 μl of 50 mM monobromobimane (mBBr) dissolved in acetonitrile [prepared ahead and stored in freezer]. Shake and let react 10 min in the dark.

(3) Add 18 μl of 60 mM cysteine [made fresh daily]. Shake and let react 5 min in the dark.

(4) Add 72 μl of 1.0 M acetic acid and vortex a few seconds. Decant into amber vials for autosampler on HPLC.

HPLC Method

This method uses two solvents:

Solvent A: 1.61 ml of 17.4 M glacial acetic acid, 4.7 ml of 1.0 M sodium acetate, 135 ml acetonitrile, 0.2734 g tetraoctylammoniumbromide, Q-water, and dropwise 1.2 N HCl to bring the pH to 4.2 in a total volume of 780 ml. This is filtered through a 0.2 μm polycarbonate membrane filter and degassed before use.

Solvent B: 100% acetonitrile.

A Beckman Instruments System Gold HPLC with a 200 ml loop on the autosampler, a Gilson 121 fluorometer, and an Adsorbosphere HS C18 5 μm (250 mm) column were used. The HPLC method was as follows:

Running Time	% Solvent A	% Solvent B	Ramp Time
0 min	100	0	--
0-15 min	60	40	15 min
15-42 min	52	48	27 min
42-72 min	15	85	30 min
72-102 min	100	0	2 min

N.B. The method presented here is largely derived from work done by Dr. Beth Ahner and is repeated here for completeness.

Appendix B

Additional Data from the Northern Appalachian Mountains

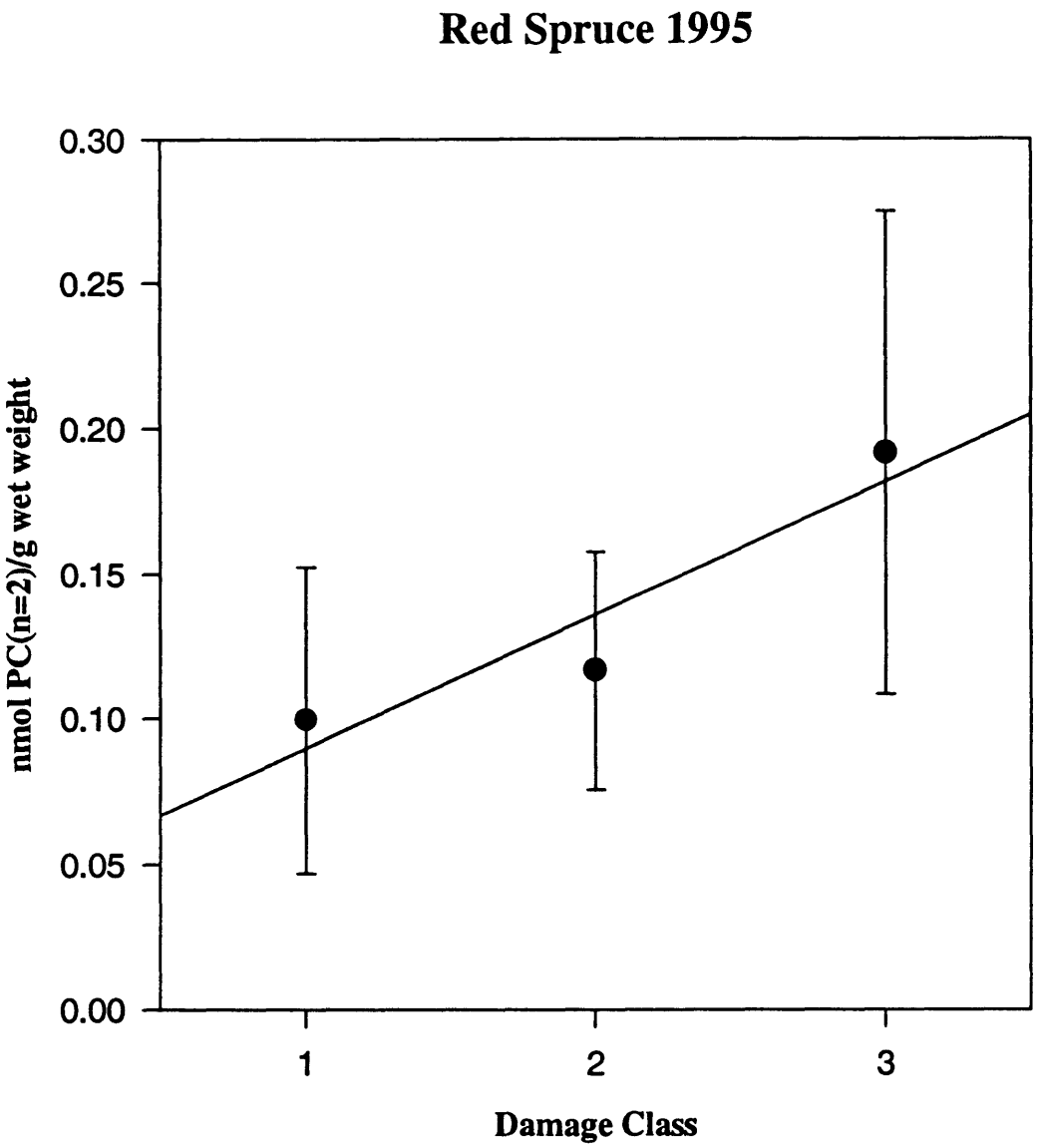

Figure B.1: Phytochelatin concentrations normalized to wet weight as a function of damage classification in foliage taken in July, 1995, from red spruce trees at 1000 m on Whiteface Mountain, NY.

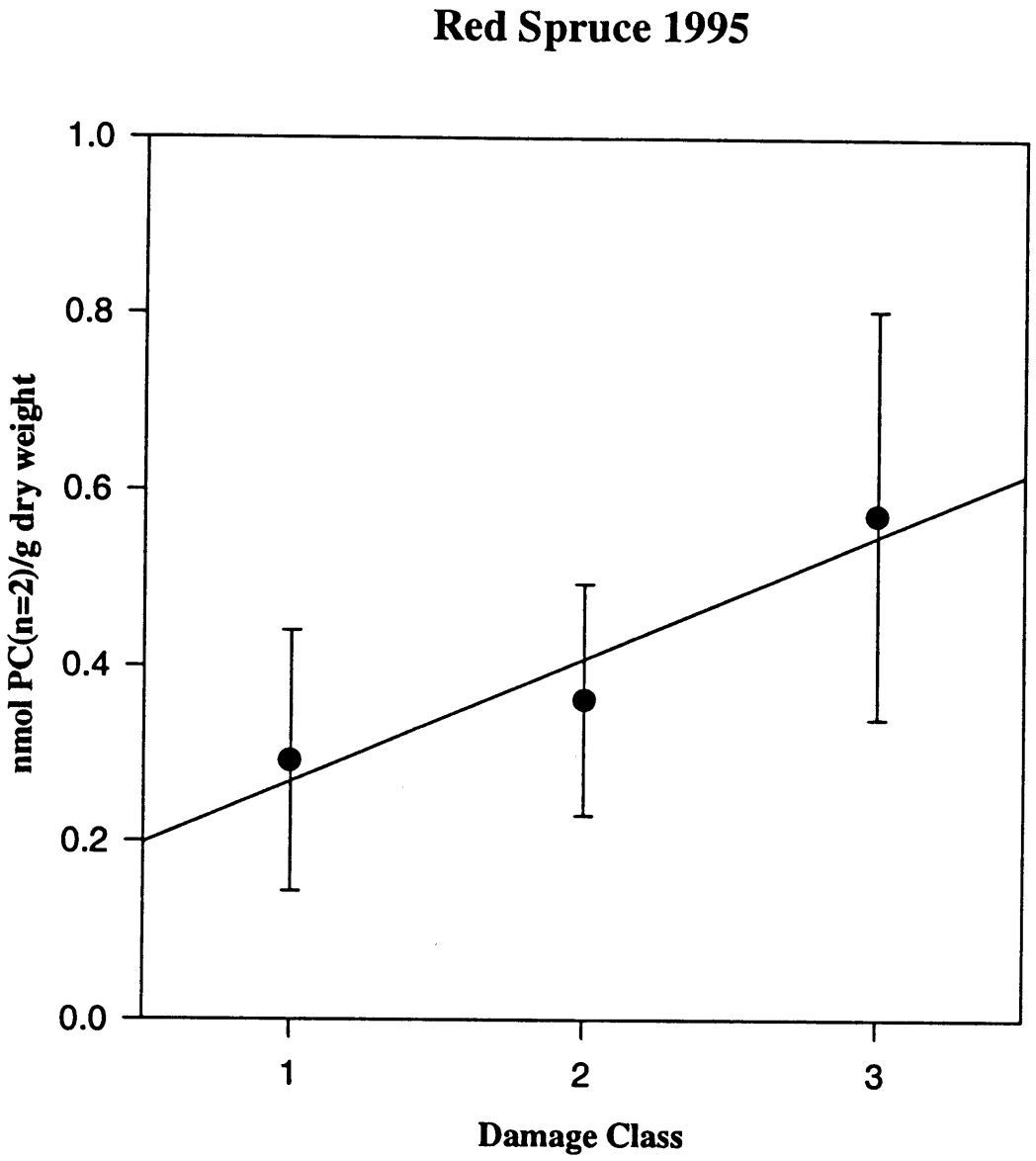

Figure B.2: Phytochelatin concentrations normalized to dry weight as a function of damage classification in foliage taken in July, 1995, from red spruce trees at 1000 m on Whiteface Mountain, NY.

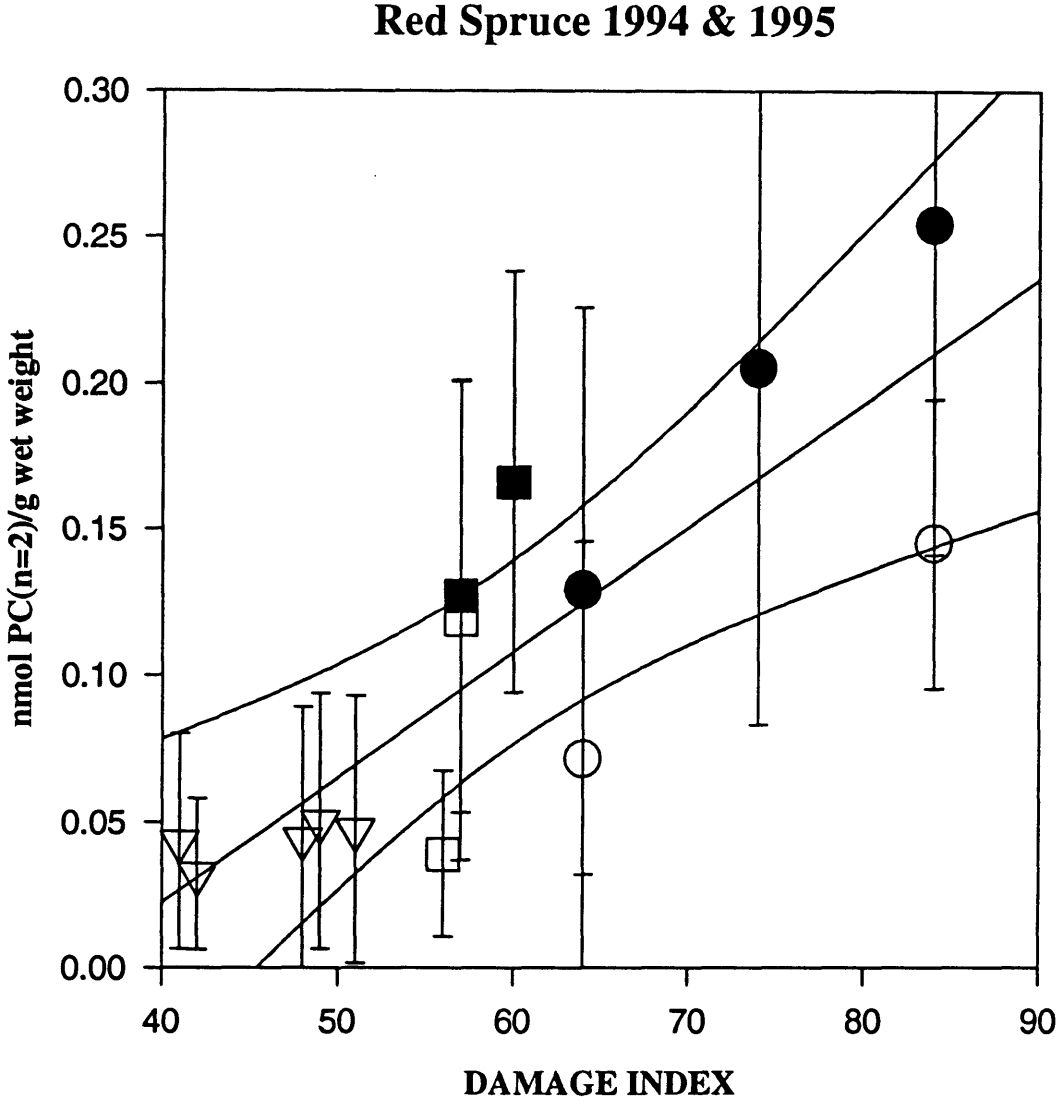

Figure B.3: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1994 and 1995 from red spruce trees at 1000 m on eleven different mountains in the Northeast, compared to red spruce damage index in the stand.

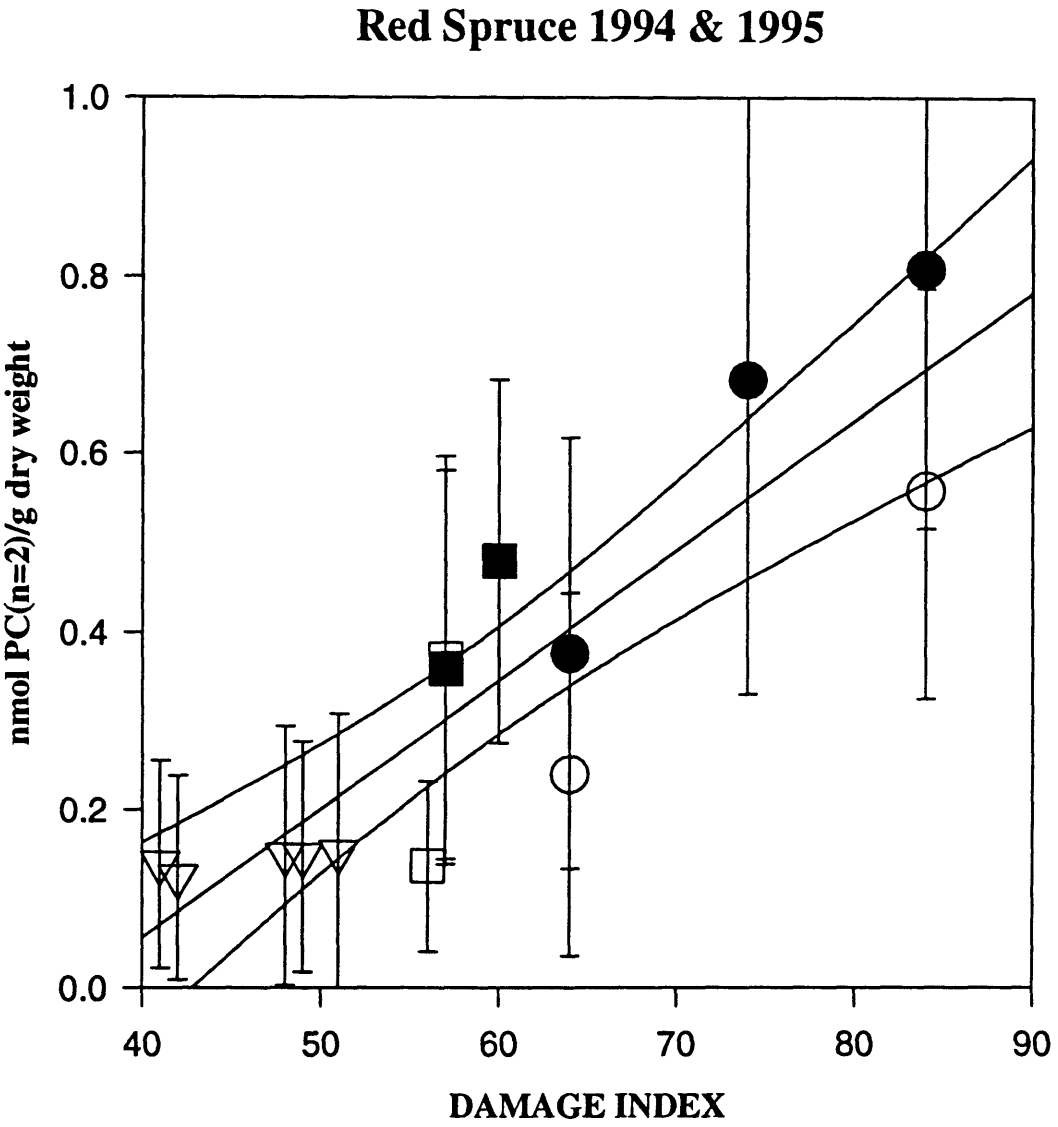

Figure B.4: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1994 and 1995 from red spruce trees at 1000 m on eleven different mountains in the Northeast, compared to red spruce damage index in the stand.

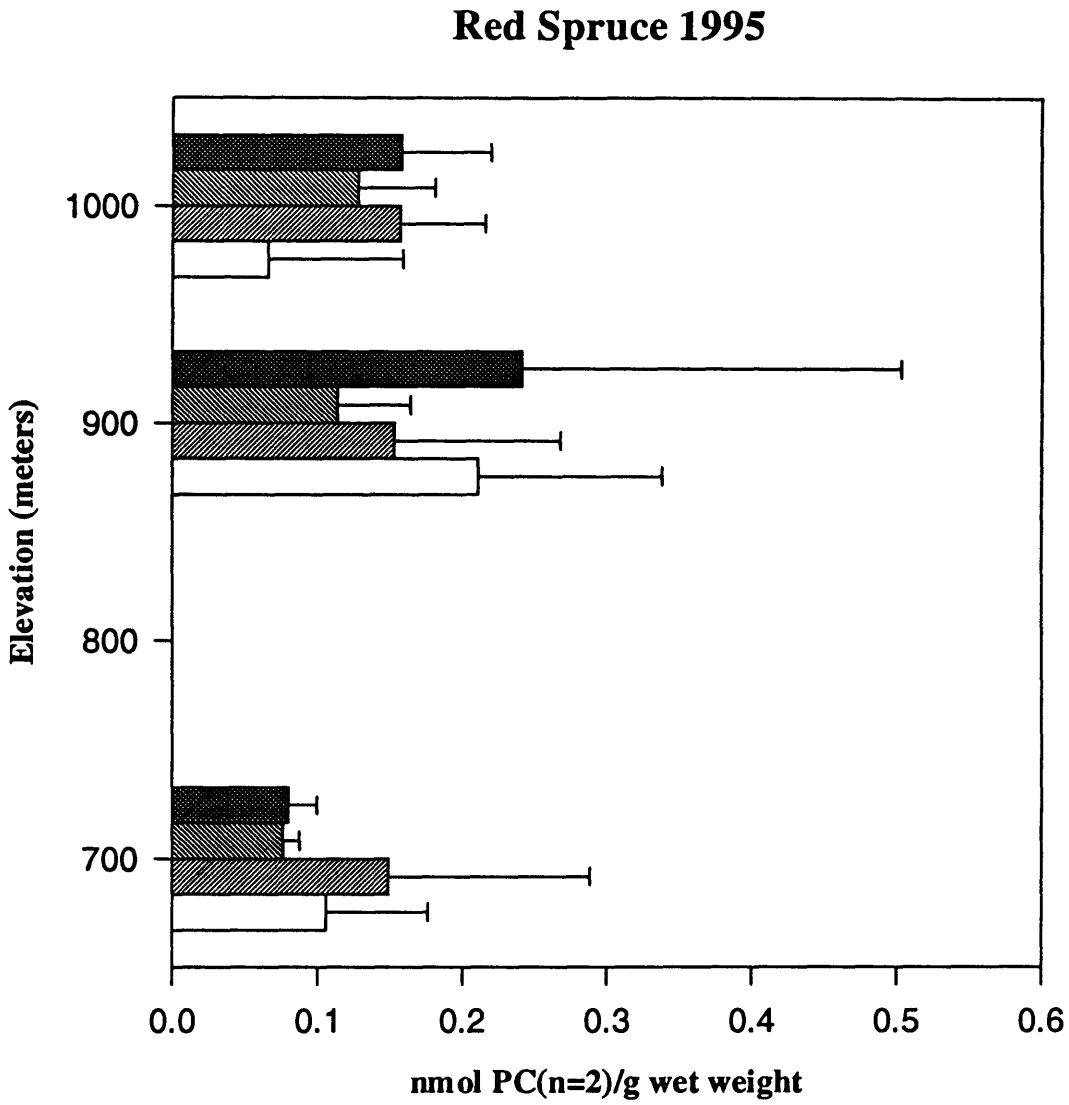

Figure B.5: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1995 from red spruce trees at different elevations on Whiteface Mountain, NY.

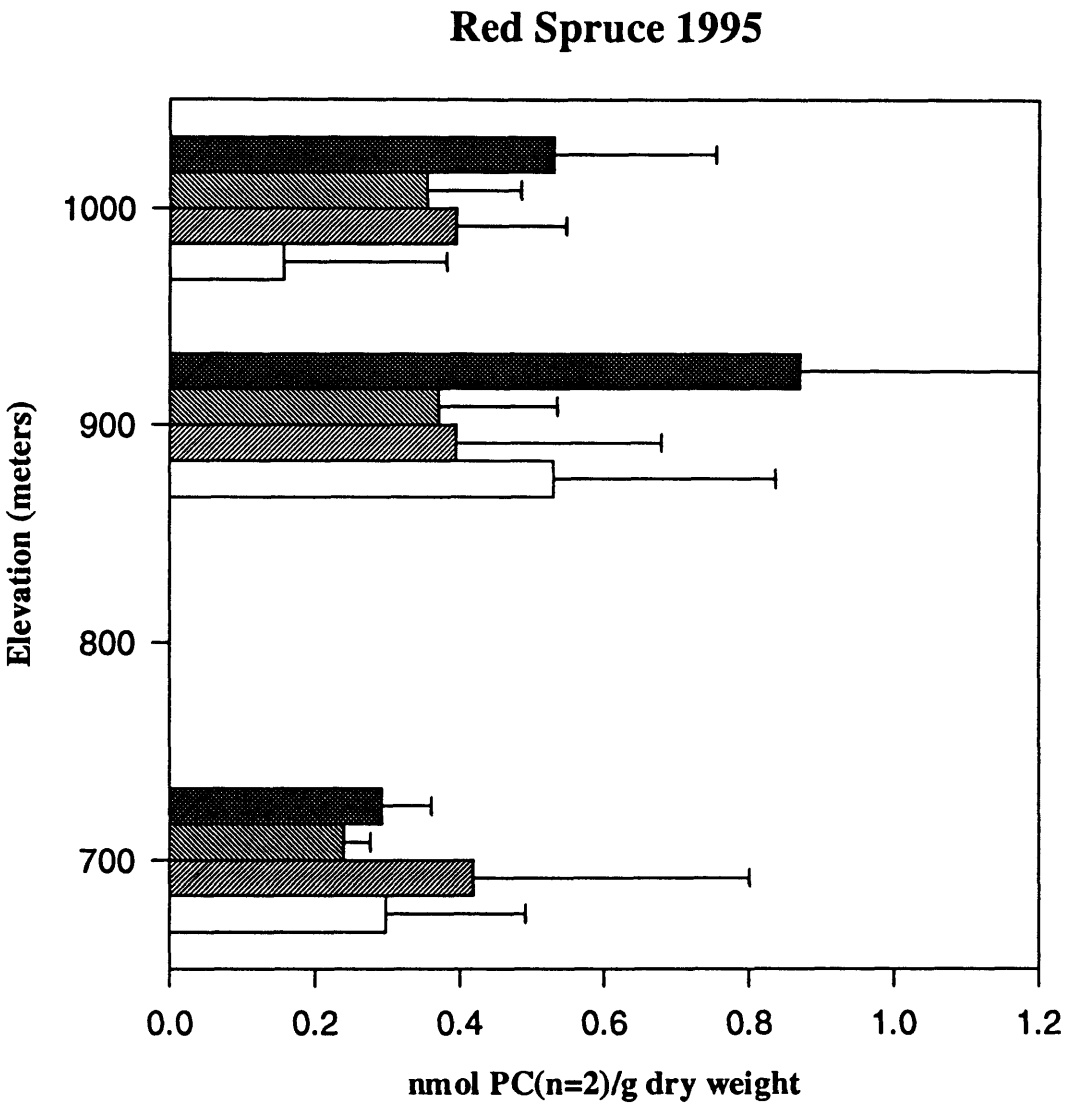

Figure B.6: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1995 from red spruce trees at different elevations on Whiteface Mountain, NY.

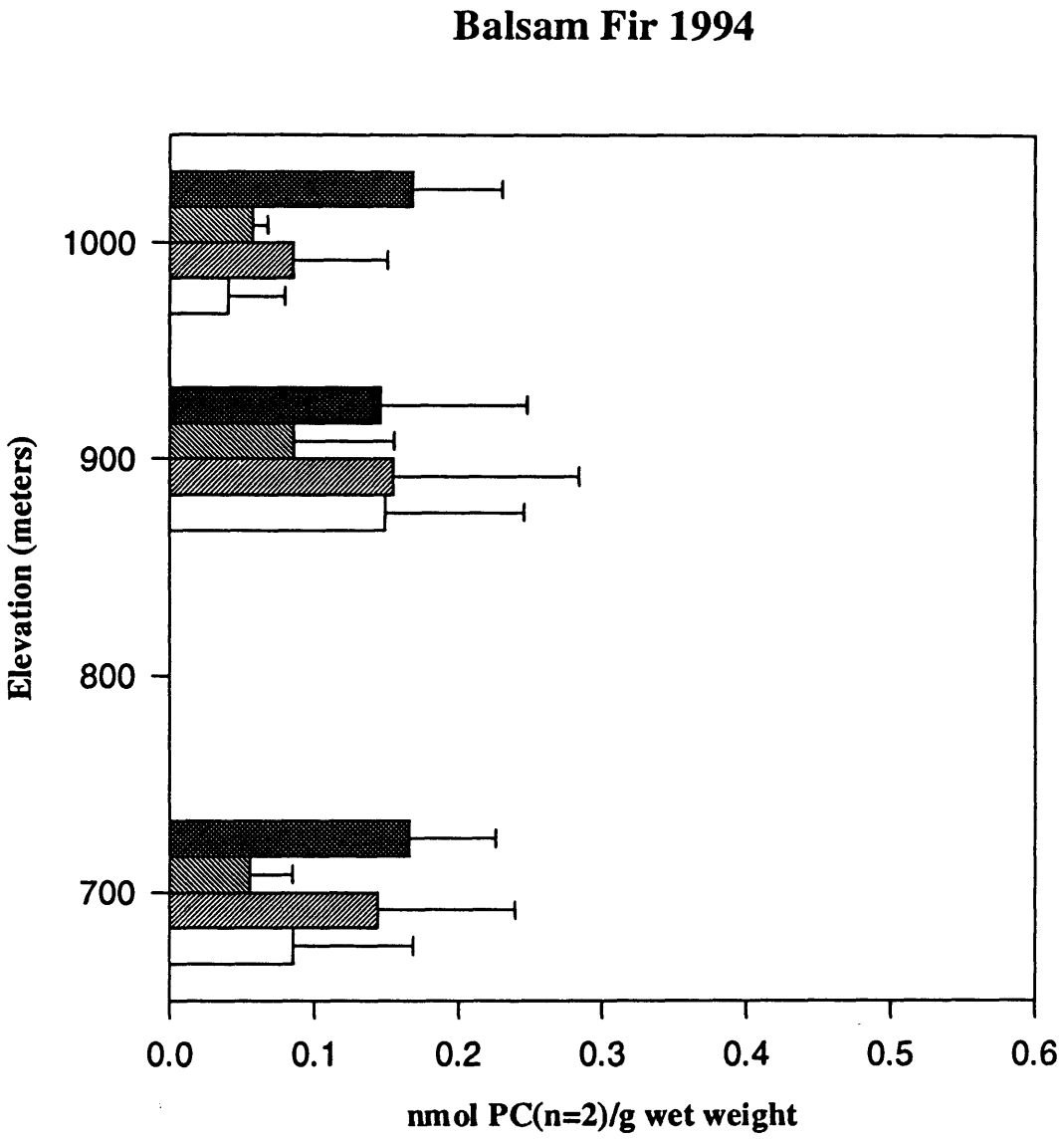

Figure B.7: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1994 from balsam fir trees at different elevations on Whiteface Mountain, NY.

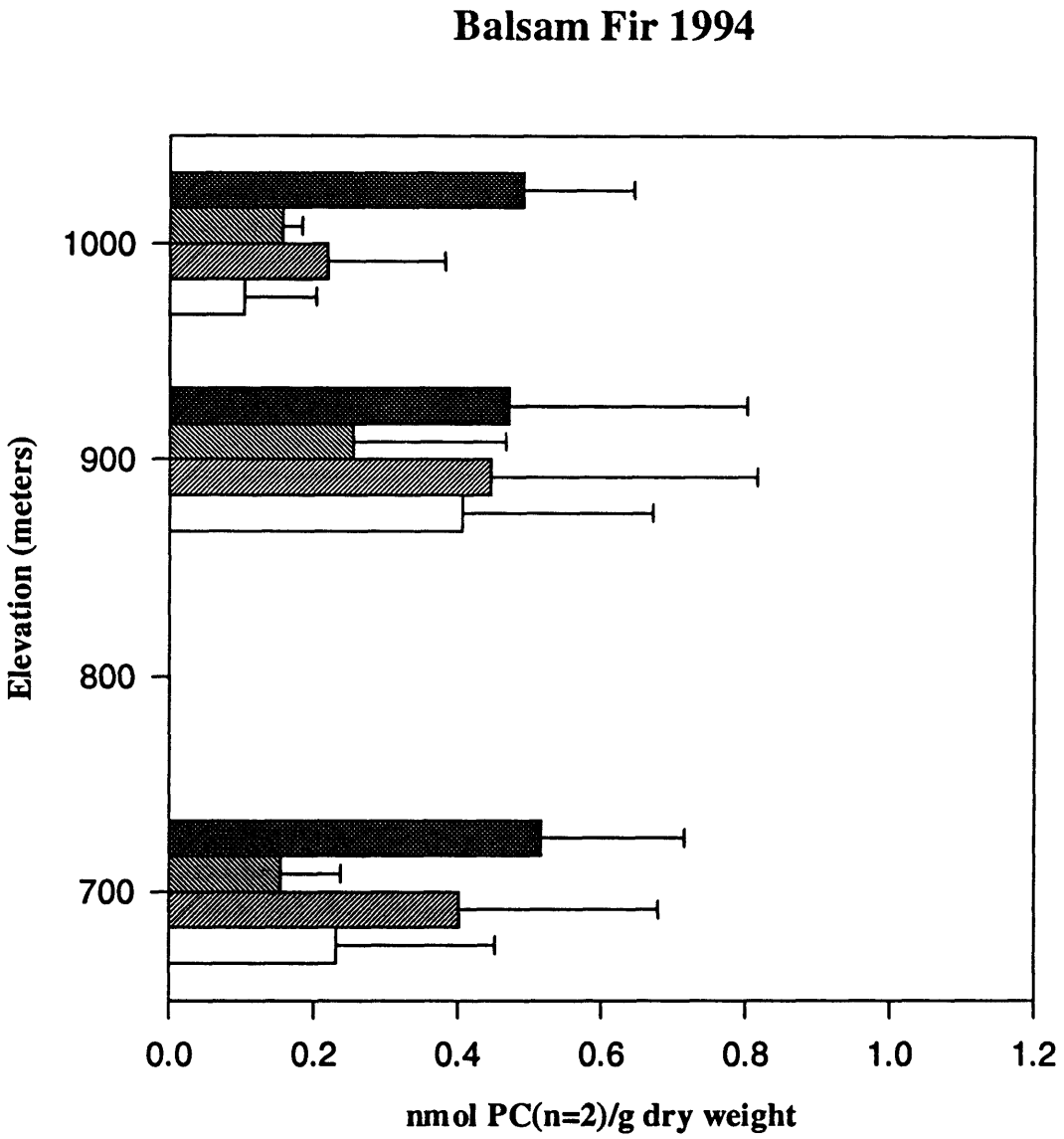

Figure B.8: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1994 from balsam fir trees at different elevations on Whiteface Mountain, NY.

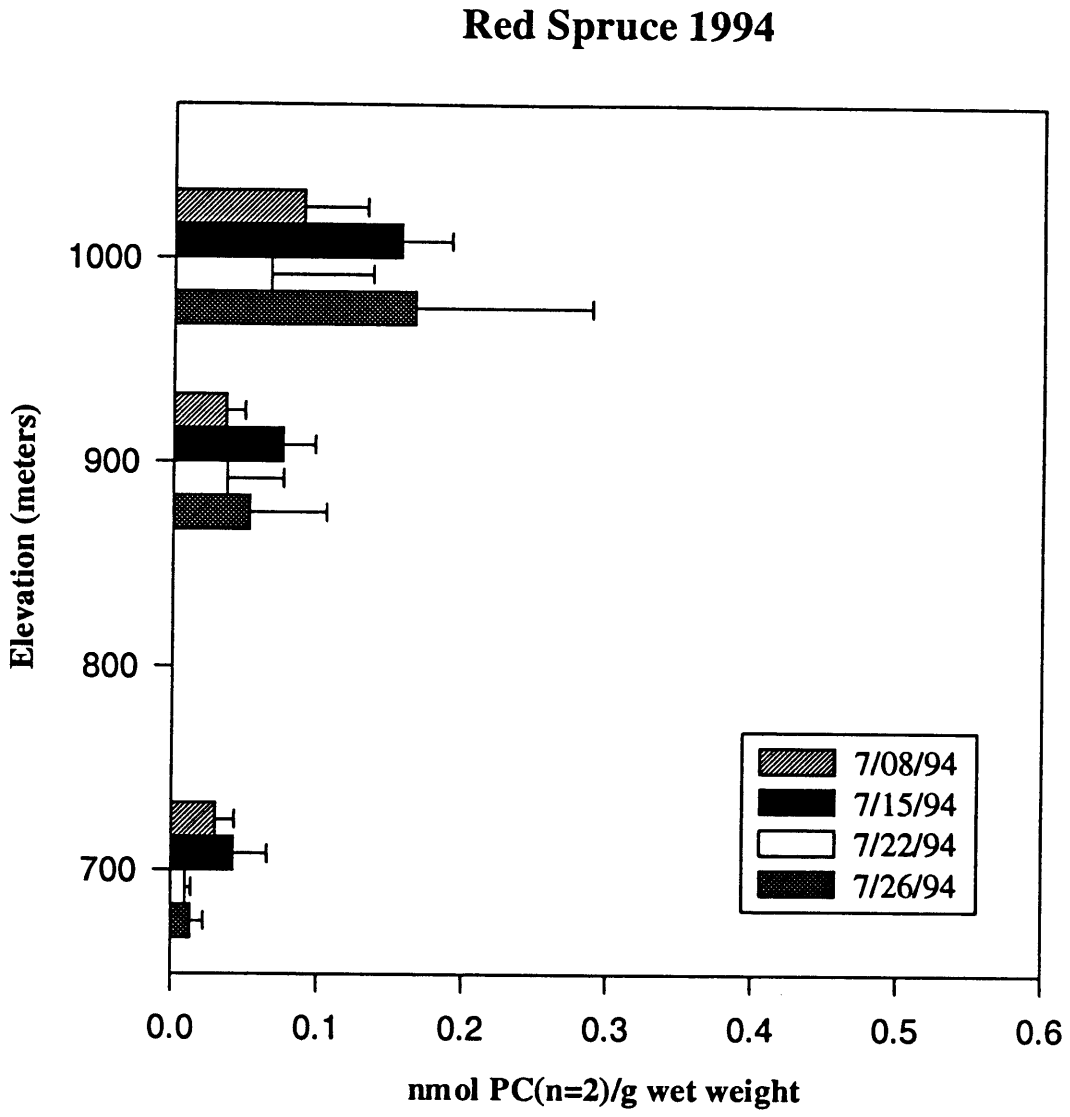

Figure B.9: Phytochelatin concentrations normalized to wet weight, in foliage taken in 1994 from red spruce trees at different elevations on Whiteface Mountain, NY.

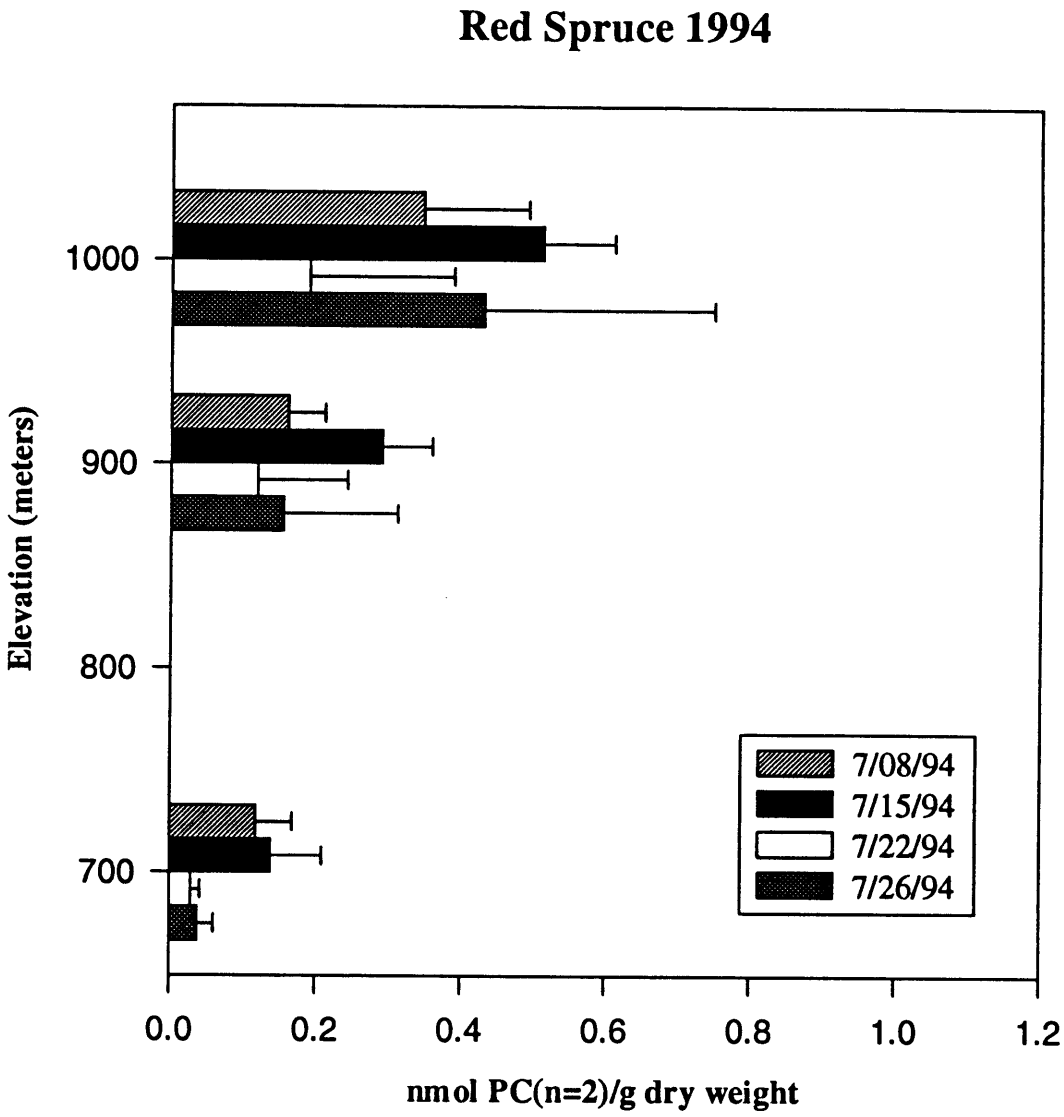

Figure B.10: Phytochelatin concentrations normalized to dry weight, in foliage taken in 1994 from red spruce trees at different elevations on Whiteface Mountain, NY.

	Giant Mt., NY	Mt. Mansfield, NY	Mt. Abraham, NY	Mt. Clay, NH	Mt. Monroe, NH	Boott Spur, NH	Wildcat Mt. E, NH	Mt. Jefferson, NH		Giant Mt., NY	Mt. Mansfield, NY	Mt. Abraham, NY	Mt. Clay, NH	Mt. Monroe, NH	Boott Spur, NH	Wildcat Mt. E, NH	Mt. Jefferson, NH
Station #1	2	3	1	2	1	2	2	1	Station #6	3	4	5	1	2	4	2	2
	2	4	5	2	1	3	4	1		2	2	5	1	3	5	3	1
	1	3	2	5	1	4	2	1		2	1	5	3	3	2	2	1
	1	3	2	1	2	2	1	5		2	2	5	5	3	1	1	1
Station #2	3	4	5	2	1	3	1	5	Station #7	4	2	4	1	1	3	1	2
	2	5	3	2	2	4	2	1		2	2	5	5	1	3	1	3
	3	1	5	1	1	4	2	1		3	1	5	5	1	2	1	1
	2	2	5	2	1	1	1	4		3	5	4	1	1	2	3	3
Station #3	1	4	5	2	1	3	1	2	Station #8	5	3	5	2	1	3	2	1
	1	5	5	1	2	2	1	1		2	4	2	3	1	2	1	1
	2	3	5	2	4	2	2	1		5	4	2	3	1	1	3	5
	1	1	5	2	1	4	5	5		4	5	5	5	1	2	4	2
Station #4	2	1	2	1	3	4	2	4	Station #9	3	3	5	1	1	2	1	1
	3	4	5	1	4	2	4	5		5	4	5	2	5	1	1	1
	3	5	4	5	1	1	3	2		2	5	1	5	5	2	2	2
	3	1	4	1	1	5	1	5		2	3	5	1	4	2	2	5
Station #5	4	4	5	5	5	1	1	5	Station #10	4	5	5	1	5	3	2	4
	4	5	5	1	5	1	5	1		5	3	5	1	2	1	2	1
	2	3	1	4	1	1	2	2		5	5	5	4	1	2	1	2
	2	2	5	5	1	1	2	2		5	2	5	5	2	5	2	2
% dead red spruce	27.5	45.0	77.5	30.0	20.0	22.5	12.5	27.5	damage index	56.0	64.0	83.5	51.0	41.5	49.0	40.5	47.5

Table B.1: Visual assessments of damage to foliage on red spruce trees at 1000 m on eight mountains during the summer of 1993.

Appendix C

Nature Paper

Role for heavy metals in forest decline indicated by phytochelatin measurements

James E. Gawel*, Beth A. Ahner†, Andrew J. Friedland‡ & François M. M. Morel†

* Civil and Environmental Engineering, 48-320, MIT, Cambridge, Massachusetts 02139, USA
† Geology Department, Princeton University, Princeton, New Jersey 08544, USA
‡ Environmental Studies Program, Dartmouth College, Hanover, New Hampshire 03755, USA

Forest decline in the United States and Europe has been documented for a number of tree species[1,2] and atmospheric pollutants from industrial sources, such as acids or oxidants, are thought to be partly responsible[1-4]. Heavy metals have also been implicated because their deposition pattern is correlated with forest decline[5-11], but so far there has been no direct evidence for a physiological link between tree damage and exposure to metals. Here we use the concentrations of phytochelatins, which are intracellular metal-binding peptides that act as specific indicators of metal stress[12,13], to show that metals are indeed likely to be a contributing factor in the decline of forests in the northeastern United States. Phytochelatin concentrations in red spruce, a species in decline, are higher than in balsam fir, a species which is not. Concentrations increase with altitude, as does forest decline, and they also increase across the region in forest stands that show increasing levels of tree damage.

We collected current-year foliage from red spruce and balsam fir trees at 1,000 m on Whiteface Mountain in New York throughout the 1993 growing season to measure phytochelatin concentrations in the needles. Phytochelatins, intracellular metal complexing agents with the formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where $n = 2$ to 11, are produced specifically by plants as a response to sublethal concentrations of heavy metals[14,15]. As can be seen from Fig. 1, mean foliar phytochelatin concentrations in red spruce were consistently higher than in balsam fir from June to August, with the largest and most significant ($P < 0.001$) difference occurring in mid-July at the peak of the growing season. In addition, the phytochelatin concentrations in red spruce in mid-July are significantly higher ($P < 0.001$) than at any other time sampled. (The relatively high standard deviations for the phytochelatin data reflect natural tree-to-tree variability, not analytical error; see Fig. 1 legend.) Balsam fir do not exhibit this peak, but rather stay at a consistently low level throughout the season. The peak phytochelatin concentration measured in red spruce (0.5 nmol per g dry wt) is comparable to that reported for the leaves of sycamore maple growing on mine tailings (although the root concentrations in these trees were about 100 times higher — 4 and 500 nmol per g dry wt, respectively)[13].

The number of standing dead red spruce trees increases sharply with elevation[16,17]. If heavy metals are partly responsible for this trend, we would expect to see a similar increase in the phytochelatin concentrations in red spruce needles. Our measurements of current-year foliage from visually healthy red spruce trees confirm this expectation (Fig. 2). Phytochelatin concentrations, which again peaked in mid-July, were highest at 1,000 m and decreased systematically and significantly ($P < 0.001$) with decreasing elevation. No such trend was seen in balsam fir (data not shown).

As a further test of the relationship between heavy metals and the decline of forests in the northeastern United States, we sampled red spruce stands showing varying degrees of decline at 1,000 m elevation on nine mountains spanning New York, Vermont and New Hampshire. New foliage from five healthy trees was collected once a week from each location during July 1994 and all values for each site were pooled together. The percentage of red spruce trees that were standing dead was used as a measure of forest decline[17]. The data presented in Fig. 3 show a systematic and significant ($P < 0.001$) increase in phytochelatin concentrations that corresponds to the extent of tree damage in the stand. The highest concentrations of phytochelatins measured were from sites in the Green Mountains of Vermont and the

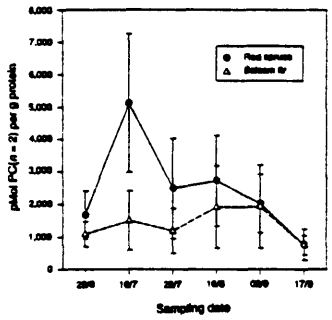

FIG. 1 Phytochelatin (pc) concentrations in current-year foliage from red spruce (*Picea rubens*) and balsam fir (*Abies balsamea*) in 1993 on the Whiteface Mountain massif, Adirondack Mountains, New York. Phytochelatin concentrations are given in picomoles (10^{-12}) of the $n = 2$ chain length (that is, $(\gamma\text{-Glu-Cys})_2\text{-Gly}$) normalized to grams of soluble protein, determined by BCA protein assay (Pierce Chemical) on needle extracts. Longer chain lengths of phytochelatins were not detected. Samples were collected from opposite sides of six red spruce and three balsam fir trees in the same stand at 1,000 m elevation using a pole pruner. Foliage from both aspects were pooled into one sample and stored immediately in liquid nitrogen before analysis. Samples were pulverized in liquid nitrogen in a ceramic mortar and pestle, and then homogenized on ice in 0.01 M methanesulphonic acid using a ground-glass pestle and tube (Glas-Col). The homogenate was centrifuged at 14,000 r.p.m. for 30 min at 4 °C and the supernatant refrigerated. Quantification of phytochelatins using reverse-phase HPLC with fluorometric detection is described in ref. 24. Error bars are calculated as standard deviations from the mean values. Replicate analyses of the same samples showed less than 10% relative error, whereas samples taken from different branches on the same tree and from different trees in the same stand produced up to twofold and fivefold differences, respectively. (Such variability is also found in metal concentrations measured in red spruce foliage[9].) Statistical analyses of variation between tree species for a given sampling date were calculated using the unpaired Student's *t*-test; ANOVA analysis was used to determine the variation within a species over time.

Adirondack Mountains of New York, the areas most severely affected by forest decline[7]. These data strongly imply that metal stress is a cause, rather than an effect, of tree damage. Because phytochelatin concentrations were measured in visually healthy red spruce trees in each stand, they do not simply reflect the extent of individual tree damage. Rather, these high phytochelatin concentrations reveal that trees in the more affected stands, including the healthy ones, are under metal stress. This stress probably reflects increased exposure to heavy metals, although we cannot rule out the indirect effects of some other primary causes such as calcium or magnesium deficiency[18].

The most parsimonious interpretation of all the available field data, which show consistency between the pattern of phytochelatin concentrations and the pattern in tree damage according to tree species, elevation and geographic distribution, is that heavy metals are a contributing cause of forest decline in the northeastern United States. A more direct study of the relationship between heavy metal exposure, phytochelatin production, and growth in red spruce is necessary to establish the degree of 'metal stress' indicated by our measurements of phytochelatin concentrations. A consensus is now emerging that multiple causative factors are involved in forest decline, probably in conjunction with freezing injury[1,19]. Oxidant damage, recently shown to be a contributing factor in the reduced growth of loblolly pine in the southeastern United States[20], has also been implicated in red spruce decline in northeastern forests[21,22]. It is known that glutathione depletion from the production of phytochelatins results in an increased sensitivity to oxidative damage in plants[23]. It thus seems not unlikely to us that forest decline in the northeastern United States is the result of a synergistic effect of toxic metals and oxidants originating in air pollution[3]. In any case, elevated phytochelatin levels in red spruce provide *prima facie* evidence that trace-metal stress is contributing to their decline. Thus, abatement of atmospheric heavy-metal pollution should probably be considered as one of the elements of a policy directed at reversing forest decline in this region. Further, phytochelatin measurements in other forested regions of the world may allow us to determine whether the role of heavy metals in forest decline is a general phenomenon or limited to the northeastern United States. □

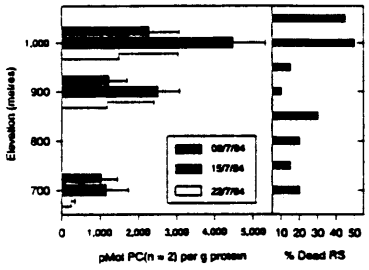

FIG. 2 Phytochelatin (PC) concentrations in new foliage from visually healthy red spruce (that is, showing less than 10% needle loss or discolouration) in 1994 along an elevational gradient (1,000, 900 and 700 m) on the Whiteface Mountain massif, Adirondack Mountains, New York, compared with per cent standing dead red spruce density. Dead red spruce as a per cent of total standing red spruce density was taken from ref. 16. Sample collection (from five red spruce trees at each elevation), extraction and analysis are described in Fig. 1 legend, with the substitution here of a Brinkmann Instruments' Polytron homogenizer for sample preparation. Similar results, as compared to those shown here and in Fig. 3, were obtained by normalizing to both fresh weight (0.05–0.15 nmol PC per g fresh wt) and dry weight (0.2–0.6 nmol PC per g dry wt). Error bars are calculated as standard deviations from the mean values. ANOVA analysis was used to determine the significance of phytochelatin variation over time and as a function of elevation.

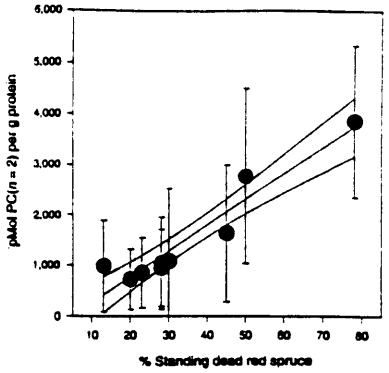

FIG. 3 Phytochelatin (PC) concentrations in new foliage from visually healthy red spruce in 1994 at 1,000 m on nine mountains in the northeastern United States, showing different degrees of forest damage. Sample collection (from five red spruce trees at each site), extraction and analysis are described for Fig. 2. Samples were taken once a week for four weeks in July 1994. All samples collected were pooled for one seasonal value at each site. The mountains sampled were, from left to right: Wildcat E, Monroe, Boott Spur and Jefferson (all in New Hampshire), Giant (New York), Clay (New Hampshire), Mansfield (Vermont), Whiteface (New York) and Abraham (Vermont). Per cent standing dead red spruce values were determined along 250 m transects following the 1,000 m elevation contour on all mountains, excluding Whiteface. Point-centred quarters were designated every 25 m, and the nearest standing red spruce stem larger than 10 cm in diameter in each quadrant was assessed as either dead or alive. Data for Whiteface Mountain were taken from ref. 16. Normalization to protein concentrations, dry weight and fresh weight all produced similar results. Error bars are calculated as standard deviations from the mean values for each mountain. ANOVA analysis was used to determine the significance of the correlation between phytochelatin concentrations and per cent standing dead red spruce for all samples, and the 99% confidence intervals are shown.

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CORRESPONDENCE and requests for materials should be addressed to J.E.G. (e-mail: weserboy@rnt.edu).

Appendix D

Laboratory Experiments

Methods

Picea rubens seeds, which were collected from trees in New York, were obtained from Mountain Farms - Herbst Seed (Fletcher, NC) and stored in the freezer. Seeds were surface sterilized by submerging for 10 min in 5.25% sodium hypochlorite (Clorox) and 2 min in 70% ethanol, and then rinsing with Q-water for 10 min. These were sowed evenly on autoclaved vermiculite in glass dishes with inverted glass watch glasses, and placed in a 20°C constant temperature incubator until after the opening of the first whirl of needles. Seedlings were then transferred to sterile hydroponic growth chambers described below.

These chambers [shown in Figure D.1] consisted of clear polycarbonate buckets with tight fitting lids in which a polypropylene rack covered with a Teflon mesh was placed for holding the seedlings. Compressed air, after passing through an activated charcoal trap and three 0.1 μm filters (Vacu-Guard by Whatman), was bubbled around the bottom of this chamber from perforated Tygon tubing. Quick-disconnect tubing connections allowed the removal of individual buckets to a laminar flow hood prior to opening or sampling. Approximately 1 L of aqueous medium (half-strength Murashige-Skoog medium as described in Murashige and Skoog 1962, modified by excluding the "organic constituents," increasing the EDTA concentration to 0.06 mM, and using $Fe(III)Cl_3$ in place of $Fe(II)SO_4$) was added to the chambers to cover the roots, and then bubbled continuously in an incubator with a 15/9 hr. light/dark cycle. Daytime conditions were 22°C and 400 μEinsteins/m^2s light intensity, with nighttime temperatures of 20°C. After initial equilibration for one week, the medium was replaced with fresh medium

spiked with varying concentrations of equimolar Cd and EDTA. Free-Cd^{2+} ion amounts were calculated using MINEQL+ (© Copyright 1994 Schecher).

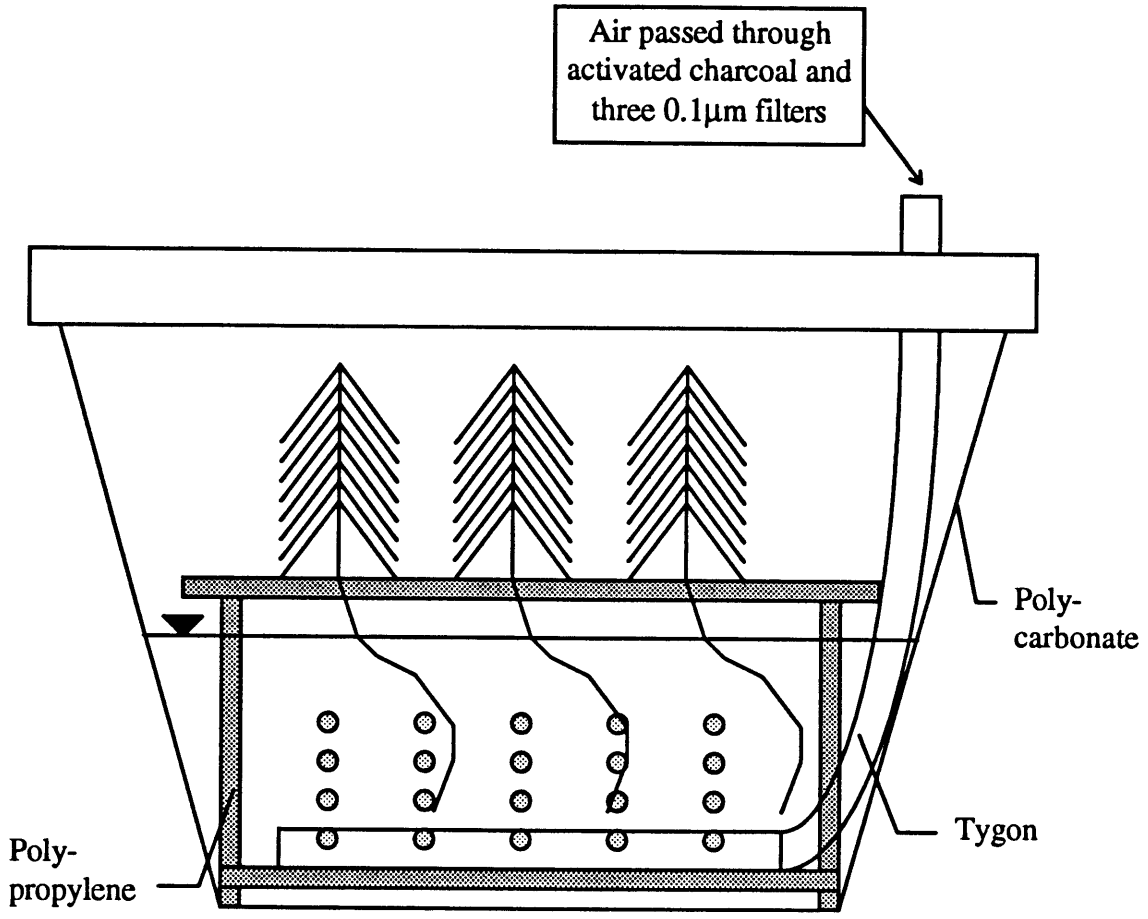

Figure D.1: Trace metal-clean growth chambers used in laboratory experiments.

An initial experiment was conducted using 16-17 week old seedlings. Seedlings were exposed to medium containing ~10^{-7} M free Cd (Cd^{2+}) or no added Cd. Initial samples were taken prior to Cd addition (Day 0), and then once a day for eleven days afterward. Six seedlings were removed from each treatment per day, and the needles from groups of three seedlings and roots from all six

were pooled in order to make enough material to analyze for each sample. Seedlings were chosen randomly and roots and shoots were placed immediately in separate cryovials in liquid nitrogen prior to analysis. Roots were not sampled on Day 0 or Day 1.

Chlorophyll 'a' measurements were performed by adding approximately 0.025 g fresh needles to 3 ml 90% acetone in a closed vial. These were placed in a box in the freezer for one week and then analyzed after American Public Health Association et al. (1976) using a spectrophotometer. Corrections were made for pheophytin 'a' and sample turbidity. Phytochelatin and protein analyses are described in Chapter 2 and Appendix A.

Results

Phytochelatin concentrations normalized to protein and wet weight did not change significantly over time in the roots [Figures D.2 and D.3] or the needles [Figures D.4, D.5, D.6, and D.7] for either treatment. Chlorophyll 'a' and pheophytin 'a' concentrations also remained the same over time in the needles, and mean concentrations in the two treatments were almost identical. However, mean phytochelatin concentrations in the roots and needles averaged over the whole experiment were significantly higher ($P < 0.05$ and $P < 0.10$, respectively) in the Cd-spiked treatment than in the Cd-free treatment.

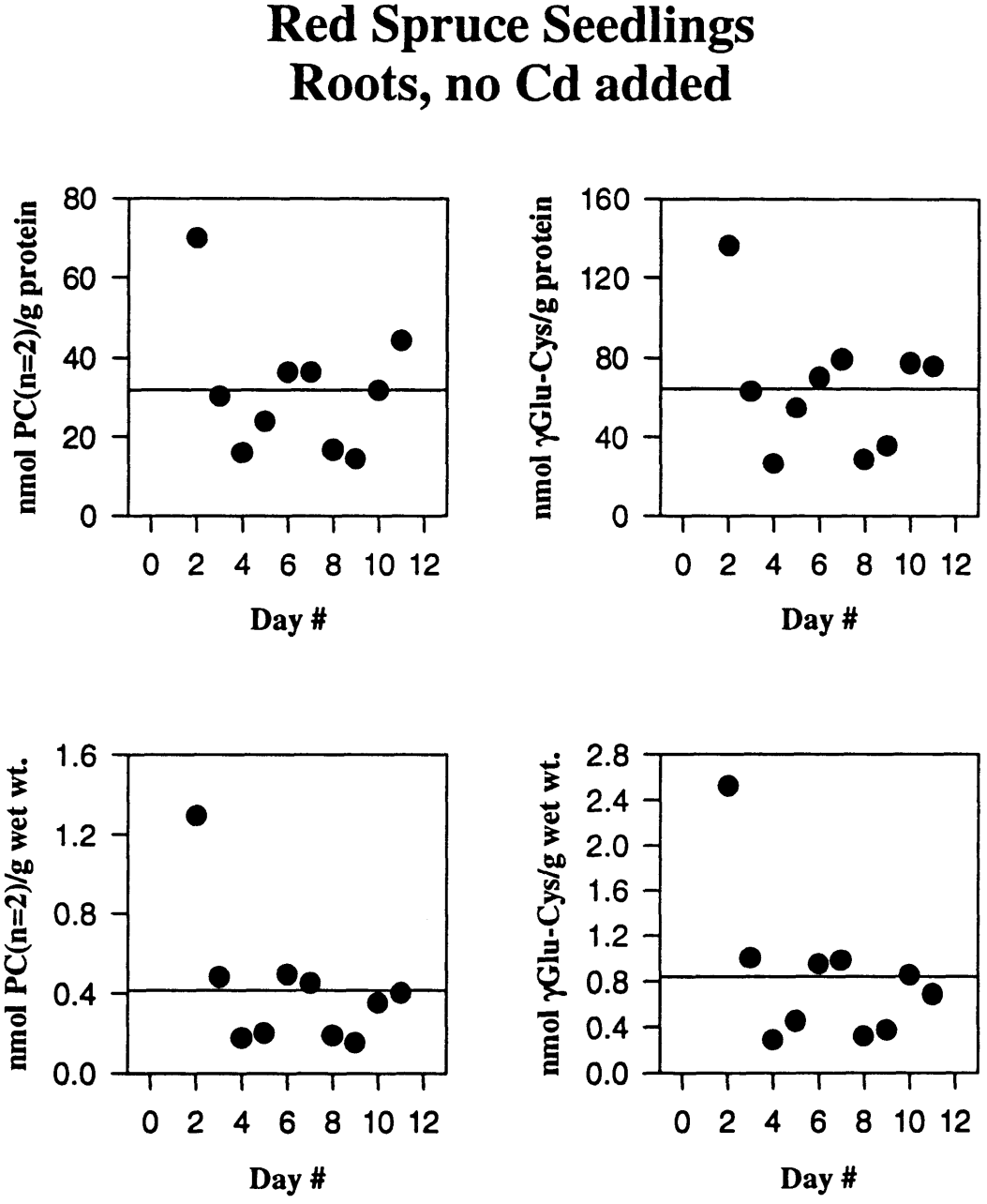

Figure D.2: Phytochelatin concentrations normalized to protein and wet weight in roots of red spruce seedlings from laboratory experiments with no added Cd.

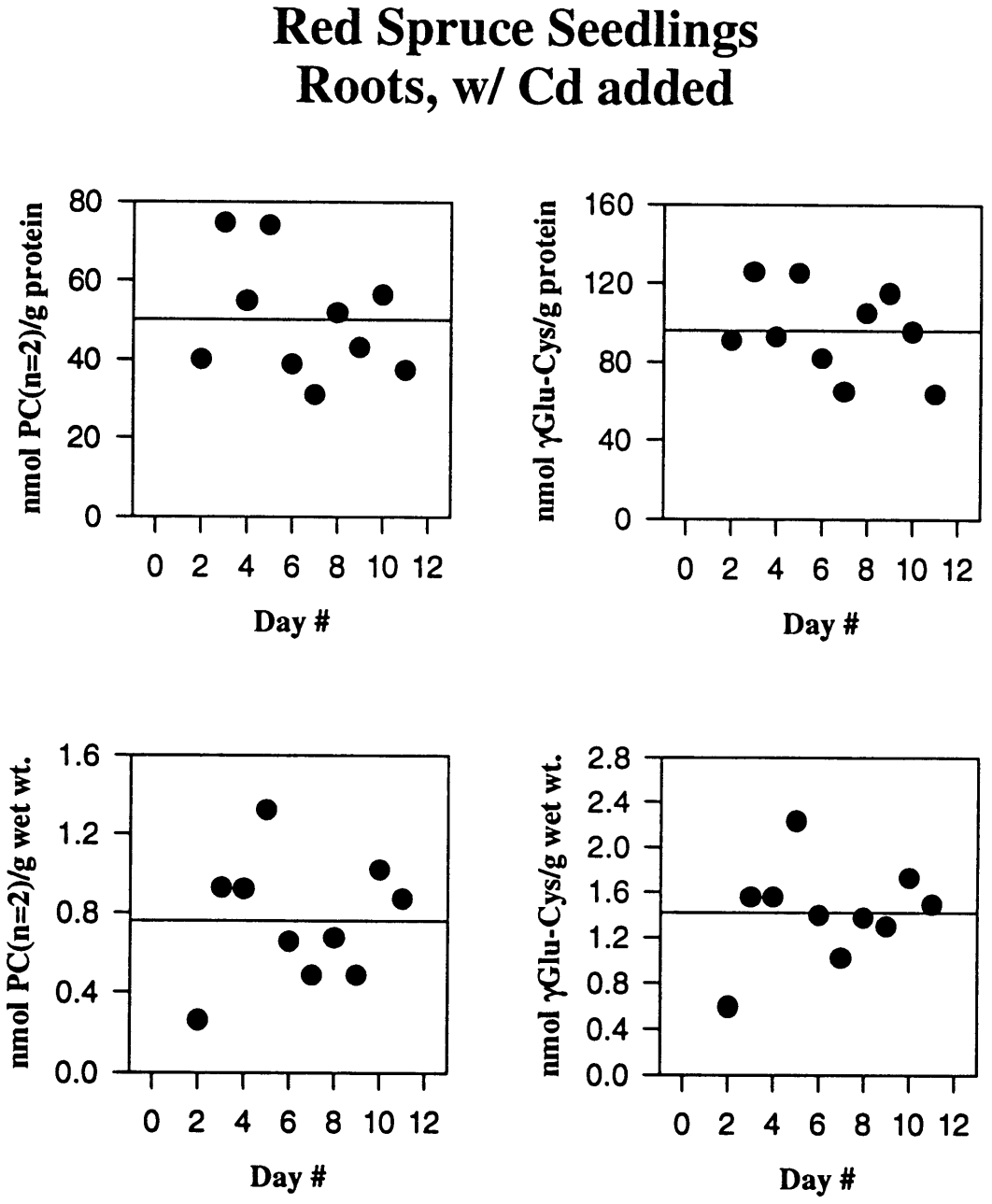

Figure D.3: Phytochelatin concentrations normalized to protein and wet weight in roots of red spruce seedlings from laboratory experiments with 10^{-7} M free Cd.

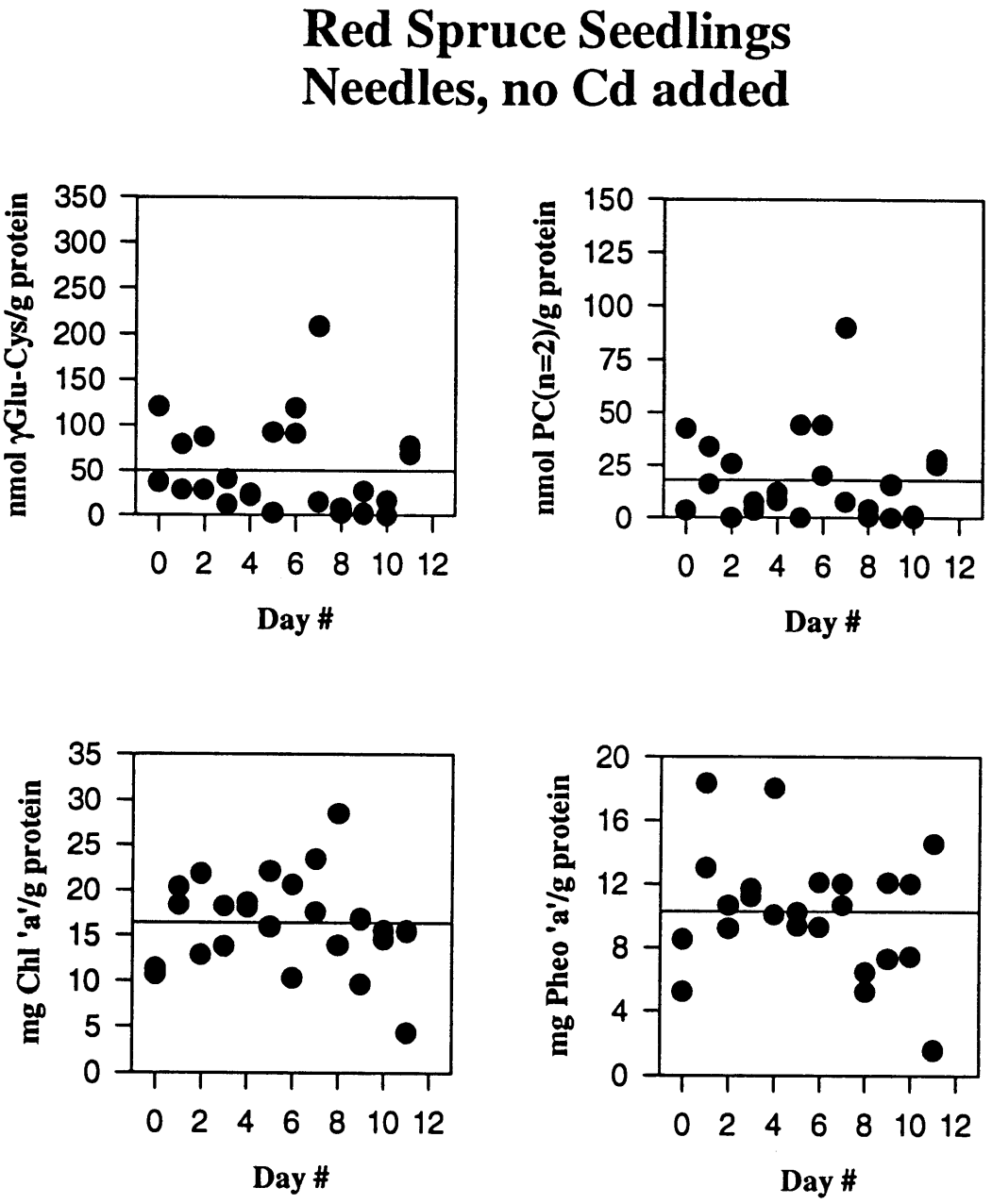

Figure D.4: Phytochelatin, chlorophyll 'a', and pheophytin 'a' concentrations normalized to protein in needles of red spruce seedlings from laboratory experiments with no added Cd.

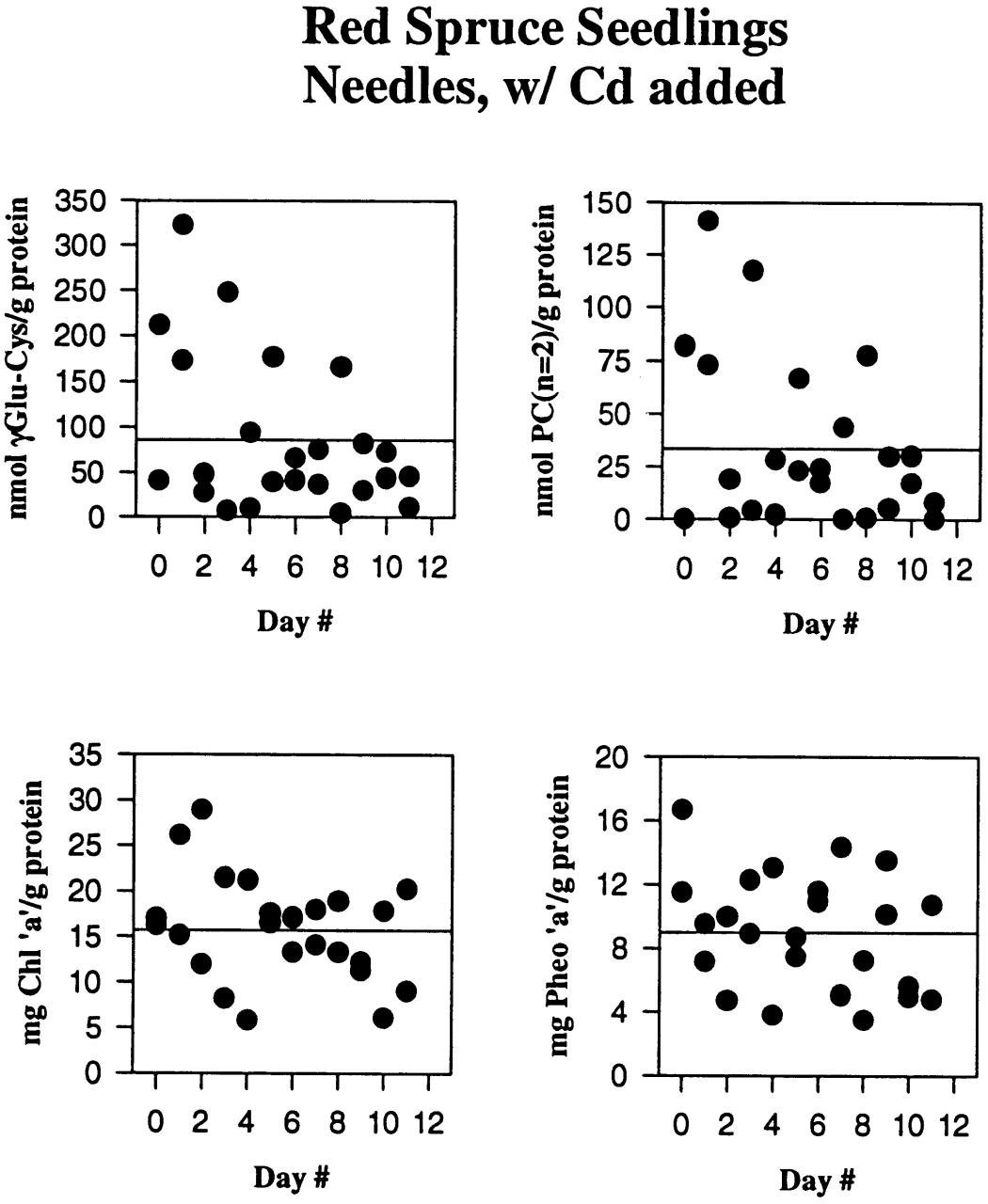

Figure D.5: Phytochelatin, chlorophyll 'a', and pheophytin 'a' concentrations normalized to protein in needles of red spruce seedlings from laboratory experiments with 10^{-7} M free Cd.

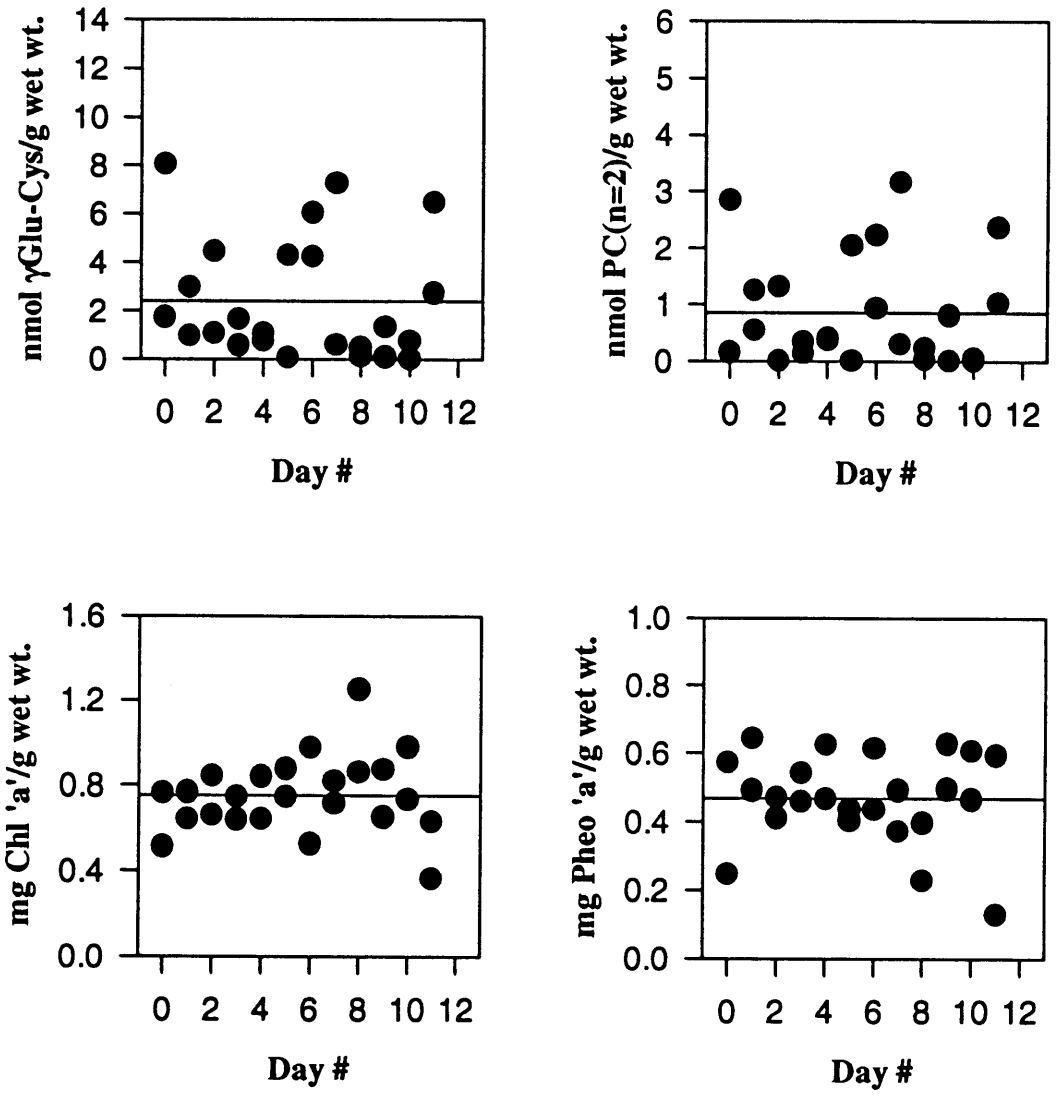

Figure D.6: Phytochelatin, chlorophyll 'a', and pheophytin 'a' concentrations normalized to wet weight in needles of red spruce seedlings from laboratory experiments with no added Cd.

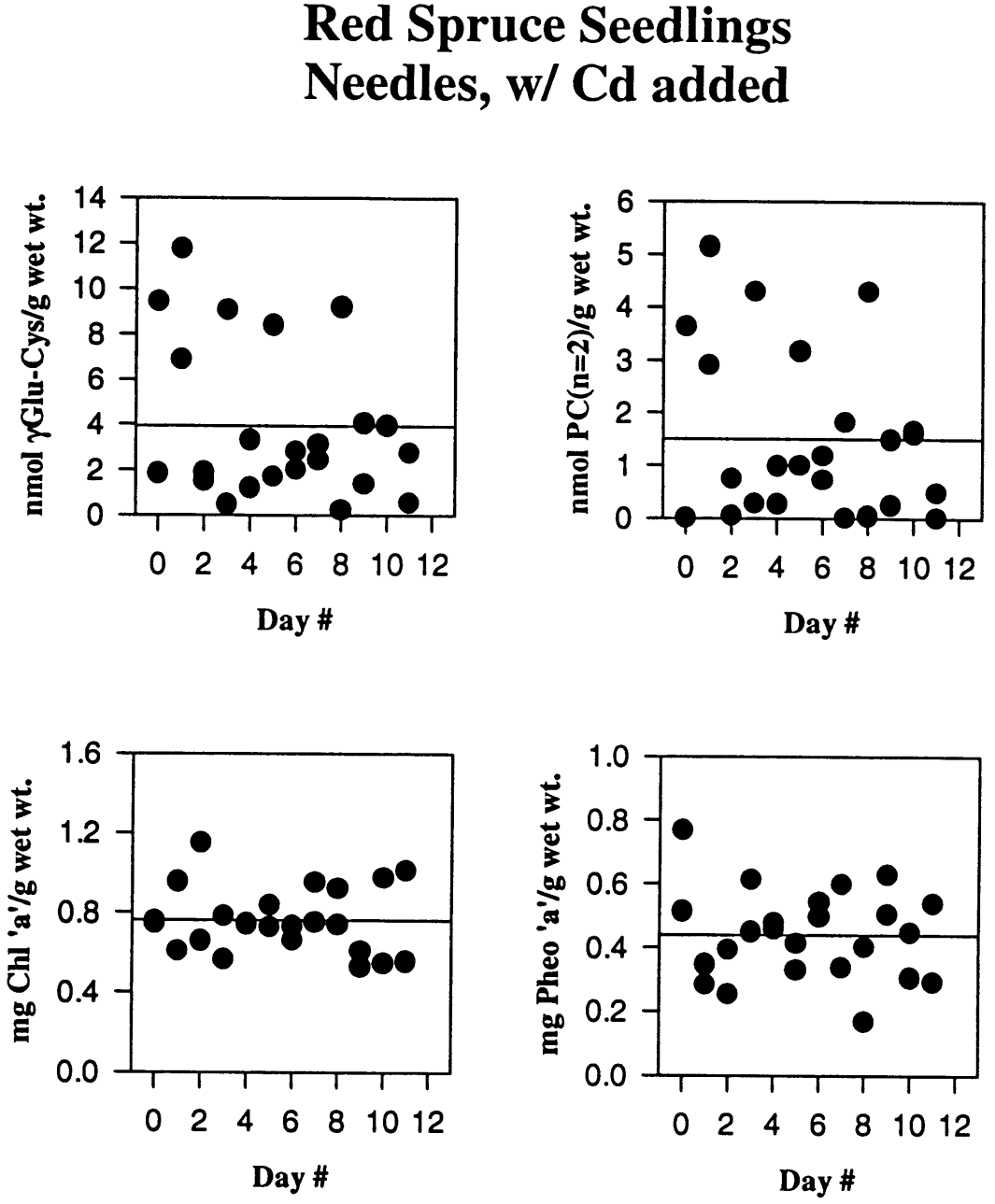

Figure D.7: Phytochelatin, chlorophyll 'a', and pheophytin 'a' concentrations normalized to wet weight in needles of red spruce seedlings from laboratory experiments with 10^{-7} M free Cd.

Discussion

The lack of a temporal trend in our data from seedlings in buckets with added cadmium may be the result of rapid phytochelatin production and equilibration within the plants. In addition, since free-cadmium concentrations are maintained at a relatively constant value in the medium by using high levels of EDTA, no decrease in phytochelatin concentrations is expected to occur until total cadmium is significantly depleted. More concentrated sampling over a short time scale (i.e. less than 24 hr) and repeated sampling over longer periods (i.e. greater than 11 days) is needed if temporal trends are to be evaluated quantitatively.

Phytochelatin levels in the Cd-free treatment were still high in comparison to trees in the field (up to two orders of magnitude greater). This may be due to germination in vermiculite, since rinsing with Q-water could not remove all of the particles and carryover may have been a problem. Bacto-Agar (Difco Laboratories) was later substituted for vermiculite (following the advice of D.L. Godbold), both because it was less damaging to roots during transplanting and because any excess on the roots would dissolve in the first change of medium, leading to less carryover interference in the experiments. However, sample analyses have not been completed for determining the effectiveness of this change in germination medium.

Fungal infection of seeds and seedlings was also a problem. Another method for sterilization was implemented to try to reduce the impact of fungal infections. Seeds were surface sterilized by soaking overnight in 3% hydrogen peroxide with a drop of Micro detergent, then with 20%

hydrogen peroxide for 1 hr, and then rinsed in a sterilized metal tea strainer with sterilized Q-water (following the advice of D.L. Godbold). However, this did not prove to be any better than the previous method. Further work needs to be done to control fungal infections, especially since it is known that some strains may produce phytochelatins themselves.

Bibliography

American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1976. *Standard Methods for the Examination of Water and Wastewater*, Fourteenth Edition, APHA, Washington, DC.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiologia Plantarum*, **15**:473-497.