Historical-scale Biochemical Markers of Oxidant Injury & Exposure in Pines

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EXECUTIVE SUMMARY

Due to the precursor sources and transport dynamics of California, photochemical oxidant injury to forests has been of concern for over 40-years (Miller et al., 1997). Ozone acts first at the biochemical level, so that biochemical markers in foliage have utility in controlled studies, but in the field, injury is best assessed by visible foliar damage (i.e., chlorotic mottle). Unfortunately, leaves are usually lost within five years, so field assessments of ozone injury must be updated periodically. This limits injury assessment to the duration of the study and to a small number of assessment sites. On the other hand, if there were *permanent, biochemical* markers of ozone injury, then both restrictions could be removed. A *permanent* marker would ultimately mean that long-term forest injury records could be established, freeing resources to assess a larger number of sites. Such a marker, that is also *biochemical* in nature means that links to the mechanism(s) of injury and hence links to the photochemical oxidant species most injurious to trees, might be established under field conditions.

Analysis of wood chemical structures from annual tree rings (i.e., dendrobiochemistry; cf. Fan and Higashi, 1999), has the potential to fill this need. In a 1994-96 seed project funded by the University of California, Davis (UCD), Center for Ecological Health Research, in cooperation with Michael Arbaugh and Paul Miller of the U.S. Department of Agriculture, Forest Service in Riverside, California. We chemically analyzed tree ring segments from ponderosa pines in the San Bernardino National Forest (NF). The technique we used is an advanced wood biochemistry tool known as pyrolysis-gas chromatography/mass spectrometry (pyro-GC/MS) (Faix et al., 1987). Since this method does not require pre-choosing the analytes, it is not limited by our current knowledge (or ignorance). Briefly stated, our pyro-GC/MS survey found two markers that correlate strongly to trees growing in ozone-injury sites northeast of the Los Angeles Basin in the post-World War II period. The two markers were the ratio of H-type to G-type lignins, and moieties of phytoalexins (a class of plant defense chemicals) bound to the wood matrix. It is reasonable to conjecture that more markers will be found in these extremely information-rich datasets.

Therefore, the objective of this project was to more definitively correlate tree ring biochemical markers with ozone injury indices at established ozone impacted sites. We set out to "map," by pyro-GC/MS analysis of tree rings, a matrix of established ozone-impact and control stands of ponderosa pines in two studies: first in the San Bernardino NF, and secondly at multiple sites in the Sierra Nevada. Initial efforts focused on the newly found H:G lignin ratio and phytoalexin markers. Parallel analyses were conducted on the lignin, phytoalexin, and antioxidant biochemistry of the needles to probe possible mechanisms of injury that may result in the wood marker response.

In the first phase study, radial tree-ring cores from ponderosa pine (*Pinus ponderosa)* trees at seven long-term study sites in the San Bernardino NF were analyzed by pyro-GC/MS. Fifty biochemical markers were screened for changes in post-1945 rings relative to pre-1945 rings, from which two markers were chosen for further study. For direction-of-change (Böolean) of the markers, there were statistically significant results. Where historical ozone exposure was the highest, trees with severe crown injury (sensitive individuals) showed decreases (denoted by [-]) in H:G lignin ratio and increases (denoted by [+]) in stilbene markers over time. Trees with slight crown injury (tolerant individuals) had opposite trends, echoing tree responses observed at a reference site with no crown injury (i.e., Silverwood Lake). At sites with intermediate historical ozone exposure, trees exhibiting slight injury showed mixed trends in the two markers. At such sites, it is possible that the marker response thresholds were insufficient to override individual differences in injury resistance or the interaction with other perturbations such as drought, pathogens, and nitrogen deposition.

In the second phase study, radial tree-ring cores were sampled at seven sites in the Sierra Nevada:

- Manzanita Lake -- Lassen Volcanic National Park (the northernmost);
- White Cloud Campground – Tahoe National Forest;
- Wawona -- Yosemite National Park;
- Shaver Lake – Sierra National Forest;
- Grant Grove -- Kings Canyon National Park;
- Giant Forest -- Sequoia National Park; and
- Mountain Home – Sequoia National Forest (the southernmost).

These analyses focused on the two markers established in the first phase study using pyro-GC/MS analysis. Selected intact cores were also subjected to analysis by reflective Fourier-transform infrared spectroscopy (FTIR), which has the potential to yield data on an annual ring-basis. Compared to the first phase study in the San Bernardino NF, the individual trees at these sites did not have a well-documented injury status; at the more northerly sites of Lassen Volcanic National Park (NP) and White Cloud, individual trees of known historical status did not appear to be available. Thus the Sierra Nevada investigation sought to apply the lessons learned from the first phase study in an attempt to establish grove-level patterns of the two pyro-GC/MS markers at these sites.

To this end, the pyro-GC/MS data showed increases in the percentage of trees exhibiting either or both [-] H:G lignin and [+] stilbene responses, from northernmost to the southernmost sites in the Sierra Nevada. One possible interpretation of this trend is that, at the grove-level, pines are historically more impacted by oxidants in a southerly vector. Relationships at the individual tree-level could not be compared statistically between sites, because individual assessments were not available for all trees at all sites. However, these data are available in this final report for comparison with existing records of the tagged individual trees, and duplicates of all tree cores are stored at UCD for future analysis. Another marker, “stilbene3,” was found to have a clear trend in its *magnitude* (not just the sign, as with the other two markers) along the southerly vector, with reasonably small standard deviations. Thus, the bipolar magnitude of stilbene3 may be an indicator of oxidant exposure at the individual-level. In addition, at the grove-level, the two individual-level bioeffect makers ([+] stilbene and [-] H:G lignin) appear to also be oxidant exposure markers, in terms of percentage of trees. As with the first phase study, and unlike stilbene3, their magnitudes varied wildly, and appear to be of limited use.

Analogous to the "intermediate" impacted sites in the first phase study, the second phase study sites in the Sierra Nevada were generally considered to be intermediate ozone-exposure sites and exhibited variable responses in stilbene and H:G lignin markers on a tree-by-tree basis. It is therefore possible that the oxidant exposure profiles at such sites represent threshold levels for these grove-level exposure markers.

With regard to the biochemical basis of these observations, data complexity, combined with a limited understanding of the constituents seen by pyro-GC/MS, precludes a simple interpretive analysis. Nevertheless, these findings appear to be mechanistically relevant since ozone fumigation of pines has been shown to induce stilbene levels as well as affect enzymes of lignin synthesis in leaves. Thus, it is plausible that wood retains chemical "records" of these effects, and that it is the biochemical basis of the findings reported here. If so, it is not surprising that the sensitivity range of the markers overlap nicely with the oxidant damage in the pines, since these grove-level exposure markers are

individual-level oxidant bioeffect markers as well. Further morphological and biochemical analyses of archived cores and samples could yield further evidence to support/deny these views.

Lastly, FTIR microspectroscopy was investigated as an alternative to pyro-GC/MS. While technically feasible, it proved to be impractical due to the requirements of skilled/tedious sample preparation and skilled/tedious spectral acquisition. The spectroscopy is potentially very time-consuming on equipment that is of significant cost (> $120,000).

In conclusion, it appears that the direction-of-change (sign) of the [+] stilbene and [-] H:G lignin markers are indicators of bioeffect (needle injury) at the individual-level, while the bipolar magnitude of the stilbene3 marker may represent an indicator of oxidant exposure (ambient ozone concentration at the site). In addition, both [+] stilbene and [-] H:G lignin may also be exposure indicators when averaged to the grove-level, however, their magnitudes vary wildly and appear to be of limited use. These concepts are summarized in the table below:

Summary of Wood Biochemical Responses

Marker	Characteristic	Level	Bioeffect	Oxidant Exposure
Stilbene	Increase over time [+]	Individual	Yes	No
"	Increase over time [+]	Grove	No	Yes
"	Bipolar magnitude	Individual	No	No
"	Bipolar magnitude	Grove	No	No
H:G Lignin	Decrease over time [-]	Individual	Yes	No
"	Decrease over time [-]	Grove	No	Yes
"	Bipolar magnitude	Individual	No	No
"	Bipolar magnitude	Grove	No	No
Stilbene3	Increase over time [+]	Individual	No	No
"	Increase over time [+]	Grove	No	No
"	Bipolar magnitude	Individual	No	Yes
"	Bipolar magnitude	Grove	No	Yes

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. BACKGROUND & PROBLEM

Ozone-induced foliar injuries such as chlorotic mottle and accelerated senescence have been well documented for both conifer and crop plants (Miller et al. 1963; Luethy-Krause et al., 1990; Price et al., 1990; Beyers et al., 1992). However, ozone (O_3) may not be the only phytotoxicant present in air pollution, and other environmental conditions such as drought and nitrogen (N) deposition can affect biochemical and physiological states contributing to "vigor," which in turn may affect responses to phytotoxicant exposure.

In principle, it is feasible to resolve the exposure and impact of many different stresses on plants, provided there is a sufficient number of biomarkers analyzed. Although there are no guidelines to estimate a "sufficient" number of markers, it is, for example, possible to distinguish drought from O_3-induced stress responses by examining the changes in just a few amino acids, particularly proline, asparagine, glycine, and serine. Proline accumulation is characteristic of drought stress but not for O_3 stress while increases in the concentration of the other three amino acids have been reported for both stresses (Bender et al., 1990; Manderscheid et al., 1991). This illustrates that the pattern of biochemical responses, and not just individual markers, is the key to identifying multiple stress factors. The more markers available, the more reliable is the pattern (Huggett et al., 1992).

Multiple stresses, of course, are common in forests. In California, N-deposition from nitrogen oxides (NO_x) or ammonia sources co-occurs with photochemical oxidants, the effects of which have been documented in California forests for over 40-years (Miller et al., 1997). In remote field sites, injury is best assessed by visible foliar damage (i.e., chlorotic mottle). Unfortunately, even evergreens such as pines will lose their leaves within five to seven years, so field assessment of ozone injury must be conducted frequently and repeatedly. This limits injury assessment to the duration of the study and to a small number of assessment sites.

On the other hand, if there were *permanent, biochemical* markers of ozone injury and other stresses, then the many restrictions of prior approaches could be removed. A *permanent* marker would mean that long-term forest injury records could be established using very infrequent sampling, freeing resources to assess a large number of sites. Such a marker that is also *biochemical* in nature, could be linked to the mechanism(s) of injury, and hence reveal the photochemical oxidant species most injurious to trees under field conditions. Certainly, it is well-known in ecotoxicology that biochemical markers tend to be the most sensitive as well as more reliable markers of specific toxicants than other types of biomarkers (Huggett, 1992). However, the National Research Council (1989) stated that "to date no readily detectable, pollutant-specific single marker for identifying the effects of air pollution on forests or trees has been identified," which still holds true today.

II. RATIONALE & RELATED WORK PRIOR TO THE CONTRACT

To address the above, tree ring biochemical analysis has the potential to reveal *permanent, biochemical* markers. Past work by groups worldwide have concentrated either on morphological characterization, or on pollutant levels in tree rings. In the meantime, no one has looked at the biochemistry of the tree ring wood itself.

In a 1994-96 seed project funded by the University of California, Davis (UCD), Center for Ecological Health Research, in cooperation with Michael Arbaugh and Paul Miller of the U.S.

Department of Agriculture (USDA), Forest Service in Riverside, California, we chemically analyzed tree ring segments from ponderosa pines at sites in San Bernardino National Forest (NF). The technique we used is an advanced wood biochemistry tool known as pyrolysis-gas chromatography/mass spectrometry (pyro-GC/MS) (Faix et al., 1987; cf. Figure 1). Since this method does not require pre-choosing the analytes, it is not limited by our current knowledge (or ignorance).

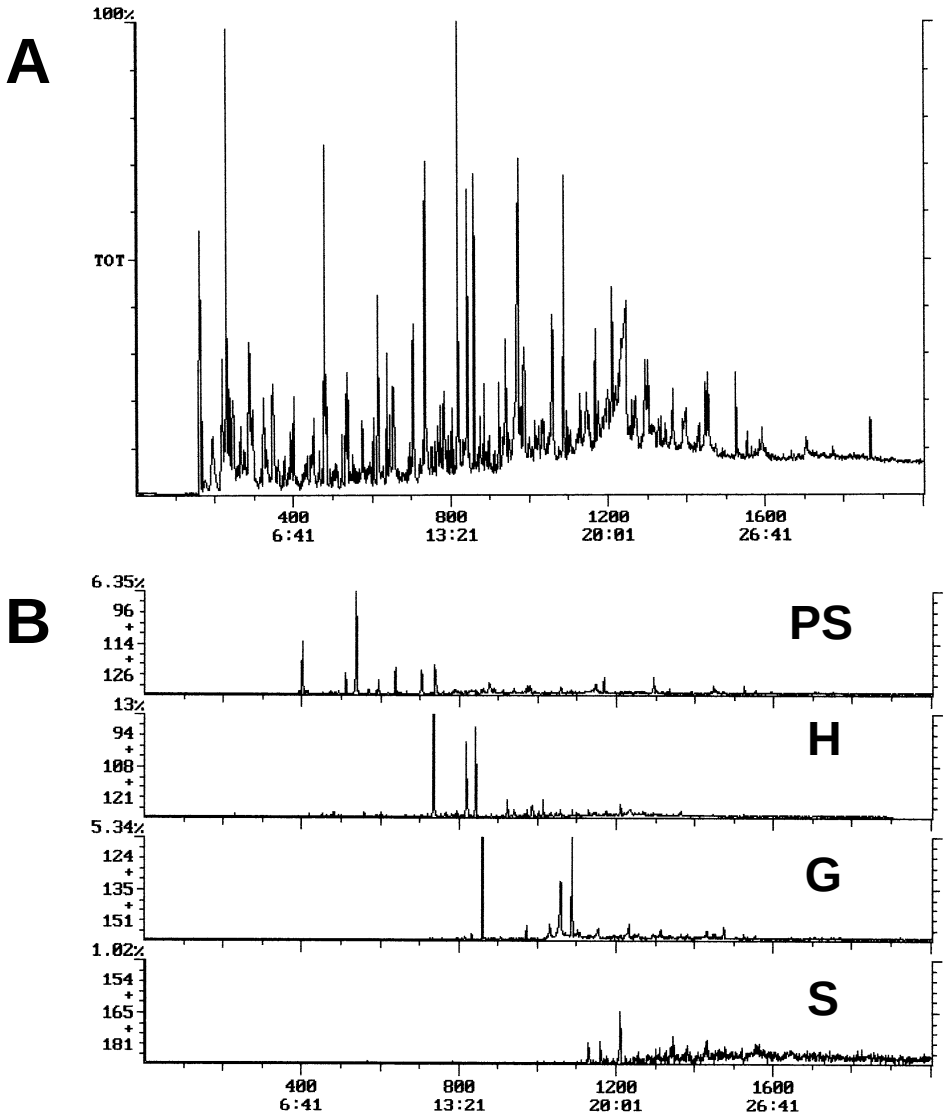


Figure 1. Pyrolysis GC-MS Chromatograms of Whole-wood. (A) Total ion chromatogram, showing all wood constituents (thermolytic fragments) at once. (B) Selected ion chromatograms, which are subsets of A, that depict thermolytic fragments from polysaccharides (PS) and H, G, and S-type lignin structures. Each chromatogram is normalized to its largest peak, and is therefore plotted on a different ordinate scale. Part B shows how different peaks in the Part A complex might arise from PS as well as H, G, and S-type lignin. Totaling the peak areas in each constituent category yields results of the type shown in Figure 2.

Just as important, pyro-GC/MS is very practical to perform. Tree cores are sampled in standard fashion, and put under dry-ice temperature for transport (it is possible that the dry ice is not needed). The cores are freeze-dried and ground to fine powder, or for resinous wood, ground under liquid

nitrogen when they are extremely brittle. The ground samples are inserted into quartz tubes and into the pyro-GC/MS instrument.

Briefly stated, our pyro-GC/MS survey found two marker categories – ratios of H-type to G-type lignins and moieties of phytoalexins (a class of plant defense chemicals) bound to the wood matrix. These markers correlate very strongly to tree responses in established ozone-injury sites, northeast of the Los Angeles Basin, in the post-industrialized period following World War II (Higashi et al., 1995) (Figure 2). It is reasonable to conjecture that many more markers will be found in these extremely information-rich data sets; for example, one wood chemist has estimated that there are over a million biochemical markers present in these datasets, of which we have examined only a dozen to date.

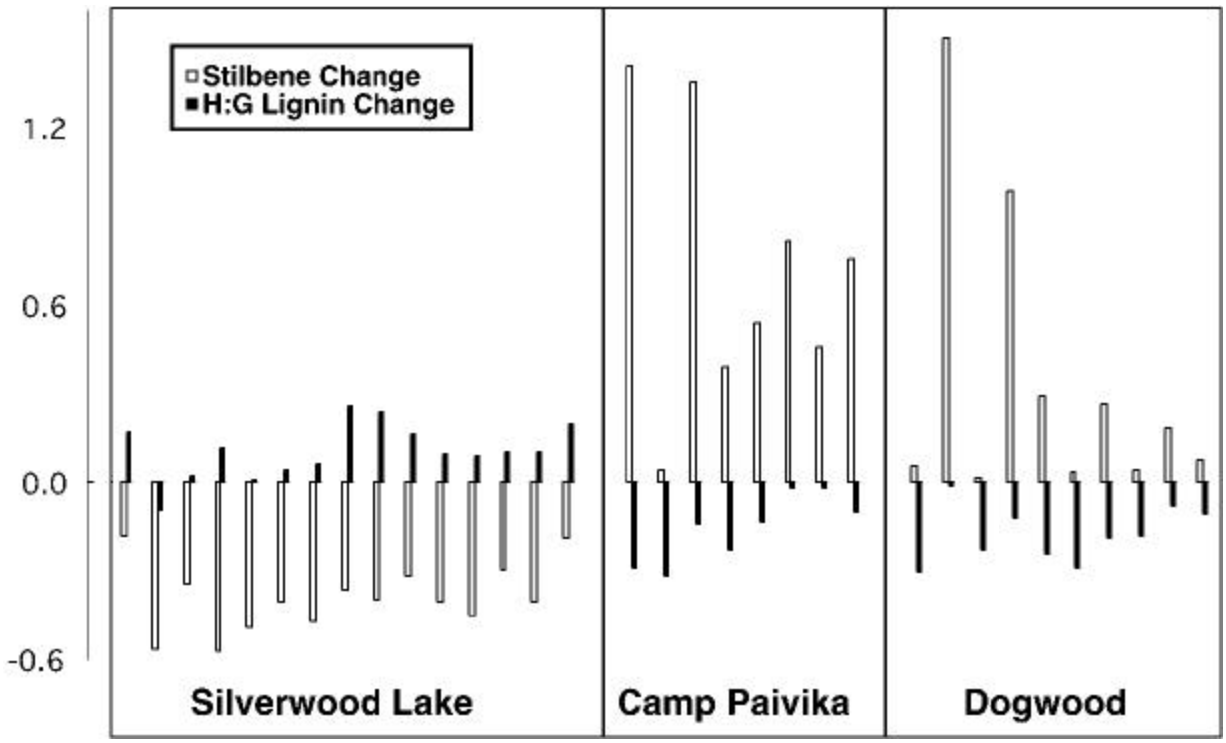


Figure 2. Differences in H:G Lignin Ratio and Stilbene Content in Ponderosa Pine Wood (each pair of bars represents an individual tree) between 1900-1940 (pre-war) and 1950-1995 (post-war). The ordinate is the relative change (post-war ÷ pre-war) of stilbene or H:G lignin ratio. Data are derived from analyses of the type shown in Figure 1. Silverwood Lake is an interior site that has no symptoms of ozone damage, and Dogwood and Camp Paivika are impacted sites along the rim with severe ozone crown damage for over 20 years (Miller, 1992). Note that, although the *magnitude* of the wood constituent changes from pre- to post-World War II eras vary among individual trees, their *direction* is very consistent within a site. The individual magnitudes may reflect genetic and/or microsite differences. (cf. Higashi et al., 1995)

III. OBJECTIVES

Based on preliminary findings, the project objectives were:

(1) To correlate pyro-GC/MS tree ring biochemical markers with phytotoxicant injury indices for ponderosa pine (*Pinus ponderosa*) at established ozone impact sites (Note: At the start of this contract, a survey of these sites was initiated as a pilot project with USDA, Forest Service, with no support for personnel or supplies);

(2) To uncover, through multivariate data analysis, potential pyro-GC/MS markers of other environmental factors such as nitrogen deposition and drought at the sites from (1);

(3) To determine, through comparative studies, whether archived tree cores could be used for analysis – if successful, this could eliminate a great deal of sampling costs; and

(4) To explore a second tree ring biochemical analysis technique, Fourier-transform infrared microspectroscopy (μFTIR), which has the same advantages as pyro-GC/MS, but is based on a different physical principle.

IV. MATERIALS & METHODS

IV.A. Sampling Design & Sample Preparation

The sampling design was critical since the overall objective was to refine and test the preliminary air pollutant markers we have assigned in ponderosa pine tree rings, while also attempting to discover new ones. These biochemical markers may range from individual compounds to patterns of chemical structures in the wood. In addition, it was also important to distinguish other factors that may be coincident with the air pollution, which may interfere with the marker assignment.

Therefore, the investigation proceeded in two phases. In the first phase, we tested and calibrated our pyro-GC/MS technique at sites in southern California using trees with well-characterized oxidant injury, but also exposed to high levels of nitrogen (N) deposition. In the second phase , we applied the techniques tested in the first phase to considerably less characterized sites in the Sierra Nevada.

For the first phase, our collaborators, Michael Arbaugh and Paul Miller of the USDA, Forest Service, identified ponderosa pine study sites for the proposed project. As stated previously, at the start of this contract, we had initiated a pilot study with them (no support for personnel or supplies) that grew into the first phase of this study. The study consisted of sampling selected trees from a matrix of six forest sites in the San Bernardino National Forest, three urban-facing and three interior sites (Figure 3). At each site were individual ponderosa pine trees that had been scored over the last two decades with regard to their sensitivity to oxidant injury (Miller, 1992). The first phase study was thus designed to distinguish between exposure markers, which would be site-dependent (regardless of injury symptoms), and bioeffect markers, which would be symptom-dependent (and less strongly site-dependent).

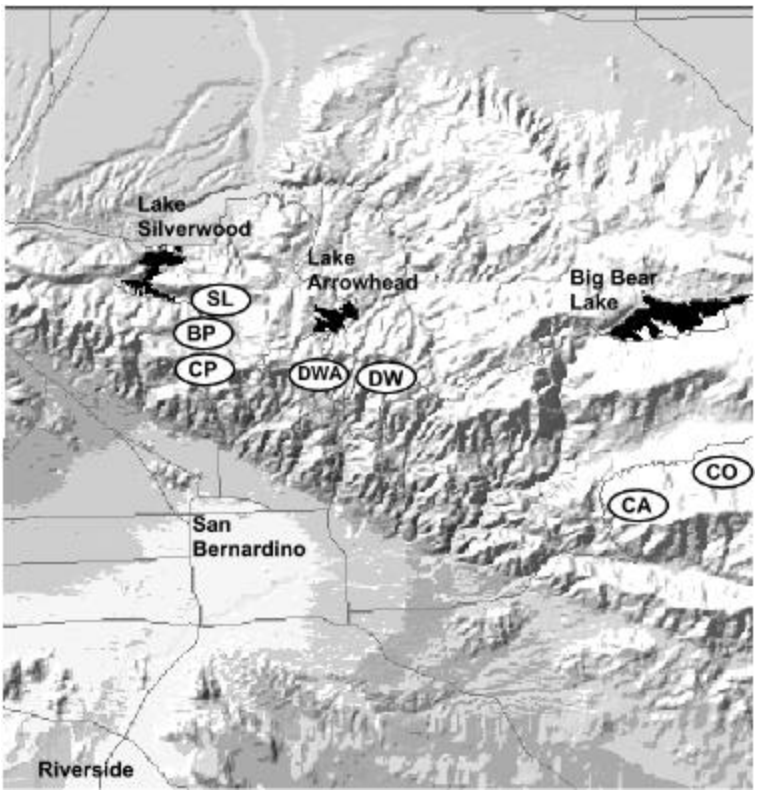


Figure 3. Location of the Tree-ring Core Sampling Sites in the First Phase Study. Site designations are as stated in the text. Sites CP (Camp Paivika) and DW (Dogwood) were used for the preliminary study, while BP (Breezy Point), DWA (Dogwood A), CA (Camp Angeles), and CO (Camp Osceola) sites – inscribing a west-to-east decreasing ozone gradient – were used for the first phase study. For both studies, site SL (Silverwood Lake) was the no-measurable ozone impact reference site. [Map adapted from Miller (1992) and generated by the Information Center for the Environment at UCD]

There were additional reasons for choosing these sites. The presence of ozone injury to ponderosa and Jeffrey pines has been documented at these sites since the early 1950's. Field experiments at these sites (Miller et al., 1963) and later ozone fumigation of ponderosa pine seedlings (Richards et al., 1968) confirmed that ozone was the cause of chlorotic mottle symptoms and early abscission of affected needles. In addition, the study sites were among a system of 18 long-term study plots of the San Bernardino Mountains Gradient Study (SBGS) established in 1973-74 and reexamined in 1978, 1984, 1988 and 1994. As shown in Figure 3, these sites were Camp Paivika (CP), Dogwood Campground (DG), Breezy Point (BP), Dogwood Campground A (DWA), Camp Angeles (CA), Camp Osceola (CO), and Silverwood Lake (SL).

For the second phase of the study, we sampled and analyzed cores of trees from sites in the Sierra Nevada range where oxidant injury had been documented (Peterson et al., 1989; cf. Table 1 and Figure 4). Because of restrictions in analysis, pyro-GC/MS dictated dividing the samples into two time-periods. Whenever possible, we sampled trees that had had dendrochronological analysis performed in the past. However, recent analyses were not available in most cases.

Table 1. Tree-ring Core Sampling Sites in the Sierra Nevada.			
Location[1]	Elevation (m)	No. of Trees Studied[2]	Ambient Ozone Data
Manzanita Lake – Lassen Volcanic National Park (LV)	1,768	150 (in 3-plots)	Yes
White Cloud (WC) – Tahoe National Forest	1,326	150 (in 3-plots)	Yes
Wawona – Yosemite National Park (YW)	1,219	150 (in 3-plots)	Yes
Shaver Lake (SHL) – Sierra National Forest	1,828	150 (in 3-plots)	Yes
Grant Grove (GG) – Kings Canyon National Park	1,981	150 (in 3-plots)	Yes
Giant Forest (GF) – Sequoia National Park	1,920	150 (in 3-plots)	Yes
Mountain Home (MH) – Sequoia National Forest	1,890	150 (in 3-plots)	Yes

[1]cf. Miller et al. (1996). [2]Number of trees tagged in an earlier effort for dendrochronology analysis. Value represents the total number of trees available for examination in the present study.

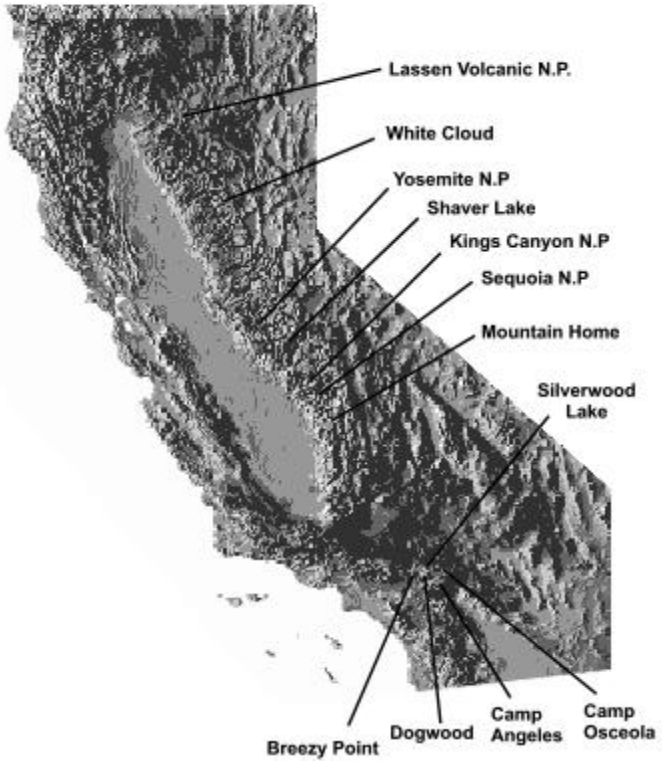


Figure 4. Location of the Sierra Nevada Tree-ring Core Sampling Sites in the Second Phase Study. Site designations are as stated in the text and in Table 1, and include the locations of the San Bernardino Gradient Study (SBGS) Sites. [Adapted from maps generated by the Information Center for the Environment at UCD].

Tree cores were sampled using standard Teflon-coated increment borers, cleaned with isopropanol and free of organic residues (e.g., coring waxes were not used). Sampling trees in this fashion is considered to have "low-impact" on tree health (cf. Lewis, 1995). Tree cores were obtained from the trees using standard methods, frozen immediately and transported under dry ice to ensure that chemical changes were minimized. They were then freeze-dried and stored under desiccated conditions at -70

°C until use. In the event a particular tree has not had prior dendrochronological analysis, we sampled a duplicate core for that purpose, to be analyzed in the future.

For pyro-GC/MS analysis, tree cores were cut into two or three segments representing periods of major air pollution change at the site. For example, the study divided the tree rings into two periods, pre- and post-World War II, representing eras prior to and during the industrial boom in the Los Angeles basin (Figure 2). The segments were ground in a delrin-milling chamber to < 3µm particles using a reciprocating micro-ballmill. This sample collection and processing procedure ensured the chemical structure integrity of the wood material, mixing it such that a sub-milligram aliquot (sample loading maximum for pyro-GC/MS) was representative of the entire wood segment.

For µFTIR analysis, wood cores were sliced longitudinally, and chemical data were obtained from *every* annular ring available.

IV.B. Pyrolysis-Gas Chromatography/Mass Spectrometry (pyro-GS/MS) Analysis

The technique of pyro-GC/MS provides detailed chemical structure information on complex wood constituents and has detection limits in the 10^{-15} molar range. Figure 5 shows a schematic of the GC/MS instrumentation that was used in this study, and the instrument currently consists of 100% off-the-shelf components, and required no further hardware development. The utility of the method is illustrated in Figures 1 and 2 (Higashi et al., 1995).

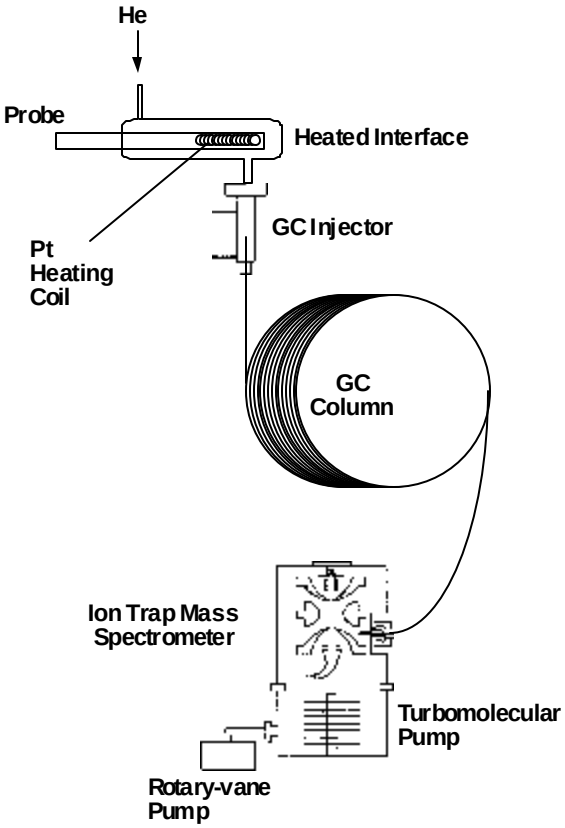


Figure 5. Schematic Diagram of the Pyro-GC/MS System (Note: the sample tube is placed in the heating coil).

To analyze wood samples, a sample tube containing approximately 0.3-mg of wood powder was placed in the platinum (Pt) heating coil swept by a helium (He) stream. The sample is then heated at 1°C/ms to 650 °C, causing thermolysis of chemical bonds according to their relative strengths, which generates volatile products. The products are swept by the He stream into a GC column, which causes the components to elute sequentially. The components eluting from the column were examined by mass-spectral detection; the identity of many fragments is often traceable to the wood structure from which they arose. Thus, the pattern of the fragments is indicative of wood components and structure.

Conditions for analysis were as follows: the sample was pyrolyzed (at 650 °C) under the He gas stream, which sweeps the thermolyzates into a high-resolution GC, typically employing sample splits of 50:1 (i.e., 98% of the sample is discarded). Temperature-programming the GC column causes components to elute sequentially into the mass spectrometer for detection and structural characterization.

The analytical pyrolysis system (Pyroprobe 2000/AS2500, CDS Inc., Oxford, Pennsylvania) was interfaced to a Hewlett-Packard (Palo Alto, California) Model 5890 GC/5971A mass-spectral system outfitted with a non-polar column (0.15-mm i.d. x 50-m, BPX-5 5% phenyl-methyl silphenylene siloxane copolymer, SGE Inc., Austin, Texas). The quartz sample tube was dropped by gravity into the pyrolysis chamber, followed by a 3-second delay to purge out residual air. The pyrolysis carrier gas path was then switched to on-line with the GC/MS, and the pyrolysis probe was heated to 650 °C for 10-s. The volatile thermolyzates were swept by a He stream into the GC for 1-min, after which the pyrolysis system was switched to off-line for thermal cleaning at 20 ml/min He flow for the duration of the analysis. The remainder of the analysis was conventional, with the thermolyzates eluting sequentially into the mass spectrometer for detection. The pyrolysis injector block temperature was fixed at 280 °C, GC injector at 280 °C, He carrier gas velocity kept constant at 40 cm/s, injector split was 1:10, column was temperature-programmed from 40 °C with a 4-min hold to 290 °C at 10 °C/min, and the mass spectrometer interface temperature was fixed at 300 °C. The mass spectrometer conditions were: electron ionization mode, 70 eV electron energy, source/manifold temperature at 180 °C, electron multiplier voltage was 1,458 V, acquisition from m/z 40-400, three spectra averaged into one to yield one spectrum/sec, centroid processing of data to yield the mass histograms, and the system was calibrated to perfluorotributylamine using the “Autotune” function of the software.

Each sample was run twice to ensure data integrity. Using the built-in software, the 25 largest peaks, excluding carbon dioxide, were quantified and corrected for the sample mass. If the two runs differed by > 25% in any peak, a third analysis would be run. In practice, this never occurred. In addition to samples, purified components such as microcrystalline cellulose (Sigma, St. Louis, Missouri), indulin AT, and trans-stilbene were analyzed for comparison. Standard paper filters (Whatman No. 451) were found to be highly reproducible and was analyzed periodically to assess changes in instrument analytical response; changes were negligible (< 3%) for the duration of the study, therefore the data warranted no instrument response correction.

Initial analysis of data sets consisted of comparison to available standards for thermolytic products using structural matching with the aid of a 78,000-compound NIST/NIH/EPA mass spectral library. Following the initial data reduction, we drew on the considerable database of pyro-GC/MS data of wood and cell wall constituents (e.g., Faix et al., 1987; Saiz-Jimenez et al., 1987; Schulten et al., 1989; Pouwels and Boon, 1990; Scheijen and Boon, 1991; Galletti and Reeves, 1991). These approaches enabled us to invoke data reduction procedures to identify wood constituents or chemical class, and the

quantities of these constituents were correlated with the documented impact of each tree at each site (Table 2). For example, in Figure 2, we assumed, based on the literature, that lignin and stilbene components might be affected. This enabled us to process the data set in established ways (as in Figure 1B) to arrive at the relationship shown in Figure 2.

A broader approach to data analysis was also used, taking advantage of the fact that most wood constituents (even those that no one knew existed) are analyzed by pyro-GC/MS. This is fortunate because we don't know, *a priori*, what biochemistry – as affected by air pollution – is recorded in the wood. This is especially important for techniques geared to analyze complex wood structures (such as the two proposed here), because they do not generate simple numerical lists amenable to conventional descriptive statistics. For example, in pyro-GC/MS, a given macromolecular wood structure is potentially represented by peaks distributed throughout the entire chromatogram -- this concept is illustrated in Figure 1B for polysaccharides and lignin sub-structures.

In order to see if we can take advantage of this rich data set for pyro-GC/MS, we investigated reducing the data set from each analysis into a form useable by conventional statistics. Since the Chemstation software was necessary for instrument data acquisition, but not intended for convenient data export, the types of data reductions presented below are completely the result of the particular data formats and limitations of the software. We explored the following ways of data reduction:

Type 1 – Quantify the total area of single ion mass chromatograms (SIMC), which will yield a simple list of up to about 300 numerical values per sample.

Type 2 – Separately quantify just the major peaks present in each SIMC, yielding a set of x-y data (intensity, ion mass).

Type 3 – Quantify the Type 2 data set plus the peak retention time, yielding a set of x-y-z data (intensity, position, and ion mass).

Type 4 - Quantify chemically coherent sets of SIMC (for example, m/z91 + m/z105 + m/z119 which are alkyl-phenols), which was the approach taken to arrive at Figure 2. As already mentioned, this requires pre-judgment as to which wood constituents are of interest.

Type 5 - Addition of all mass spectra acquired during a GC run, in effect producing a single mass spectrum.

In all cases, each marker, including unknowns, was represented by a single numerical peak area value. Throughout this study, we found a linear relationship of the acetic acid 60 Dalton peak area with sample mass (data not shown). Since << 1-mg sample was difficult to weigh into the narrow pyrolysis tubes, we judged the weighed mass to be less reliable than the acetic acid peak area. Accordingly, all marker peak areas were normalized to that of the corresponding acetic acid peak area.

The mass-normalized peak areas were then converted to fractional change for pre- and post-1945 (first phase study) or 1955 (second phase study) periods prior to analysis using the following approach:

$$Y = [(X_{post} - X_{pre}) \div X_{pre}] \quad (1)$$

where Y is the response variable used in the analysis, X_{pre} is the value from analysis of material pre-period, and X_{post} is the value from analysis of post-period. For the sign (direction-of-change) test analysis, if Y was within ± 0.05 then it was designated as "0" change.

For the first phase study, two sets of analyses were conducted comparing the consistency of changes in the two-biochemical markers, H:G lignin ratio and stilbene, implicated as promising in the preliminary study. The first set tested the hypothesis that the biochemical markers were indicators of air pollution exposure, and not necessarily of bioeffect. This was assumed to be true if the sign changes (+, -, 0) of either biochemical marker was consistent with preliminary results, regardless of historical crown injury or basal area changes of individual trees. For example, based on the preliminary study results (Figure 2), patterns similar to Dogwood and Camp Paivika would be expected for all trees at high and intermediate sites (Table 2), while patterns similar to Silverwood Lake might be expected for all trees at low exposure sites (Table 2).

The second set of analyses was to test whether the two markers indicate biochemical effects of air pollution injury. For the biochemical effects analysis, two groups of trees were identified prior to field sampling (i.e., sensitive and tolerant), based on the pattern of foliar injury evaluations from 1973-94. Historical injury scores were translated into Forest Pest Management (FPM) indices (Pronos et al., 1978) for this study. The FPM system scores the youngest annual needle whorl to have visible ozone injury. Low values (0-1) signify severe crown injury and large values (4-5) signify low crown injury. Classifications of sensitive (S), intermediate (I), or tolerant (T) were based on the amount of crown injury shown by the tree relative to the average site injury.

Following the 1997 crown evaluation conducted in this study, each sample tree was reassessed and assigned to a final group. Approximately 55% of all trees remained in their original classification, and 30% were reclassified from sensitive to tolerant, or sensitive to intermediate. About 5% of trees were reclassified from tolerant to sensitive, and 10% were reclassified from tolerant to intermediate. The larger numbers of reclassified sensitive-trees were the result of generally lower levels of crown injury (Table 3) in 1997, relative to historical injury between 1974-94. The intermediate category was created when it became apparent that some of the original sample trees showed both tolerant and sensitive ratings at different evaluation periods, and some substitute trees (replacements for trees we were unable to locate) did not have the extreme injury of the original sampling group. Data analysis was conducted both including the intermediate group, as well as by combining the intermediate and tolerant groups into a single (e.g., tolerant) group.

Changes in average annual bole increment were also calculated for each tree. Total ring-width to the nearest 0.01-mm was measured using a digital micrometer and the number of annual rings was counted for the two time periods (i.e., pre- and post-period after standard cross dating techniques were used to accurately associate each tree ring with a year). Ring-widths were converted to basal area and the change in average annual basal area was calculated for the pre- and post-periods by site (Table 2) and by crown sensitivity group. Both direction-of-change (sign) and t-tests were conducted to determine if either the sign or the amount of change in basal area between periods is associated with crown condition groups at each site.

IV.C. Fourier-transform Infrared (FTIR) Microspectroscopy Analysis

Fourier-transform infrared (FTIR) spectroscopy is one of the cornerstone techniques for modern organic structure identification, and is used extensively in wood structure research, partly because of its ability to discriminate the very complex structures of wood (e.g., Jung and Himmelsbach, 1989; Banerjee and Lee, 1991; Friese and Banerjee, 1992). In addition, it requires little or no sample preparation for analysis, and is thus, well suited for tree-ring analysis. Despite these features, we know of no literature on this application of FTIR.

Conventional FTIR would have still required cutting segments and grinding as with pyro-GC/MS. However, we explored an advanced form of FTIR, known as FTIR *micro*spectroscopy (µFTIR; Morris, 1995). It uses a light-type microscope coupled to a FTIR spectrometer to obtain spectra from areas as small as 10-µm x 10-µm for applications such as mineral identification in tiny granules (Rintoul and Fredericks, 1995). In our case, this ability was coupled with a programmable mapping stage that has 1-µm reproducibility in sample position.

For tree ring analysis, the potential of µFTIR over conventional FTIR is considerable. The technique is capable of non-destructively analyzing complex wood constituents in *intact* cores at a rate of less than 2-min per analysis, at a resolution that readily enables the *analysis of individual rings,* and furthermore links each spectra directly to the image of the core. Figure 6 shows that there were significant differences in the spectra of different tree rings, and therefore held great potential for relating these spectral features to the documented impact of each tree at each site, or to meteorological and ozone data for each year.

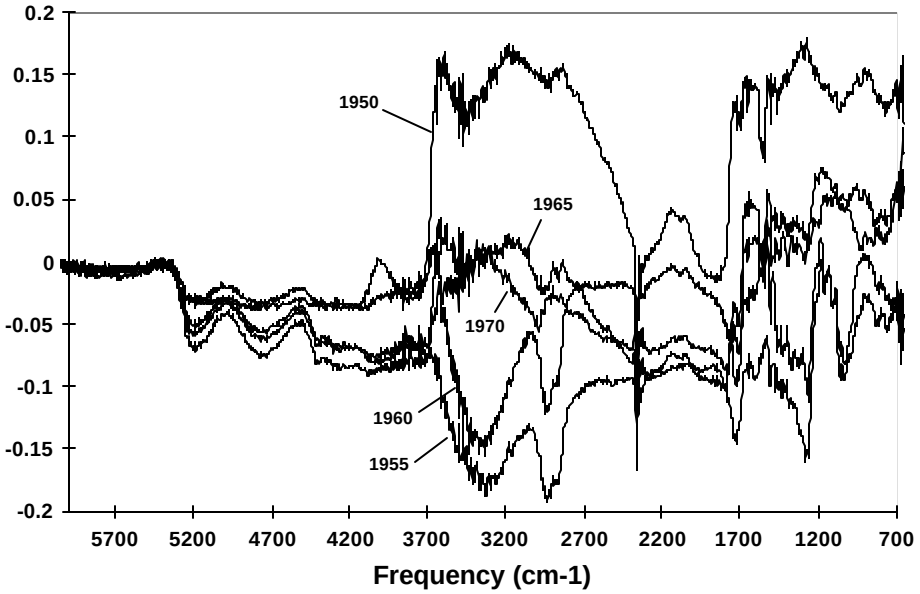


Figure 6. Difference in µFTIR Spectra Relative to 1946. [The spectrum from the 1946 segment was subtracted from spectra of selected rings. This tree clearly expresses significant differences in wood chemistry, even over short time periods. Although caution must be exercised for any interpretation of the natural products biochemistry involved, these peaks and valleys can be compared with known environmental conditions (e.g., ozone, drought, and nitrogen deposition) on an annual basis at the proposed "calibration" sites. Thus, these differences are best considered to be "exposure" markers. Separate physiological experiments on seedlings will be required to determine the chemical identity of these wood constituents and their mechanism of response to air pollutants.]

V. RESULTS

V.A. Pyro-GC/MS Datasets

A typical chromatogram from tree core analysis is shown in Figure 1A. As described in the Methods section, peak areas can be obtained either from conventional integration of individual peaks, or integration of entire single-ion chromatograms putatively representing a wood constituent substructure, or some variations and combinations of these approaches. In particular, we hypothesized, that lignin (Galliano et al., 1993) and stilbene (Rosemann et al., 1991) components in the wood might be affected by oxidants. Earlier literature (Faix et al., 1987) plus our comparative analysis of cellulose and indulin AT indicated that areas under the single-ion chromatograms of 94, 108, and 121 Daltons, and 123, 135, and 151 Daltons, should be summed to obtain H-type and G-type lignin, respectively (Figure 2). Stilbene, based on our analysis of authentic trans-stilbene, could be quantified using the 104 Dalton single-ion chromatogram peak of styrene at 890-s retention time (example not shown). This approach enabled us to process the data to arrive at the relationship shown in Figure 2.

Specifically, we quickly ruled out the Type-1 data reduction, as it was extremely labor intensive using the necessary instrument software. Type-2 data reduction lacked the dynamic range and failed to satisfactorily reduce even the preliminary data set. Type-3 data reduction was ruled out due to the normal slight irreproducibility in peak retention time, which played havoc with reliable assigning of peaks in the extremely crowded chromatograms. Therefore, for this study, we used Type-4 and Type-5 data reduction, which is how the preliminary study was done to produce the results in Figure 2.

For Objective 3, we also investigated whether stored cores from previous studies could be used for analysis. In the vast majority of cases, cores are treated with wood-preserving stain (e.g., varnish), that would add considerable artifactual "noise" to the data sets. Since it may be possible to analyze the centers of stained cores, where the preservative has not penetrated, we devised a simple test using duplicate cores from Silverwood Lake that were either frozen in liquid-nitrogen and stored at -70°C, placed under dry ice and stored at -70°C, or kept at ambient temperature for two weeks before freeze-drying, pulverization, and analysis. The first two sample-handling schemes yielded comparable chromatograms, however, the last was dramatically different in several key aspects. For example, the H-lignin was much reduced in the ambient-stored samples, possibly due to the more chemically labile nature of H-lignin substructures in air. This factor alone would be reason to reject ambient-stored wood from consideration in such biochemical analyses, therefore, we dropped from consideration the analysis of previously sampled cores.

V.B. First Phase Study using Pyro-GC/MS

In the preliminary survey summarized in Figure 2, only sensitive trees with severe crown injury were selected from the "impacted" sites, so that we were unable to distinguish whether H:G lignin ratio and stilbene changes were indicators of exposure (site-level effects) or effect (severe crown injury). Therefore, four additional sites from among the 18 San Bernardino Gradient Study (SBGS) sites (Breezy Point, Dogwood A, Camp Osceola, and Camp Angeles) were sampled to determine if these two markers have potential as indicators of either oxidant exposure or damage to ponderosa pines, particularly if they can be associated with the severity of crown damage or changes in radial stem growth rates of ponderosa pine. In order to assess this, the main study consisted of individual trees tagged and included in the SBGS, previously documented to have varying resistance to oxidant injury

(Coyne and Bingham, 1982; Grulke, 1999). The number of trees sampled at each site and number of core samples taken is summarized in Appendix A.

V.B.1. Patterns of Growth & Crown Injury

Tree ages and growth rates differed among sites (Table 2). Trees in the eastern sites (CO and CA) were older, but had smaller average basal area increments in recent years relative to those from pre-1945. Trees at western sites were younger and average basal area increments were larger post-1945 relative to pre-1945. Changes in basal area for individual trees were highly variable at all sites, such that when averaged, the value was close to zero (Table 2).

Table 2. Pine Tree Data Measured in 1997[1].

Site [# of Trees]	Relative Ozone Exposure	DBH[2] (cm)	Basal Area[3] Pre-1945 (cm^2/yr)	Basal Area[3] Post-1945 (cm^2/yr)	Basal Area[3] Change in ABA	No. of Rings Pre-1945	No. of Rings Post-1945	FPM[4] 1997
Camp Paivika (CP) [8]	High	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Breezy Point (BP) [17]	High	49.0 (13.0)	718 (359)	1,213 (696)	0.03 (0.48)	31.7 (4.4)	53.8 (7.5)	1.6 (0.8)
Dogwood (DW) [10]	High/Int.	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Dogwood A (DWA) [14]	High/Int.	70.7 (20.3)	1,302 (1,502)	1,529 (1,204)	-0.18 (0.35)	27.4 (19.0)	44.7 (8.2)	2.3 (0.9)
Camp Angeles (CA) [13]	Int./Low	68.6 (12.1)	2,162 (720)	1,154 (508)	-0.04 (0.43)	93.3 (35.4)	51.0 (4.6)	2.9 (0.9)
Camp Osceola (CO) [13]	Low	53.1 (15.7)	1,319 (936)	839 (274)	0.38 (1.00)	70.8 (27.0)	50.7 (4.6)	4.3 (0.9)
Silverwood Lake (SL) [15]	Int.	69.8 (16.8)	N/D	N/D	N/D	46.3 (23.8)	50.0 (0.0)	5.0 (0.0)
All [90]	All	60.2 (17.7)	1,380 (1,078)	1,196 (777)	0.04 (0.62)	55.7 (36.7)	50.1 (7.1)	2.7 (1.2)

[1]Numeric values represent the Mean (± standard deviation), and "N/D" indicates no data was recorded or available at the site for a specific variable. [2]DBH (diameter at breast height) is the tree diameter at 1.4 m. [3]Change in ABA (Annual Basal Area) = (post-1945 minus pre-1945). A positive "change" denotes an increase in ABA over time. [4]FPM (Forest Pest Management) is a crown injury scoring system for pines, where 0 = high needle injury and 5 = low needle injury.

Branches of sample trees evaluated in 1997 (Table 2) indicated that the same relative pattern of ozone injury existed among the sample sites as was identified in previous studies (Miller, 1992). Average FPM scores were generally much higher for the latter survey, which corresponds to reduced ambient ozone in recent years relative to historical exposure (1997 had the lowest recorded ozone levels in the San Bernardino Mountains).

FPM scores were used to separate trees at all sites into three crown condition categories: trees that are sensitive (S), intermediate (I), and tolerant (T) to ozone injury. In this study, we included both 'avoiders' and 'tolerators' as defined by Grulke (1999) in the T group. It is likely that trees with intermediate injury (I) have some endogenous tolerance or avoidance responses to ozone (e.g., lower stomatal conductance), but not as much as tolerant individuals. The mean FPM scores were significantly different ($p < 0.05$) for the S and T groups (Table 3). At some sites the results were variable (Table 3) due partially to the low sample sizes of some groups. In general, the greatest difference between crown conditions was found at the high-intermediate ozone exposure site, Dogwood A, and lowest at the least impacted sites,Camp Osceola and Silverwood Lake (Table 3).

Table 3. Average Forest Pest Management (FPM) Injury Scores by Crown Condition Category[1].

Site [# of Trees]	Relative Ozone Exposure	← Crown Condition Category[3] →			All
		Sensitive (S)	Intermediate (I)	Tolerant (T)	
Camp Paivika (CP) [8]	High	N/D	N/D	N/D	N/D
Breezy Point (BP) [17]	High	0.50	0.80	1.07	0.75
Dogwood (DW) [10]	High/Int.	N/D	N/D	N/D	N/D
Dogwood A (DWA) [14]	High/Int.	0.27*	0.9	2.05*	0.96
Camp Angeles (CA) [13]	Int./Low	0.40*	1.29	1.40*	1.21
Silverwood Lake[2] (SL) [15]	Int.	N/D	N/D	5.00	5.00
Camp Osceola (CO) [13]	Low	1.08	1.16	1.40	1.25
All Sites [90]	All	0.57*	1.12	1.30*	1.04

[1]Values are the mean of 1974, 1978, 1988, and 1998 crown evaluations, where 0-1 = severe, 2-3 = intermediate, and 4-5 = low crown injury. [2]Not included in overall average because crown injury was evaluated only in 1998. [3]Asterisk (*) indicates a significantly different FPM score among crown condition categories at a site ($p < 0.05$); N/D = no data.

V.B.2. Relationship of Biochemical Indicators to Oxidant Exposure

Initial examination of the biochemical marker results indicated that there was no consistent pattern of changes related to the sites. For example, comparing the T-rated trees (the only crown condition category that was present at all sites), there was no trend with either of the biochemical markers attributable to differences in historical ozone exposure. This was confirmed by subsequent sign test results (data not shown). Thus, neither marker appears to be reliable assessment of ozone exposure; in pine needles, chronic exposure to ozone did not result in significant changes to total lignin or phenolic content (Booker, 1996).

V.B.3. Relationship of Biochemical Markers to Crown Condition

When all trees were grouped according to their crown condition, regardless of the site, consistent responses were found for both stilbene and H:G lignin marker changes from pre- to post-1945 (see results for "All Sites", Table 3). Specifically, the majority of H:G lignin marker changes were negative for S-rated trees, and positive for T-rated trees, while the relationship was reversed for the stilbene marker, consistent with the preliminary study (Figure 2). Considering data for only the high and high-intermediate ozone exposure sites Camp Paivika, Breezy Point, Dogwood, and Dogwood A, the H:G lignin marker changes were significant, while stilbene marker changes were significant except at Breezy Point (Table 4). For the intermediate-low ozone exposure sites (Camp Angeles, Camp Osceola), located at the eastern side of the mountains (Fig. 3), neither biochemical marker was consistent with crown condition category (Table 4). It is possible that the response threshold of the markers were insufficient to override individual differences in factors such as injury resistance, interactions with other perturbations (e.g., pathogens, and N-deposition), differences in total ozone exposure, composition of air pollutants relative to western sites, tree age, or precipitation. At eastern sites, N-deposition is proportionally lower (Fenn and Bytnerowicz, 1993), and precipitation is also about 50% lower (Miller, 1992) than at western sites.

Table 4. Significance of Pre- to Post-1945 Direction-of-Change (sign) for Stilbene and H:G Lignin Ratio[1].

Site	Relative Ozone Exposure	Crown Condition Comparisons	Stilbene	H:G Lignin	Basal Area Sign Test	Basal Area T-test
Camp Paivika (CP)[2]	High	S	***	***	NS	NS
Breezy Point (BP)	High	S *vs* T	NS	*	NS	NS
" " "	"	S *vs* (T+I)	NS	*	NS	NS
Dogwood (DW)[2]	High/Int.	S	***	***	NS	NS
Dogwood A (DWA)	High/Int.	S *vs* T	**	***	NS	NS
" " "	"	S *vs* (T+I)	*	NS	NS	NS
Camp Angeles (CA)	Int./Low	S *vs* T	NS	NS	NS	NS
" " "	"	S *vs* (T+I)	NS	NS	*	NS
Silverwood Lake (SL)[3]	Int.	T	***	***	NS	NS
Camp Osceola (CO)	Low	S *vs* T	NS	NS	NS	NS
" " "	"	S *vs* (T+I)	**	NS	NS	NS
All Sites	-----	S *vs* T	***	***	NS	*
" "	-----	S *vs* (T+I)	***	***	NS	*

[1]Tests to compare the average normalized changes from pre-1945 to the post-1945 era. Comparisons were made for trees assigned to sensitive (S) vs. tolerant (T) crown condition categories (S vs T), and sensitive vs. all other crown condition categories (S vs (T+I)); both the sign test and t-test were used to assess changes in basal area. Asterisk (*) indicates a significant differences in direction-of-change at a given site at $p < 0.10$, ** indicates $p < 0.05$, *** indicates $p < 0.01$, and NS = not significant. [2]All trees at Camp Paivika and Dogwood were sampled in the preliminary study and rated sensitive (S). [3]All trees sampled at Silverwood Lake were rated tolerant (T).

As an exercise, I-rated trees were included with T-rated trees, but this made little difference in the results, except for Dogwood A and Camp Osceola (Table 4). The change in results at Dogwood A is likely due to the intermediate crown condition categories being closer to a S-rating than to T-rating, according to the FPM scores (Table 3). Changes in the Camp Osceola results reflect the lack of difference in FPM scores between crown condition categories (Table 3), thus there is less confidence in assigning trees to a crown condition category at this site.

In summary, where historical ozone exposure was the highest, trees with severe crown injury (S-rated) showed decreases of H:G lignin ratio and increases of stilbene markers over time (from pre- to post-1945 periods), while trees with slight crown injury (T-rated) had opposite trends, echoing those at the Silverwood Lake reference site with no crown injuries (also T-rated).

V.B.4. Relationship of Biochemical Markers & Crown Condition with Basal Area Changes

Overall crown injury of all trees was correlated with changes in basal area ($r = 0.57$), but not at individual sites, and not with changes in either H:G lignin changes ($r = 0.27$) or stilbene changes ($r = 0.04$). There were no significant differences in the amount of change (t-test) or direction-of-change (sign test) of average annual basal area increment among crown condition categories (Table 4), although changes were generally smaller for the sensitive crown condition group.

Overall changes in basal area between pre- and post-1945 were significant with crown condition groups (Table 4), and therefore also consistent with both biochemical markers. This may indicate that for larger geographical areas, some relationship exists between reduction in wood production due to air pollution effects that can be detected from biochemical constituents of the wood.

V.B.5. Relationship of Any Biochemical Markers to Precipitation and N-Deposition

As stated in Objective 2, we examined whether any markers appeared to correspond to the precipitation or nitrogen deposition pattern of the SBGS sites. For the reasons stated previously, Types 1-3 data reductions were rejected as infeasible. Therefore, the initial approach, as with the H:G lignin and stilbene for oxidant relations, was Type-4 data reduction where we targeted wood constituents as the analytes for data reduction. Following mass spectral recommendations in the literature (e.g., Galliano et al., 1993; Faix et al., 1987; Schulten et al., 1989), we examined markers for protein, polysaccharides, levoglucosan (subclass of polysaccharides), chitin, suberin, cutin, and separately H, G, and S lignins. We found no significant relationship with SBGS N-deposition or precipitation gradients for any of these markers. The principal confounding factor for all of these markers was the very high variability within sites, exceeding 1,000%, that was far larger than differences between sites. Similarly, Type-5 data reduction yielded only highly variable data sets.

V.B.6. First Phase Study Conclusions

Both H:G lignin and stilbene marker changes over time have potential for use as biochemical effects markers of chronic ozone injury to tree crowns. Neither marker was significant for oxidant exposure (site-dependent). Other biotic or abiotic influences are likely to affect the magnitude of these markers in wood. It may be especially important to understand the role of other components of air pollution (such as N-deposition) and the impact of the atmospheric and other environmental factors on wood biochemistry. The method appears promising for use in future studies because of the intrinsic data-richness of pyro-GC/MS (the 50-markers surveyed here represent only a fraction of the

total markers present in the datasets), and sub-milligram sample requirement makes finer time resolution feasible.

V.C. Second Phase Study using Pyro-GC/MS

V.C.1. Description of Sites

The approximate location of the sites are shown in Figure 4. As in Table 1, all of the sites chosen were either actual stands of trees from, or nearby, Project Forest sites in the Sierra Nevada, as follows:

- Manzanita Lake at Lassen Volcanic National Park (LV) – we were not able to locate the precise trees used in previous studies, so we sampled 10-trees in the immediate area. The approximate middle of the grove was 40° 32.282' N, 121° 33.277' W.
- White Cloud campgrounds (WC) – 10-trees were sampled in the immediate area. The approximate middle of the grove was 39° 19.144' N, 120° 50.693' W for WC.
- Wawona at Yosemite National Park (YW) -- we sampled 15 tagged trees at the grove near the Wawona wastewater treatment facility.
- Shaver Lake (SHL) -- we sampled 15 tagged trees in Plot #2.
- Grant Grove at Kings Canyon National Park (GG) -- we sampled 10 tagged trees in Plot #2.
- Giant Forest at Sequoia National Park (GF) -- we sampled 10 tagged trees in Plot #1.
- Mountain Home State Demonstration Forest -- the Project Forest sites lacked trees of sufficient age, however, Mr. John Pronos of the USDA, Forest Service, guided us to one of his FPM-measuring sites, Mountain Home/Slick Rock (MH). Only six trees were available for coring at this site, therefore this is the only site in this study that had less than ten trees analyzed. The specific trees sampled (that were tagged) are listed in Figures 7-13, and the number of cores are listed in Appendix A.

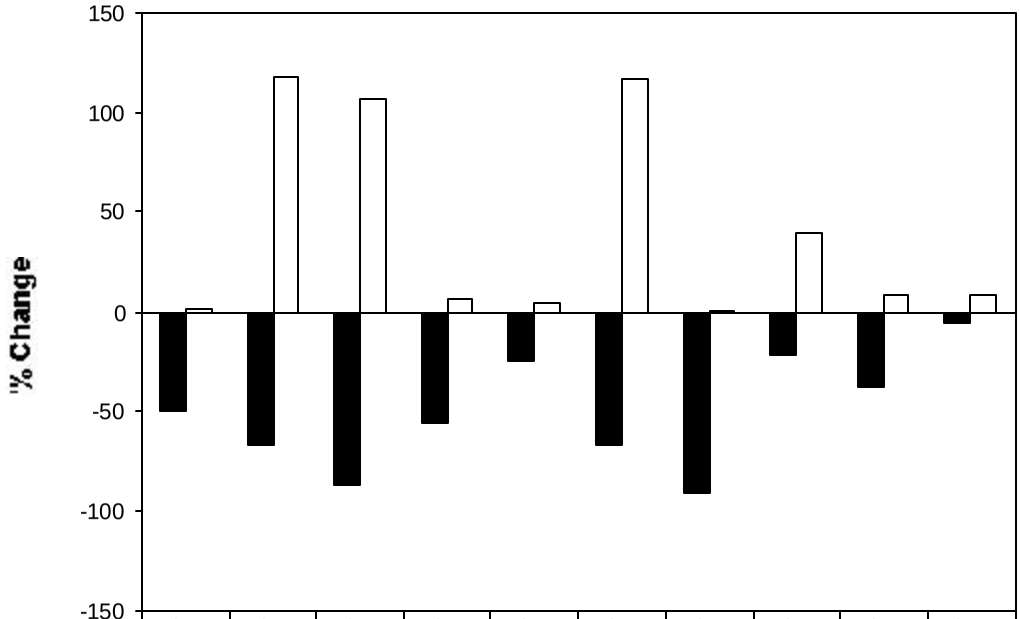
V.C.2. Stilbene and H:G Lignin Ratio Marker Results

As described above, and in the same manner as the first phase study in the San Bernardino NF, stilbene and H:G lignin marker data were obtained for the Sierra Nevada sites. We present the results for each individual tree for two reasons:

- the first phase study showed that these two markers were not relatable to sites (exposure), but were correlated with injury (effects) at the individual level; and

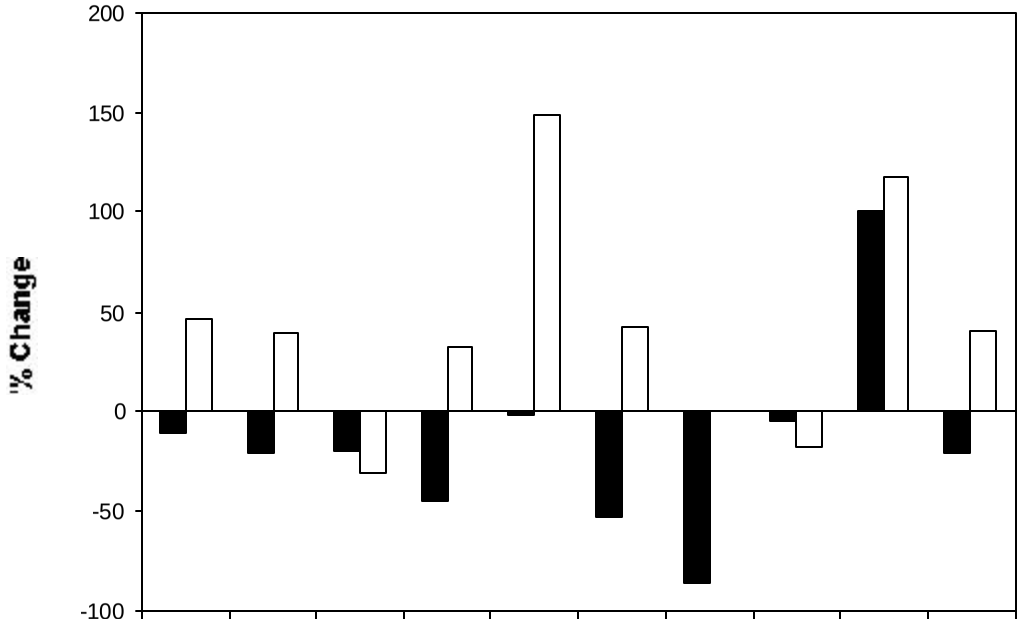
- recent or extensive oxidant injury assessments were unavailable for tagged trees, while tagged trees could not be located at LV and WC, so the marker results could not be correlated to individual oxidant injury as in the first phase study.

Each figure is similar in presentation to Figure 2, in which the stilbene and H:G lignin are paired to each other for individual trees. Tree identification and numerical values of the markers are given in the table below the graph. Preliminary interpretations are given in each figure legend.



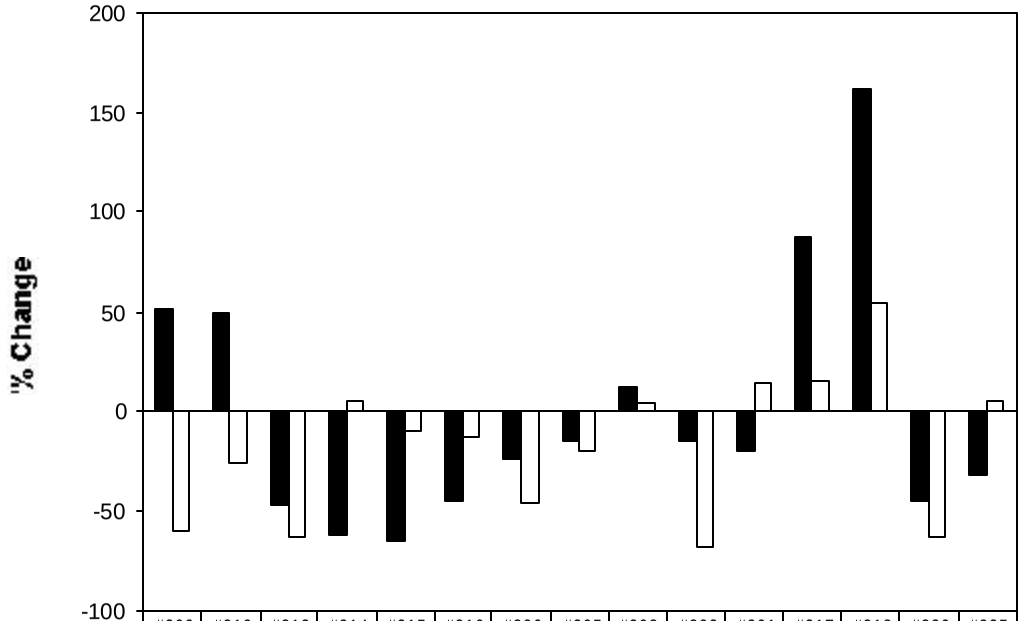
	lv1	lv2	lv3	lv4	lv5	lv6	lv7	lv8	lv9	lv10
Stilbene	-50.40	-67.53	-87.33	-55.50	-24.60	-67.40	-91.47	-21.09	-38.10	-5.30
H:G Lignin Rati	0.96	118.19	106.92	7.23	4.21	116.96	0.70	39.78	8.33	8.28

Figure 7. Individual Tree Results for Stilbene and H:G Lignin Markers at Manzanita Lake (LV). None of the trees show an oxidant effect -- stilbene (0 of 10) are [+] and H:G lignin (0 of 10) are [-]. The trees at LV resemble those at the oxidant unimpacted site at Silverwood Lake (Figure 2).



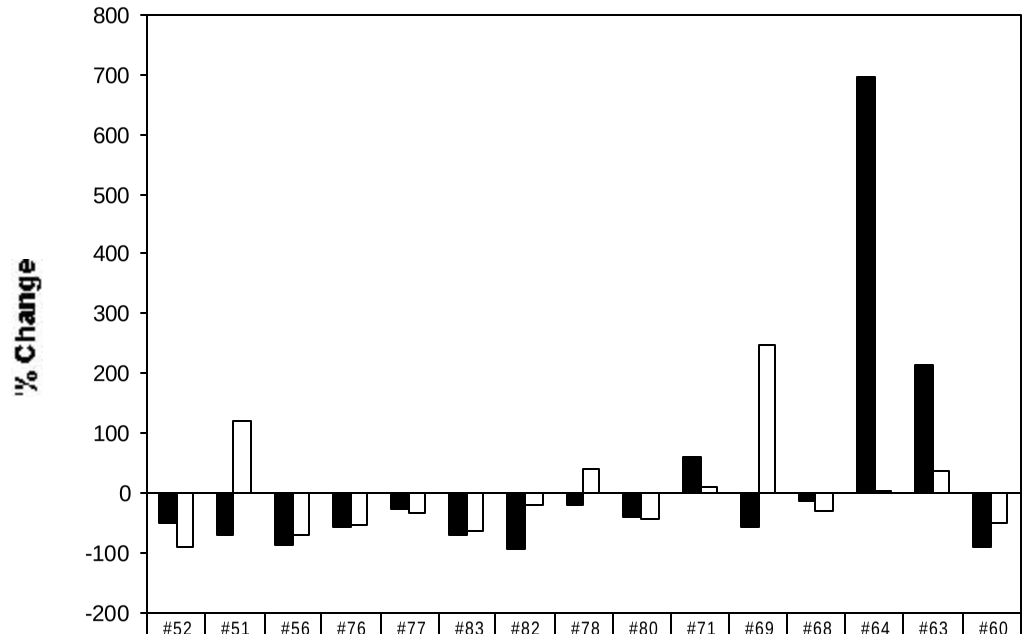
	wc1	wc2	wc3	wc4	wc5	wc6	wc7	wc8	wc9	wc10
Stilbene	-10.55	-21.50	-20.43	-44.17	-2.15	-53.29	-86.43	-4.79	100.19	-21.06
H:G Lignin Rati	46.77	39.15	-30.74	32.99	148.86	42.64	0.93	-18.21	118.19	41.01

Figure 8. Individual Tree Results for Stilbene and H:G Lignin Markers at White Cloud (WC). Almost all trees show a lack of an oxidant effect -- stilbene (1 of 10) are [+], and H:G lignin (2 of 10) are [-]. No individual tree shows both markers of an oxidant effect. The trees at WC somewhat resemble those at the oxidant unimpacted site at Silverwood Lake (Figure 2).



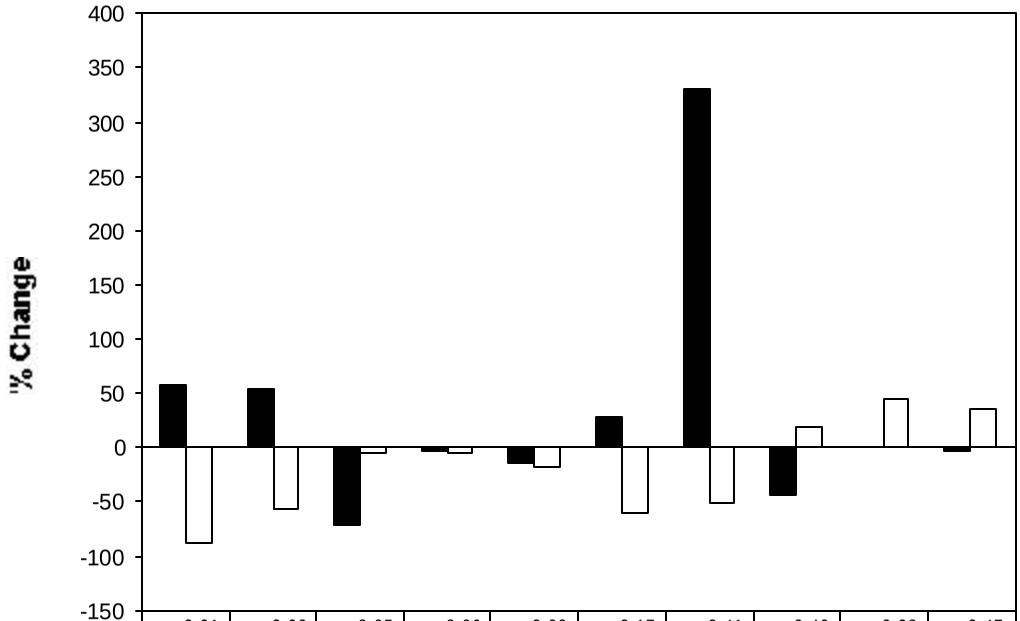
	#209	#210	#213	#214	#215	#216	#206	#205	#203	#202	#201	#217	#218	#220	#225
Stilbene	51.25	49.90	-46.88	-62.00	-65.32	-45.04	-23.65	-15.01	12.33	-14.85	-20.73	87.35	162.19	-44.85	-31.34
H:G Lignin Rati	-59.71	-26.33	-63.20	5.09	-9.99	-13.02	-46.17	-19.98	4.71	-67.44	14.38	15.08	54.81	-63.20	5.09

Figure 9. Individual Tree Results for Stilbene and H:G Lignin Markers at Wawona (YW). Trees at this site are mixed with respect to the sign change of stilbene and H:G lignin. The trees at YW are highly variable among individuals, similar to those at CA and DWA -- the intermediate ozone sites from the First Phase Study.



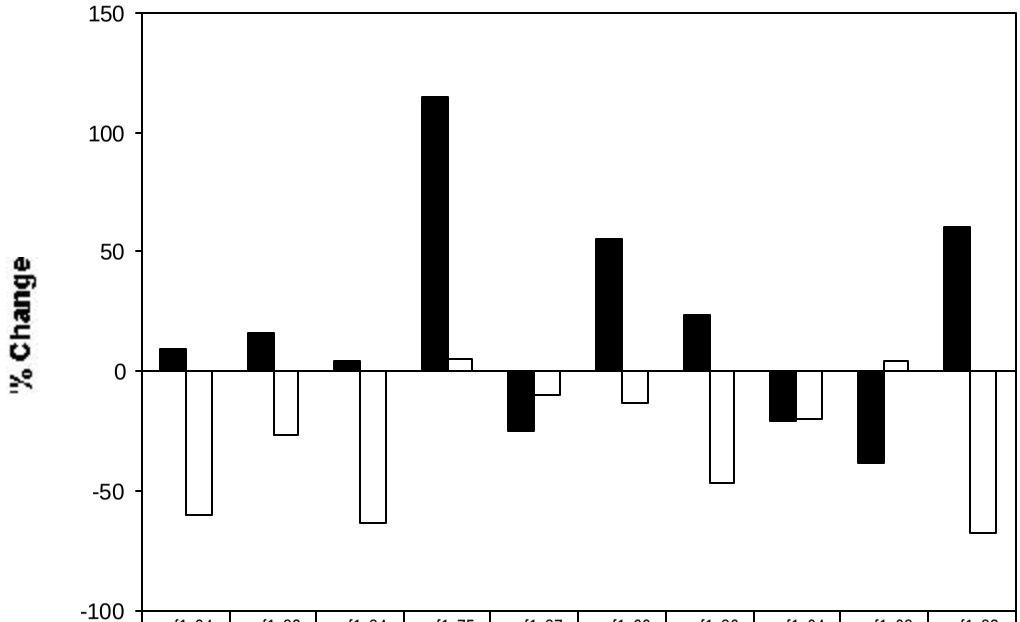
	#52	#51	#56	#76	#77	#83	#82	#78	#80	#71	#69	#68	#64	#63	#60
Stilbene	-50.40	-67.53	-87.33	-55.50	-24.60	-67.40	-91.47	-21.09	-38.10	60.27	-53.95	-13.05	694.87	214.01	-88.94
H:G Lignin Rati	-90.52	118.19	-67.33	-52.48	-33.13	-63.84	-19.52	39.78	-45.83	8.28	248.81	-31.82	3.42	34.62	-49.40

Figure 10. Individual Tree Results for Stilbene and H:G Lignin Markers at Shaver Lake (SHL). The trees at this site are mixed with respect to the sign change of stilbene and H:G lignin. The trees at SHL are highly variable among individuals, similar to those at CA and DWA -- the intermediate ozone sites from the First Phase Study.



	gg2-01	gg2-02	gg2-05	gg2-06	gg2-09	gg2-17	gg2-41	gg2-40	gg2-38	gg2-47
Stilbene	58.44	52.77	-72.12	-2.38	-14.41	27.96	330.46	-43.57	-0.20	-4.35
H:G Lignin Rati	-87.35	-56.60	-5.77	-6.08	-17.86	-59.67	-50.98	18.59	44.23	36.00

Figure 11. Individual Tree Results for Stilbene and H:G Lignin Markers at Grant Grove (GG). Nearly half of the trees at GG exhibit [+] stilbene (4 of 10), while most have [-] H:G lignin (7 of 10). Although the trees at GG are variable at the individual level, many show the oxidant stress patterns identified in the First Phase Study.



	gf1-94	gf1-82	gf1-84	gf1-75	gf1-87	gf1-60	gf1-86	gf1-64	gf1-68	gf1-83
Stilbene	9.38	16.25	4.25	114.43	-24.60	55.26	23.07	-21.09	-38.10	60.27
H:G Lignin Rati	-59.71	-26.33	-63.20	5.09	-9.99	-13.02	-46.17	-19.98	4.71	-67.44

Figure 12. Individual Tree Results for Stilbene and H:G Lignin Markers at Giant Forest (GF). Most of the trees at GF exhibit [+] stilbene (7 of 10) and [-] H:G lignin (8 of 10). The trees at GF show a profile similar to the high oxidant impact site (Camp Paivika) in the First Phase Study.

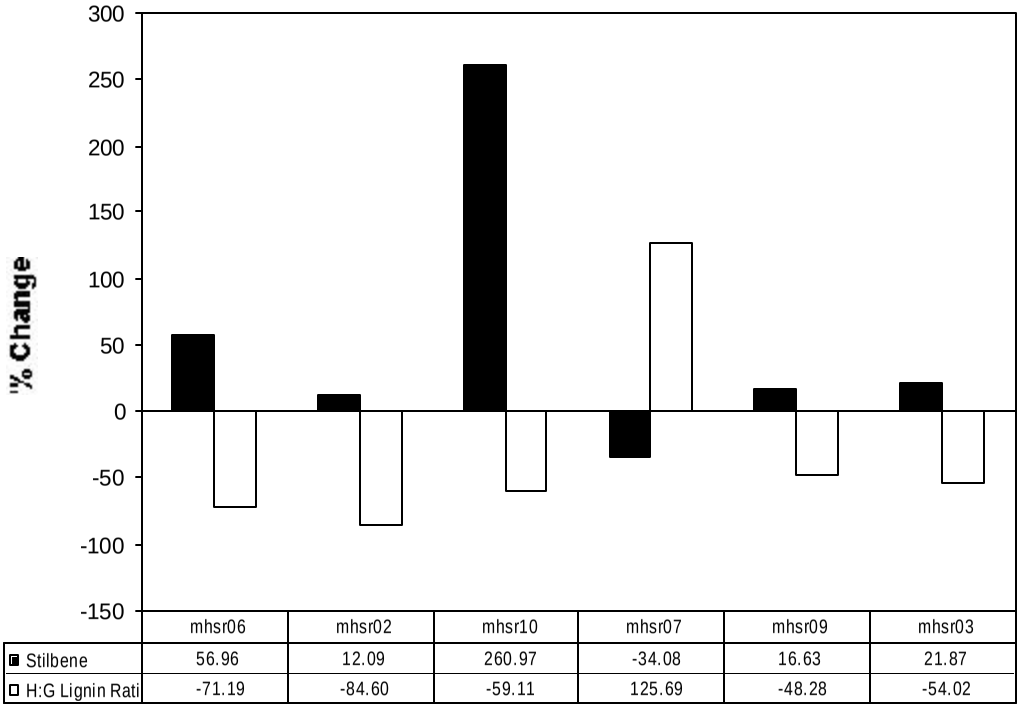


Figure 13. Individual Tree Results for Stilbene and H:G Lignin Markers at Mountain Home (MHSR). Most of the trees at MHSR exhibit [+] stilbene (5 of 6) and [-] H:G lignin (5 of 6) -- the same five trees exhibited both symptoms. The trees at MHSR show a profile similar to those at the high oxidant impact site (Camp Paivika) in the First Phase Study.

Table 5 summarizes the percentage of individuals exhibiting either or both direction-of-change (sign) markers for oxidant injury. Note that the tallies correspond well to the southerly vector of increasing ozone among these sites (Van Ooy and Carroll, 1995). This result could have been predicted based on the first phase study.

Table 5. Percentage of Trees Exhibiting Oxidant Injury Markers[1].

Site	[+] Stilbene	[-] H:G Lignin	Both
Lassen Volcanic NP -- Manzanita Lake	0.0	0.0	0.0
White Cloud	10.0	20.0	0.0
Yosemite NP -- Wawona	33.3	60.0	13.3
Shaver Lake	20.0	60.0	0.0
Kings Canyon NP -- Grant Grove	40.0	70.0	40.0
Sequoia NP -- Giant Forest	70.0	80.0	60.0
Mountain Home -- Slick Rock	83.3	83.3	83.3

[1]Plus [+] means an increase in the marker over time, from pre- to post-1955.

On the other hand, the first phase study would have also predicted that the magnitude of the oxidant injury markers would have no relation to the sites. Shown in Table 6 are the average bipolar magnitudes of the markers for the trees at each site. Surprisingly, there is a clear increasing trend for

stilbene along the increasing ozone gradient, accompanied by an equally clear decreasing trend for H:G lignin. However, it is doubtful whether such averages can reliably discriminate oxidant injury or exposure, since the standard deviations (in parentheses) are sometimes extremely large. For example, there is no such discernable trend among the sites of the first phase study.

Table 6. Average Percentage Change in Bipolar Magnitude of Oxidant Injury Markers[1].

Site	Stilbene	H:G Lignin	Stilbene3
Lassen Volcanic NP -- Manzanita Lake	-33.92 (100)	41.16 (125)	-11.28 (40)
White Cloud	-16.42 (295)	42.16 (132)	-3.85 (69)
Yosemite NP -- Wawona	-0.44 (14262)	-17.99 (201)	-5.49 (47)
Shaver Lake	20.65 (977)	-0.05 (17044)	33.03 (57)
Kings Canyon NP -- Grant Grove	22.17 (411)	-18.55 (238)	38.66 (62)
Sequoia NP -- Giant Forest	19.91 (232)	-29.60 (94)	43.34 (63)
Mountain Home -- Slick Rock	22.30 (308)	-31.92 (245)	50.84 (38)

[1]Values in parentheses is the percent standard deviation to the nearest integer.

On the other hand, some other markers may indicate oxidant exposure. The last column of Table 6 shows the average magnitude of another stilbene peak present in the datasets, "stilbene3". This marker also exhibited a clear increasing trend of its magnitude with increasing ozone, but has a relatively small standard deviation among trees for each site, ranging from 40-70 % s.d. of the % Change value.

V.C.3. Second Phase Study Conclusions

The direction-of-change (sign) effect markers for oxidant injury, established in the first phase study, appeared to correspond to expected oxidant injury in pines on the basis of historical ozone concentrations as these sites, in a southerly vector. (Van Ooy and Carroll, 1995). The average bipolar magnitude of these markers also appeared to relate to the southerly vector, but the deviation was often very large, severely reducing confidence in the approach. However, a different stilbene marker, stilbene3, showed a clear trend of its magnitude with the historical ozone concentrations, with reasonably small standard deviations. Thus, stilbene3 could be a marker for oxidant exposure. It is possible that other, potentially useful markers may be found in the rich data sets of wood analysis by pyro-GC/MS.

V.D. FTIR-microspectroscopy Analysis

As stated earlier, Objective 4 was to investigate the use of FTIR microspectroscopy as another analytical means. Figure 6 illustrates the potential of the technique at the outset of this investigation. However we ran into several problems while testing cores from the White Cloud site. First, the rounded surfaces of tree cores provided sufficient irregularities in specular reflection (and other effects) such that it resulted in great variability in spectra from a single ring, as the core was rotated.

Spinning the core on its longitudinal axis to average the signals was considered. However, this was rejected due to the simple fact that tree cores rarely exhibit rings perpendicular to the core, and spinning usually resulted in crossover to adjacent rings. Spinning could work for "perfect" trees that are cored "perfectly", but not for real-life samples.

Next, we reasoned that a flat surface could yield reasonable data. However, there was great difficulty in longitudinally slicing 4mm cores. Not all cores are straight when removed from the tree, or of uniform density throughout, so the cores had to be sliced manually, compensating every few millimeters. This proved to require considerable skill, and was additionally labor-intensive and time-consuming.

Flat surfaces exhibited good spectra, and spectral region-of-interest (ROI, or shape and size of spectral reading area) could be set to maximize the signal. But here, too, we ran into problems. Tree rings are irregularly spaced, even the flat surface of sliced cores were irregular in shape, and sizes of rings varied considerably. The first problem meant that each ring must be manually targeted, while the second and third problems meant that shape and size of the ROI must be manually adjusted for many (sometimes most!) rings. Difference in ROI shape and size also led to problems in normalizing spectral responses in a meaningful way.

An alternative to adjusting ROI for each ring, was to specify a very small spot in the ROI so as to ensure that it will fit well within any ring. This seemed to work for the rapid-growth, very wide rings from trees at White Cloud that allowed a large ROI. However, the spectral signal-to-noise was unacceptable for the very small ROI required to cover the slow-growth, very narrow rings from trees at Manzanita Lake in Lassen Volcanic National Park. Specifically, each ring on a Manzanita Lake tree core could take 10-15 minutes of acquisition, after which the next ring needs to be manually targeted. Thus, a 40-year transect on a single tree core from Manzanita Lake (following a tedious slicing job) would take upwards of 10 hours to complete, for just a single replicate, in effect with a spectroscopist attending continuously. For a tree core from White Cloud with wide rings, the same transect could be completed in 2-hours. For others, the narrowest ring would define the analysis rate; some tree cores from sites such as Breezy Point exhibited rings as narrow as those from Manzanita Lake.

We also considered an automated continuous-scan transect, which would yield hundreds of spectra from a single transect. This would at least relieve the need for manual targeting. But here, too, the ROI must be set smaller than the narrowest ring in order to distinguish transitions between rings. In fact, for spatial resolution, the ROI must be set much smaller than in the case of manual targeting, further increasing the analysis time. And it leaves us with, at present, unknown algorithms for sorting the hundreds of spectra from a single transect into ring-coherent sets.

Finally, we also briefly entertained the idea of FTIR analysis of pulverized tree core segments, much in the same manner as with pyro-GC/MS. However, this was rejected on the grounds that it would defeat the purpose of using FTIR microspectroscopy, namely that its ability to analyze each ring would override the poor (relative to pyro-GC/MS) information content of each data set.

VI. DISCUSSION & CONCLUSIONS

Analogous to the "intermediate" impacted sites in the first phase study, the second phase putative intermediate ozone-exposure sites in the Sierra Nevada exhibited variable and mixed stilbene and H:G

lignin ratio marker responses on a tree-by-tree basis. It is therefore possible that the oxidant exposure profiles at such sites represent threshold levels for these grove-level exposure markers.

With regard to the biochemical basis of these observations, the data complexity, combined with a lack of sufficient understanding of constituents seen by pyro-GC/MS, precludes a simple interpretive analysis. Nevertheless, these findings appear to be mechanistically relevant since ozone fumigation of pines has been shown to induce stilbene phytoalexins as well as affect enzymes of lignin synthesis in leaves. For example, effects of ozone treatment on enzymes along the phenolic pathways have been investigated, including phenylalanine ammonia lyase (the gateway enzyme to phenolic biosynthesis), cinnamyl alcohol and coniferyl alcohol dehydrogenases (key enzymes of the lignin pathway), chalcone synthase (key enzyme in the flavonoid pathway), and stilbene synthase (key enzyme in the phytoalexin pathway). Stimulation of all of these enzyme activities has been reported in ozone-treated pine needles (Rosemann et al., 1991; Heller et al., 1990; Langebartels et al., 1990). Changes in some of the enzyme activities were also accompanied by the product accumulation, including stilbene metabolites (Rosemann et al., 1991) and flavonoids (Langebartels et al., 1990). Other than being good oxyradical scavengers, many of these secondary metabolites are cell wall and wood components which respond to wounding or fungal attack (Kindl, 1985). The stilbene and H:G lignin markers found in this study could be reflecting these pathways.

Other plant defense mechanisms that have responded to ozone exposure involve the induction of β-1,3-endoglucanase and endochitinase activities (Schraudner et al., 1992). These two enzymes were shown to inhibit the growth of fungi that contain β-1,3-glucans or chitin as cell wall polymers, and a role of these hydrolases in plant defense against microbial infection was thus postulated (Bowles, 1990). In addition, a plant cell wall component (β-1,3-glucan callose) that has been known to respond to wounding and pathogen attack was found to accumulate in ozone-injured leaves (Schraudner et al., 1992). These biochemical effects are consistent with the notion that ozone exposure predisposes plants to wounding and pathogen attack which divert resources away from growth or weaken an individual's ability to cope with other stresses. It is also possible that callose is formed from non-biological injury. However, in this study, markers for chitin and callose (levoglucosan) failed to exhibit any relationship at the individual tree or grove levels.

Thus, it is plausible that wood retains chemical "records" of some these effects, and that it is the biochemical basis of the findings reported here. If so, it is not suprising that the sensitivity range of the markers overlap nicely with oxidant damage in the pines, since these grove-level *exposure* markers are individual-level oxidant *bioeffect* markers as well. Further morphological and biochemical analyses of archived cores and samples could yield further evidence to support/deny these views.

Lastly, FTIR microspectroscopy, while technically feasible, proved impractical due to the requirements of skilled/tedious sample preparation and skilled/tedious spectral acquisition. The spectroscopy is potentially very time-consuming on equipment that is of significant cost (> $120,000). In FTIR spectroscopy, it is common to invoke chemometrics (e.g., principal component analysis) to spectral data processing, and it is possible that such an approach would have helped. However, the shortcomings discussed here, on raw data quality, profoundly affected subsequent data processing. Therefore, for FTIR microspectroscopy to yield useful data, the obstacles presented here must be overcome.

In conclusion, it appears that the direction-of-change (sign) of the [+] stilbene and [-] H:G lignin markers are indicators of bioeffect at the individual-level, while the bipolar magnitude of the stilbene3

marker may represent an indicator of oxidant exposure. In addition, both [+] stilbene and [-] H:G lignin may also be exposure indicators when averaged to the grove-level, however, their magnitudes vary wildly and appear to be of no use. These concepts are summarized in Table 7. It is possible that the biochemical markers are of considerable robustness, since for both individual and grove-level markers, they were clearly manifested despite the relatively small sample sizes in this study.

Table 7. Summary of Bioindicator Responses.

Marker	Characteristic	Level	Bioeffect	Oxidant Exposure
Stilbene	[+] Increase over time	Individual	Yes	No
"	[+] Increase over time	Grove	No	Yes
"	Bipolar magnitude	Individual	No	No
"	Bipolar magnitude	Grove	No	No
H:G Lignin	[-] Decrease over time	Individual	Yes	No
"	[-] Decrease over time	Grove	No	Yes
"	Bipolar magnitude	Individual	No	No
"	Bipolar magnitude	Grove	No	No
Stilbene3	[+] Increase over time	Individual	No	No
"	[+] Increase over time	Grove	No	No
"	Bipolar magnitude	Individual	No	Yes
"	Bipolar magnitude	Grove	No	Yes

VII. LITERATURE CITED

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VIII. APPENDIX A

Number of Trees and Cores Analyzed/Archived at Each Study Site.				
Site	Trees	---------- Cores[1] ----------		
		Analyzed	Archived for Dendrology	Archived for Analysis
I. San Bernardino Gradient Study				
Camp Angeles (CA)	13	26	13	0
Breezy Point (BP)	17	34	17	0
Camp Osceola (CO)	13	26	13	0
Dogwood A (DWA)	14	28	14	0
Silverwood Lake (SL)	15	30	15	0
II. Sierra Nevada Study				
Manzanita Lake – Lassen Volcanic NP (LV)	10	20	10	10
White Cloud – Tahoe NF (WC)	10	20	10	10
Wawona – Yosemite NP (YW)	15	30	15	0
Shaver Lake – Sierra NF (SHL)	15	30	15	0
Grant Grove – Kings Canyon NP (GG)	10	20	10	0
Giant Forest – Sequoia NP (GF)	10	20	10	0
Mountain Home – Sequoia NF (MH)	6	12	6	0

[1]"Analyzed" indicates cores that are stored in pulverized form in a freezer; cores "Archived for Dendrology" are stored at room temperature in paper tubes; and cores "Archived for Analysis" are stored as intact cores in a freezer.

APPENDIX B

List of Abbreviations

BA	Basal area
BP	Breezy Point (in the San Bernardino National Forest)
CA	Camp Angeles (in the San Bernardino National Forest)
CO	Camp Osceola (in the San Bernardino National Forest)
CP	Camp Paivika (in the San Bernardino National Forest)
DG	Dogwood Campground (in the San Bernardino National Forest)
DWA	Dogwood Campground A (in the San Bernardino National Forest)
EPA	U.S. Environmental Protection Agency
FPM	Forest Pest Management
FTIR	Fourier-transform Infrared Spectroscopy
GF	Giant Forest (at Sequoia National Park)
GG	Grant Grove (at Kings Canyon National Park)
LV	Manzanita Lake (at Lassen Volcanic National Park)
MHSR	Mountain Home State Demonstration Forest -- Slick Rock
N	Nitrogen
NF	National Forest
NIH	National Institutes of Health
NIST	National Institute of Standards and Technology
NP	National Park
Pyro-GC/MS	Pyrolysis-gas Chromatography/Mass Spectrometry
ROI	Region of Interest
SBGS	San Bernardino Mountain Gradient Study
SHL	Shaver Lake (in the Sierra National Forest)
SIMC	Single Ion Mass Chromatograms
SL	Silverwood Lake (in the San Bernardino National Forest)
UCD	University of California, Davis
USDA	U.S. Department of Agriculture
WC	White Cloud Campground (in the Tahoe National Forest)
YW	Wawona (in Yosemite National Park)
µFTIR	Fourier-transform Infrared Microspectroscopy