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Responses of the lichen *Ramalina menziesii* Tayl. to ozone fumigations

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Abstract: Tropospheric ozone (O_3) is a strong oxidant, and is known to have serious negative effects on forest health. Lichens have been used as biomonitors of the effects of air pollution on forest health for sulfur and nitrogen pollutants. However, effects of O_3 on lichens are not well understood, as past fumigation studies and community analyses produced conflicting results. We fumigated the lichen *Ramalina menziesii* Tayl. with three levels of O_3 (10, 60, and 120 ppb) in two-month long experiments, testing photosynthesis, respiration, chlorophyll content, pigment degradation, and cell membrane leakage in response to the treatments. In southern California, the high ozone pollution season coincides with the warm arid summers. Many of the O_3 fumigations conducted by others were located in climates with regular rainfall events. In our fumigations, we allowed the lichens to maintain ambient water status. We found that *R. menziesii* showed no signs of physiological decline in response to low and moderate levels of O_3 fumigations and an insignificant response in some parameters to 120 ppb fumigations. Several plant species have been shown to be good indicators of O_3 pollution, while community studies of lichens and ozone have shown that lichens are not. In our estimation, the lichen *R. menziesii* is not a good indicator species for O_3 pollution, possibly due to antioxidant activity protecting the plant portion of the thallus (the algal cells within the symbiosis).

Keywords: Air quality, biological monitoring, lichen, Los Angeles, ozone

Introduction

Our work was conducted in the Los Angeles air basin, an area that encompasses the cities around Los Angeles, as well as the mountains surrounding the urban zones. Historically, the Los Angeles air basin has had sizeable ecological and human health problems associated with air quality and the occurrences of chronic air pollution and acute events have been a driving force behind air pollution legislation in California and subsequently throughout the United States. The aim of our work is to increase the capability of forestland managers around the basin to un-

derstand how air pollution is distributed in the basin by developing a better understanding of how lichens are affected by air quality issues.

Lichens have been considered excellent biomonitors of air pollution for over a century (NASH III 2008), and our understanding of the mechanisms of lichen responses to air pollution has become more sophisticated over that time. There are two reasons that lichens are thought to respond dramatically to shifts in air quality. First, they are almost entirely dependent on atmospheric moisture and airborne nutrients for their survival. Lichen physiological dependence on ambient air to meet their needs has led to adaptations that allow lichens to accumulate atmospheric molecules, including pollutants. Second, lichens are passive water regulators (NASH III 2008). When dry, they shut down metabolically and can accumulate pollutants on their surfaces, which can become a concentrated dose of toxins when it is finally wet. In arid climates, long seasonal drought can lead to very high concentrations of pollutants on lichen surfaces (NASH 2008).

Lichens are used as indicators for critical loads of pollution deposition for different ecosystems (FENN et al. 2008; GLAVICH & GEISER 2008), as indicators of cumulative loads using tissue accumulation of pollutants (GOMBERT et al. 2006; JOVAN & CARLBERG 2007), and as indicators of landscape level air quality using community composition changes (VAN HERK 1999; JOVAN & MCCUNE 2004; GEISER & NEITLICH 2007). This paper addresses the physiological responses of the lichen *Ramalina menziesii* Tayl. to ozone, a strong oxidant associated with secondary products of combustion emissions.

Ozone has been has been associated with general declines in forest health (PETERSON & DALY 1989; GRULKE et al. 1998), as well as with shifts in lichen community composition, and loss of lichen species (SIGAL 1979; SIGAL & NASH 1983). Both field and laboratory studies have demonstrated equivocal effects of ozone on lichens. In a systematic survey of five mountain ranges in the Los Angeles air basin, NASH & SIGAL (1998) demonstrated a decline in both diversity and abundance of species present in the montane regions around the basin, and found that the changes in the lichen communities were correlated with the O_3 concentration gradient. At that time, O_3 was thought to be the pollutant largely responsible for declines in forest health. On the other hand, JOVAN & MCCUNE (2004; 2005) found that climate and ammonia deposition were important drivers lichen community composition than O_3 in the Central Valley of California. Work from the US Forest Service Fire Laboratory in Riverside, California (FENN & BYTNEROWICZ 1993; BYTNEROWICZ & FENN 1996) showed that the O_3 gradient was accompanied by a strong, previously undetected nitrogen deposition gradient. Our work with the lichen *R. menziesii* (RIDDELL et al. 2008) showed that nitric acid (HNO_3), a common pollutant in the basin, can act as a strong acid on lichen thalli, and cause thallus death at high ambient levels of deposition within five weeks in both fumigation and transplant experiments. The significant effects of HNO_3 on *R. menziesii* physiology and the strong correlation between O_3 and N pollution gradients lead us to question former conclusions about the effects of ozone on lichen communities in the Los Angeles air basin. The aim of this paper is to examine the role of O_3 as a driver of *R. menziesii* distribution. To do this, we conducted O_3 fumigations using realistic concentrations found in the Los Angeles air basin, and tested the physiological responses of the lichen to the pollutant.

Methods

Fumigation chambers (constantly stirred tank reactors)

The O_3 fumigation chambers (or, constantly stirred tank reactors, CSTRs) are closed Teflon film cylindrical 1.35 m x 1.35 m chambers housed in a climate-controlled greenhouse with particulate and charcoal air filtration systems, on the University of California campus in Riverside, CA, described in detail in PADGETT et al. (2004). Ozone, generated by injecting pure oxygen into an O_3 generator (Superior Electric Co., Bristol, CT, USA), is sent to the CSTRs via flow meters (Atheson Gas Products Model 602) attached to Teflon tubes that feed into the CSTRs through a port in the upstream air duct. Air from each chamber is sampled for 6 min every hour and O_3 concentrations are measured using a Dasibi O_3 monitor (Dasibi Environmental Corp Model 1003-AH, Glendale, CA). In order to simulate real world conditions, we generated O_3 from 10:00 to 16:00 each day. To mimic light conditions under oak canopies at the collection site, the CSTRs were covered with shade cloth so that afternoon light levels are between 200 and 400 mmol m^{-2} s^{-1} PPFD. Because the high O_3 pollution season takes place during the arid, hot summer months in southern California, the lichens were only exposed to ambient moisture in the chambers. Lichen thalli were hung on blue oak (*Quercus douglasii*) branches (on which the lichen grows abundantly in the field) in the chambers.

Treatments and measurements

We conducted two-month long O_3 fumigation experiments, in November 2007 and March 2008. We had three treatments per fumigation: low O_3 (~ 10 ppb, our control), moderate O_3 levels (~ 60 ppb), and high O_3 levels (~ 120 ppb). For each treatment we used three CSTRs containing five *R. menziesii* thalli. Each thallus was sampled for tissue chlorophyll content, photosynthetic and respiratory capacity (CO_2 exchange capacity), chlorophyll fluorescence, and cell membrane integrity. November 2007 treatments were sampled on days 1, 15, 29, 43, and 57. March 2008 treatments were sampled on days 1, 29 and 60 for chlorophyll, chlorophyll fluorescence and CO_2 exchange capacity. Tissue samples were collected from the growing tips of the fumigated lichen thalli. Twenty mg of tissue were removed from each sample for chlorophyll analyses, and 0.3 to 0.5 g of tissue from each sample was used for all other analyses.

CO_2 exchange capacity

Each sample was rinsed for one minute in deionized (DI) water, and soaked for two hours in DI water. The rinse and soak solutions were then analyzed for ion concentration and conductivity, and the tissue samples were tested for chlorophyll fluorescence and CO_2 exchange capacity. Photosynthesis and respiration were measured following the procedures of LARSON & KERSHAW (1975), and are described in greater detail in RIDDELL et al. (2008). Gross photosynthetic carbon absorption (GP) and respiratory carbon emission (dark respiration, or DR) were measured three times to generate an average value for each sample, and are reported herein as μg CO_2 exchanged per gram air-dry weight per minute.

Chlorophyll and phaeophytins

We measured chlorophyll from 20 mg of tissue per sample by extraction in 5 ml dimethyl sulfoxide (DMSO) following RONEN & GALUN (1984). The samples were incubated in test tubes for 45 minutes at 60 °C, and then diluted with an additional 5 ml DMSO. Optical density (OD) was measured in a spectrophotometer at 415, 433, 645, 665, and 750 nm. Chlorophyll *a* and *b* concentrations were calculated using Arnon's equation (ARNON 1949).

Acidification of the chlorophyll *a* molecule can cause the removal of the central magnesium, converting the pigment to phaeophytin, a brown pigment utilized in the electron chain transport system of photosynthesis and reducing the photosynthetic capacity of the lichen thallus. We used an OD_{433}:OD_{415} curve developed by RONEN & GALUN (1984) to calculate the chlorophyll *a*: phaeophytin *a* ratio using Eq. 1:

$$\text{(Eq. 1)} \qquad \% \text{ phaeophytin } a = -104.8 \times \ln(OD_{433}:OD_{415}) + 39.715$$

Chlorophyll fluorescence

Chlorophyll fluorescence uses saturating light pulses to measure the integrity of photosystem II (PS II) and the electron chain transport system. Pulses of actinic and saturating light pulses are directed at the sample using a PAM 2000 fluorometer (Walz, Germany), which measures the amount of fluorescent light emitted by the sample during the episodes of minimal light (F_o) and saturating light pulses (F_m). The samples were wet with deionized water, dark adapted for 15 minutes, and patted dry in a dark chamber at 22 °C. The lobe tips of the thalli were exposed to modulated light to obtain the minimal fluorescent yield (F_o), then to a saturating light pulse for 0.6 seconds to obtain maximum fluorescent yield (F_m). The samples were then exposed to saturating light pulses at 20-second intervals to give F_o' and F_m'. F_v (where $F_v = F_m - F_o$) represents the variable fluorescence of the sample, and was used to calculate the ratio of F_v/F_m, a measure of the potential quantum yield of PS II (BILGER et al. 1995).

Cell membrane integrity and ion leakage

Ozone can, upon entering a cell, damage lipid membranes, pigments and proteins (BAIER et al. 2005). We soaked all samples in deionized water for one minute (a quick rinse) and for two hours, then analyzed the solutions for K^+, Ca^+, Mg^+, Na^+, and NO_3^- concentrations, using an ion chromatograph (Dionex Corp., Sunnyvale, CA) leached from the samples. Potassium is considered to be a good indicator of cell membrane integrity (PEARSON 1985). We also measured the conductivity of the solutions (Accumet Basic AB30 Fisher Scientific conductivity meter). We expected to find higher levels of conductivity and K^+ leakage in samples with greater damage to cell membranes (in our case, 120 ppb O_3 treatments).

Statistical analyses

Each response variable was analyzed with mixed model analyses of variance (ANOVA) in SAS for windows (SAS, 2002). Heteroscedastic data were log transformed to meet the assumption of equal variance between means. We used a mixed model ANOVA to estimate residuals and covariance parameters, and run Tukey's HSD multiple comparison tests (STEEL & TORRIE 1980).

Results

The fumigated lichen thalli did not change in appearance over the course of the experiment. The thalli remained green and the lobes maintained a soft and flexible texture. In all parameters (chlorophyll content, CO_2 exchange, F_v/F_m, and ion leakage) there were little to no significant differences between treatments. Because the results from the second O_3 fumigation were very similar to the first, this paper will present data from the first fumigation and note any different results in the second.

CO_2 exchange capacity and chlorophyll fluorescence

Net photosynthetic CO_2 gain decreased in all treatments after day 15, indicating that there was a chamber effect on photosynthesis (Fig. 1a). The decrease in respiration in the high O_3 treatment was not significantly different from day one. The potential quantum yield (F_v/F_m) decreased significantly ($p < 0.01$ for all HSD comparisons between day 1 and day 57) over time (Fig. 1b). There were no significant differences between O_3 and control treatments in either parameter. This decrease in all treatments suggests a "chamber" effect on photosynthetic vitality, which could reflect the effects on the lichen thalli of the different moisture and light regimes in the chambers from the collection site, rather than a treatment effect from the fumigations.

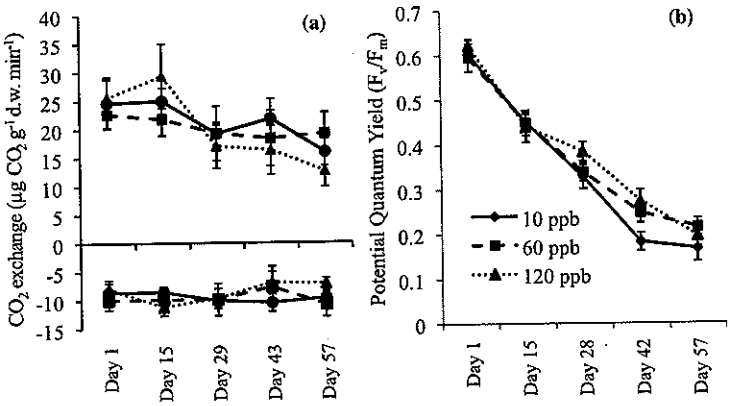

Fig. 1. Effects of ozone fumigation on **(a)**, gross photosynthesis (GP) and dark respiration (DR) and **(b)**, potential quantum yield of photosystem II (F_v/F_m), in thalli of *Ramalina menziesii*. Photosynthesis is shown in the upper portion of the graph (a) as CO_2 gain, and DR on the lower portion of (a) as CO_2 loss. Error bars represent two standard errors from the mean. No significant difference was found between treatments in GP, DR or Fv/Fm over the course of the fumigation period.

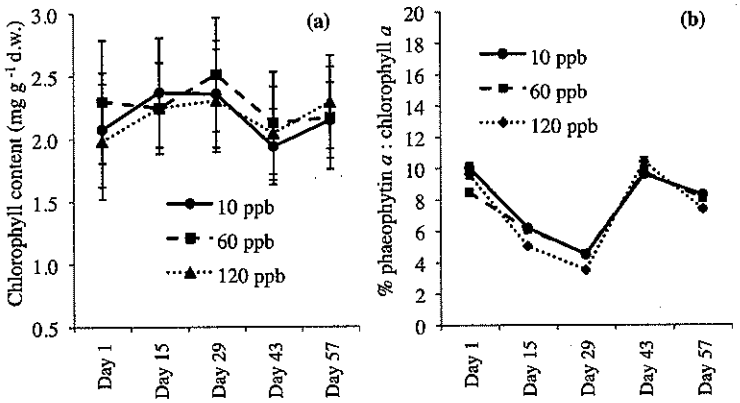

Fig. 2. Effects of ozone fumigation on chlorophyll content **(a)**, and the ratio of phaeophytin *a* to chlorophyll *a* **(b)**, in thalli of *Ramalina menziesii*. There was no significant difference between any of the treatments over time. Error bars represent two standard errors from the mean.

Chlorophyll tissue content and degradation into phaeophytins

The chlorophyll content of the tissue did not change significantly over time in any of the treatments, and the chlorophyll content of the thalli treated with moderate and high levels of O_3 fumigations was not significantly different from the control (10 ppb) treatment (Fig. 2a). The ratio of OD_{433}:OD_{415} or % phaeophytin *a*:% chlorophyll *a*, did not change over the course of the fumigations, indicating that there was no degradation of chlorophyll *a* pigments into phaeophytin *a* resulting from O_3 treatments (Fig. 2b).

Cell membrane integrity, leachate solution conductivity, and ion concentrations

The conductivity of the rinse and soak solutions increased in all treatments (Fig. 3a); however, the 60 ppb O_3 and 120 ppb O_3 treatments were not significantly different from the control. The overall increase in conductivity in all treatments again indicates that some of our effects may be related to the chambers, rather than the treatments. The increases in ion leakage from fumigated thalli in one-minute rinse, and two-hour soak solutions were not significantly different between treatments (Figs. 3b-e). In spite of the fact that on day 57, Ca^+, Mg^{2+}, K^+, and Na^+ had all increased in the O_3 fumigations compared to the control treatment, the difference was not significant.

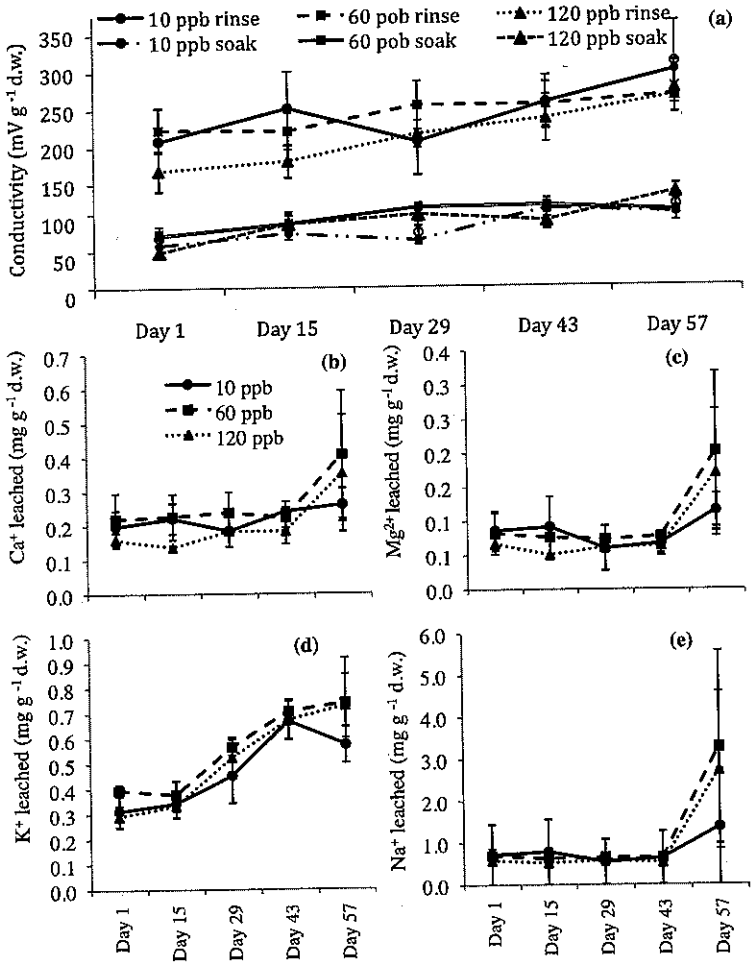

Fig. 3. Conductivity of one-minute rinse and two-hour soak solutions (**a**), and Ca^{+} (**b**), Mg^{2+} (c) K^{+} (**d**), and Na^{+} (e) ion concentrations in one-minute rinse solutions of *Ramalina menziesii* thalli fumigated with 60 ppb and 120 ppb concentrations of O_3 for 57 days. There was no significant difference in solution conductivity between treatments, although conductivity of both rinse and soak solutions and K^{+} concentrations did increase in all treatments over time.

Discussion

Overall, *R. menziesii* did not respond with a decline in physiological vitality to chronic O_3 fumigations at any level. Chlorophyll concentrations, the ratio of phaeophytin *a* to chlorophyll *a*, gross photosynthesis (GP), dark respiration (DR), the potential quantum yield of photosystem II (F_v/F_m), and cell membrane integrity

did not change relative to the control responses. In spite of the buffered environment of the greenhouse, the lichens still experience some seasonal changes in weather, and climatic conditions in the chambers. In this case, as all lichens in all treatments are responding similarly, they may be responding to chamber conditions over prolonged periods of time, rather than to chronic fumigations with O_3.

While it is well understood that lichen species respond differentially to pollutants, the lack of response of *R. menziesii* to O_3 after 57 days of fumigations at 120 ppb has several implications. In this study, O_3 levels were lower on moist days, when the lichens were more saturated with ambient water. First, as we did not regularly wet our experimental thalli, the desiccated condition of the fumigated lichens could support the suggestion put forth by RUOSS & VONARBURG (1995) that lichen vulnerability to O_3 may be linked to a metabolically active state. Because O_3 levels are low in cooler, wet weather, it is unlikely that *R. menziesii* thalli are exposed to high levels of O_3 when wet under natural conditions. Our experiments did not address the question of O_3 toxicity in relation to thallus moisture content and metabolic activity, so these statements are purely speculative. Second, the strong relationship between O_3 concentration gradients and N deposition gradients suggests that some lichens may not be responding to even extremely high levels of O_3, but instead responding to N. Our work with HNO_3 fumigations showed that *R. menziesii* was very responsive to the compound, which can act as both an oxidant and a strong acid and a strong N input.

In previous short term, high concentration O_3 fumigations (1 to 5 days at 100, 240, 480, and 784 ppb O_3), ROSS (1983) demonstrated that *R. menziesii* was less susceptible to O_3 damage than *Pseudoparmelia caperata* (now *Flavoparmelia caperata* (L.) Hale). NASH & SIGAL (1979) saw decreases in gross photosynthesis in *Parmelia sulcata* after several hours of exposure to 500 ppb O_3, and in *Hypogymnia imshaugii* after several hours at 800 ppb. These short term but acute fumigations suggest that extremely high concentrations, which have occurred in the Los Angeles air basin, may also play a role in damaging lichens. Ozone pollution has decreased significantly since the late 1980's, and these high concentrations are now less common (LEE et al. 2003). Thus, our more moderate fumigations reflect realistic conditions.

In long-term O_3 fumigations, SCHEIDEGGER & SCHROETER (1995) fumigated seven different species for 80 days, and found that each species responded differently: some with reduced chlorophyll fluorescence (CF), and some with reduced net photosynthesis (NP) or increased DR. The most common effect was collapse of algal cells. However, there was no pattern to the type of damage exhibited by each species. TARHANEN (1997) fumigated four lichen species for two-four weeks with 10 to 300 ppb O_3, and found that his most sensitive species, *Usnea hirta*, had decreased starch volume in cells, and an increase in electron opacity.

The varied responses of fumigated species suggest that morphology and secondary compounds produced by lichen species may play an important part in determining susceptibility to O_3. VALENCIA-ISLAS et al. (2007) found that reactive lichen compounds produced in the cortical region of the lichen thallus can have strong antioxidant properties, and thus may be important in determining lichen species distribution in polluted areas. However, while VALENCIA-ISLAS et al. found that usnic acid, found in *R. menziesii* and *Usnea* spp., was a strong

antioxidant, *R. menziesii* and *Usnea* species have been extirpated from polluted areas in the Los Angeles air basin. This disappearance suggests that factors besides O_3 are more important drivers of the impoverishment of the lichen communities in the basin. In fact, usnic acid can, in the presence of strong acids, facilitate the shuttling of excess H^+ ions across cell membranes (HAUCK & JURGENS 2008), and may decrease the ability of lichens containing this antioxidant to resist HNO_3 and other acidic pollutants.

Conclusions

Our results demonstrated a lack of response of *R. menziesii* to chronic O_3 fumigations, suggest that lichen species we consider to be pollution sensitive may not be responding to O_3 concentration gradients. While some O_3 fumigation studies have shown that some species respond negatively to O_3, our data show that oxidants are not affecting the most important drivers of lichen community distribution in the absence of sulfur pollutants. Several community composition studies have suggested that N compounds are more important drivers of community shifts than O_3, a finding compatible with our results, and the mixed results of O_3 fumigation studies. Our study design did not address several important questions, including responses of lichens under hydrated vs. dehydrated conditions, and in longer-term fumigations of many months to years. Also, in order to better understand the responses of lichen communities to current pollution trends, fumigations using more species and more pollutants are necessary.

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