

The effect of HNO₃ gas on the lichen *Ramalina menziesii*

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Dedicated to Prof. Dr. Drs. h.c. O.L. Lange on the occasion of his 80th birthday

Abstract

Nitric acid (HNO₃) and ozone (O₃), secondary products of photochemical reactions of nitrogen oxides (NO_x) and volatile organic compounds, are important pollutants in arid regions with large outputs from petrol combustion. In the Los Angeles (LA) air basin, nitrogen dry deposition rates in forests downwind of the urban areas can reach 35–40 kg ha⁻¹ year⁻¹, roughly equivalent to the amount of N used to fertilize agricultural fields. The marked decline in the lichen population of the LA air basin has previously been attributed to local O₃ concentration gradients, which overlaid the patterns of species extirpation. Recent research in the air basin has shown that nitrate (NO₃⁻) deposition gradients run parallel to the O₃ concentration gradient, and that deposition of NO₃⁻ and HNO₃ can have significant effects on forest health. Our research examines the effects of HNO₃ dry deposition on the lichen *Ramalina menziesii* Tayl. in an effort to understand the loss of lichen species in southern California, and increase the usefulness of lichens as biomonitors of nitrogen pollutants. We transplanted healthy *R. menziesii* thalli from a “pristine” location into fumigation chambers and exposed them to HNO₃ under humid and dry conditions, and moderate and high HNO₃ fumigations. *R. menziesii* thalli treated with HNO₃ in month-long fumigations experienced a significant decline in chlorophyll content and carbon exchange capacity compared to thalli in control chambers. Leachate conductivity, NO₃⁻ and K⁺ concentrations increased with HNO₃ fumigation levels and time. We conclude that *R. menziesii* has an unequivocally negative response to HNO₃ gas concentrations common to ambient summer conditions in the LA air basin.

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Keywords: Air pollution; Biomonitoring; Lichens; Nitric acid; Nitrogen deposition; *Ramalina menziesii*

Introduction

Just as canaries provide warnings of toxic gases to coal miners, so can the investigation of lichen communities provide information on potential deterioration of ecosystems stressed by air pollutants (Nash, 2008).

Lichen species are well known to be differentially sensitive to air pollutants. The most sensitive species may become locally extirpated in urban areas or near industrial facilities, while a few very tolerant species will survive and even flourish. Except for SO₂, the mechanisms underlying this differential sensitivity are poorly understood.

In the case of southern California, we know that approximately half the epiphytic lichen species known to

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occur in the late 1800s and early 1900s (Hasse, 1913) have subsequently disappeared (Ross, 1982; Sigal and Nash, 1983). Twenty-five years ago the apparent cause of the lichen decline in the Los Angeles (LA) region seemed clear – namely oxidant air pollutants with an emphasis on ozone (O_3). O_3 is widely recognized as the major phytotoxic air pollutant in the United States (Lefohn, 1991) in general, and in the San Bernardino Mountains in particular (Miller and McBride, 1999). Along gradients of increasing oxidants, lichen communities on both conifers (Nash and Sigal, 1998) and oaks (Sigal and Nash, 1983) exhibited marked declines in species richness and relative health.

However, over the past decade, a previously unrecognized high N-deposition pattern has been documented with a gradient overlapping the O_3 gradient (Fenn and Bytnerowicz, 1993). In fact, it is suggested that the forests near LA are N-saturated (Breiner et al., 2007; Fenn et al., 1996) and the total N-deposition, variously estimated as up to $35\text{--}50\text{ kg ha}^{-1}\text{ year}^{-1}$ (Bytnerowicz and Fenn, 1996; Padgett and Bytnerowicz, 2001), is nearly equal to the highest deposition rates observed in Europe (e.g. the Netherlands and northern Germany), but with one major difference. In southern California oxidized forms of N predominate, whereas in Europe reduced forms of N predominate (Bytnerowicz and Fenn, 1996). Major components of the southern California N-deposition components include two strong gas phase acids, nitric acid (HNO_3) and HNO_2 . Of these two, the HNO_3 gas phase occurs in the highest concentrations, and it exhibits diurnal patterns that parallel that of O_3 (Seinfeld and Pandis, 1998). Field measurements of gaseous HNO_3 in the mountains downwind of LA have measured levels as high as 27.3 ppb (24-h average), whereas remote locations elsewhere may see levels in the range of 0.00025 ppb 24-h averages (avg.) (Bytnerowicz and Fenn, 1996). Unlike O_3 , once HNO_3 is created, it no longer participates in atmospheric chemical reactions, and it rapidly deposits to exposed surfaces. Thus, our operational question is whether gaseous HNO_3 alone is sufficiently phytotoxic to contribute to the observed lichen decline in southern California. Herein, we report results of our initial experimentation with *Ramalina menziesii* Taylor, one of the most sensitive lichen species in the LA urban area. It is almost unknown today in this area (Ross, 1982; Mount Palomar to the south was the closest extant location in the late 1980s) but was abundant earlier (Hasse, 1913), as 35 herbarium specimens document its relative abundance across the LA basin in the early 1900s (Ross, 1982; Sigal and Nash, 1983). As far as we know, this is the first report on the effects of HNO_3 gas phase on any lichen and one of the first dealing with any organism.

Materials and methods

Collection and transplanting

R. menziesii thalli (vegetative bodies) were collected at the University of California Sedgwick Reserve, near Santa Ynez, California, from abundant populations on *Quercus douglasii* Hook. & Arn branches; all collections were made within a 150-m radius of one another. Lichen thalli were collected after more than a week of dry weather, and transported in paper bags to the lab on the same day. Five randomly selected thalli were placed on *Q. douglasii* branches in each treatment chamber, with no more than one thallus from any individual tree in any one chamber. Thalli were always handled with nitrile gloves.

Chambers

The treatment chambers are housed in a climate-controlled greenhouse with particulate and charcoal air filtration systems, on the University of California Riverside campus in Riverside, CA. The chambers (constantly stirred tank reactors or CSTRs) are $1.35\text{ m} \times 1.35\text{ m}$ clear Teflon cylinders, independently supplied with filtered air. The air in the CSTR is exchanged every 1.5 min, under a slightly negative pressure. Gaseous HNO_3 feeds into the CSTRs through a port in the upstream air duct, controlled with flow meters placed on the HNO_3 supply tubes. Air from each chamber is sampled for 6 min every hour and HNO_3 concentrations are measured using an electron nitrogen oxide monitor (Thermo Anderson, Franklin, MA). By converting all gaseous N-containing compounds in the chamber sample line to NO with a molybdenum converter ('Molycon' Monitor Labs Inc., Englewood, CO, USA) and comparing NO concentrations from the chamber to ambient NO concentrations, we are able to estimate HNO_3 concentrations for each chamber (Padgett et al., 2004). To mimic light conditions under oak canopies, the CSTRs are covered with shade cloth so that afternoon light levels are between 200 and $400\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ PPFD.

Treatments

Two 1-month-long fumigation experiments were conducted: The first compared HNO_3 fumigations under humidified to ambient dry conditions, and the second compared responses to moderate and high levels of HNO_3 fumigations. In the first experiment, we set up two humid control chambers, two humid HNO_3 fumigation chambers, two dry control and two dry HNO_3 fumigation chambers. HNO_3 levels varied among treatment chambers from a 24-h dosage of $7\text{--}25\text{ }\mu\text{g m}^{-3}$

24-h avg., within the range of historic levels at a high deposition site in the LA basin. Humidity was supplied to the chambers through a misting system in the air supply ducts between 6 and 10 a.m., typical of morning fog events at the collection site. Chlorophyll extractions were performed on days 1, 7, 15 and 28. Carbon exchange and membrane ion leakage were measured on days 1, 7 and 28.

In the second experiment, we used two control chambers, two chambers with “moderate” HNO_3 concentrations (19.9–25 $\mu\text{g m}^{-3}$ 24-h avg.), and two chambers had “high” HNO_3 levels (26.4–35.3 $\mu\text{g m}^{-3}$ 24-h avg.). Sampling of tissue chlorophyll content and ion leakage took place on days 1, 14 and 28.

In both experiments, samples of each thallus were selected from the distal lobes of the thalli, where the nets are finely dissected and fairly uniform. HNO_3 concentrations started at ambient background levels in the greenhouse (typically very low or zero) in the morning, with gas beginning to flow into the chambers at 9:30 a.m., and turning off at 4:00 p.m. Concentrations peaked at 2:00 p.m. and ramped down to background levels in the early evening, mimicking typical diurnal HNO_3 patterns in the LA air basin.

CO₂ exchange and membrane ion leakage

Carbon dioxide exchange was measured in the light (photosynthetic carbon uptake) and in the dark (respiratory carbon release). Samples weighing ~0.5 g were collected from the distal lobes of each treated thallus. Since damaged cell membranes are not able to regulate ion loss, we rinsed for 1 min in 50 ml deionized (DI) water, soaked for 2 h in 100 ml DI water, and analyzed the solutions for conductivity, NO_3^- , potassium (K^+) and sodium content using a Accumet Basic AB30 (Fisher Scientific) conductivity meter and an ion chromatograph (Dionex Corp., Sunnyvale, CA). High levels of ions, particularly K^+ , indicated damage to cell membranes (Pearson, 1985). The samples were then weighed and placed in clear glass sample cuvettes of known volumes (~250 ml) for carbon dioxide exchange measurements.

We measured net photosynthetic CO_2 exchange in a climate-controlled chamber under metal halide grow lamps (light level at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF, 17 °C). Carbon dioxide concentration was calculated by injecting air samples into an infrared gas analyzer (IRGA, ADC Bioscientific, Herts, UK) before the chambers were sealed, and after 8 min in the light. The thallus samples were then placed in dark sample chambers and similarly tested for CO_2 released during respiration. We measured photosynthetic and respiratory carbon exchange four times each. Oven dry weight (ODW) was obtained after drying samples for 24 h at 60 °C

(Matthes-Sears et al., 1987). Carbon dioxide concentrations were converted to $\mu\text{g CO}_2$ exchanged $\text{g ODW}^{-1} \text{min}^{-1}$ using

$$\begin{aligned} & \mu\text{g CO}_2 \text{ exchanged g ODW}^{-1} \text{ min}^{-1} \\ &= \frac{(\text{ppm}_f - \text{ppm}_i) \times \text{chamber volume} \times F \times 1.96 \times 10^{-6}}{\text{time} \times \text{ODW}} \end{aligned}$$

Chlorophyll extractions

We extracted chlorophyll from ~0.02 g samples of air-dry tissue in 10 ml dimethyl sulfoxide (DMSO) (Ronen and Galun, 1984). Chlorophyll content was calculated using Arnon' (1949) equation:

$$\begin{aligned} & \text{mg chlorophyll g dw}^{-1} \\ &= \frac{(20.2\text{OD}_{645} + 8.02\text{OD}_{665}) \times 10 \text{ ml}}{1000 \text{ ml} \times \text{g dw}} \end{aligned}$$

Acidification of chlorophyll to phaeophytins was measured by calculating the ratio between the two pigments at the optical densities 433:415. The ratios were compared to the standard curve in Moss (1967).

Statistical analyses

Each response variable was analyzed with mixed models and analyses of variance (ANOVA) in SAS for Windows[®]. Non-normally distributed or non-heterogeneous data were log transformed. We used a mixed model analysis to estimate residuals and covariance parameters, and run Tukey's multiple comparison tests (Steel and Torrie, 1980).

Results

Overall in both experiments *R. menziesii* thalli treated with HNO_3 showed visual signs of bleaching and by the end of day 28 were clearly damaged, if not dead. Thalli became brittle and yellowish brown, in contrast to control treatments where thalli remained pale yellow-green and were supple when wet.

Experiment 1: humidity and HNO_3

Rinse solution and ion loss

Rinse solution conductivity increased significantly (three-way ANOVA) with time in both humid and dry HNO_3 fumigation treatments, but remained unchanged over time in both control treatments (Fig. 1, Tukey's multiple comparison test). Rinse solutions for dry HNO_3 treatments had significantly higher conductivity than for humid HNO_3 treatments over time, and samples from both humidified and dry HNO_3 treatment chamber

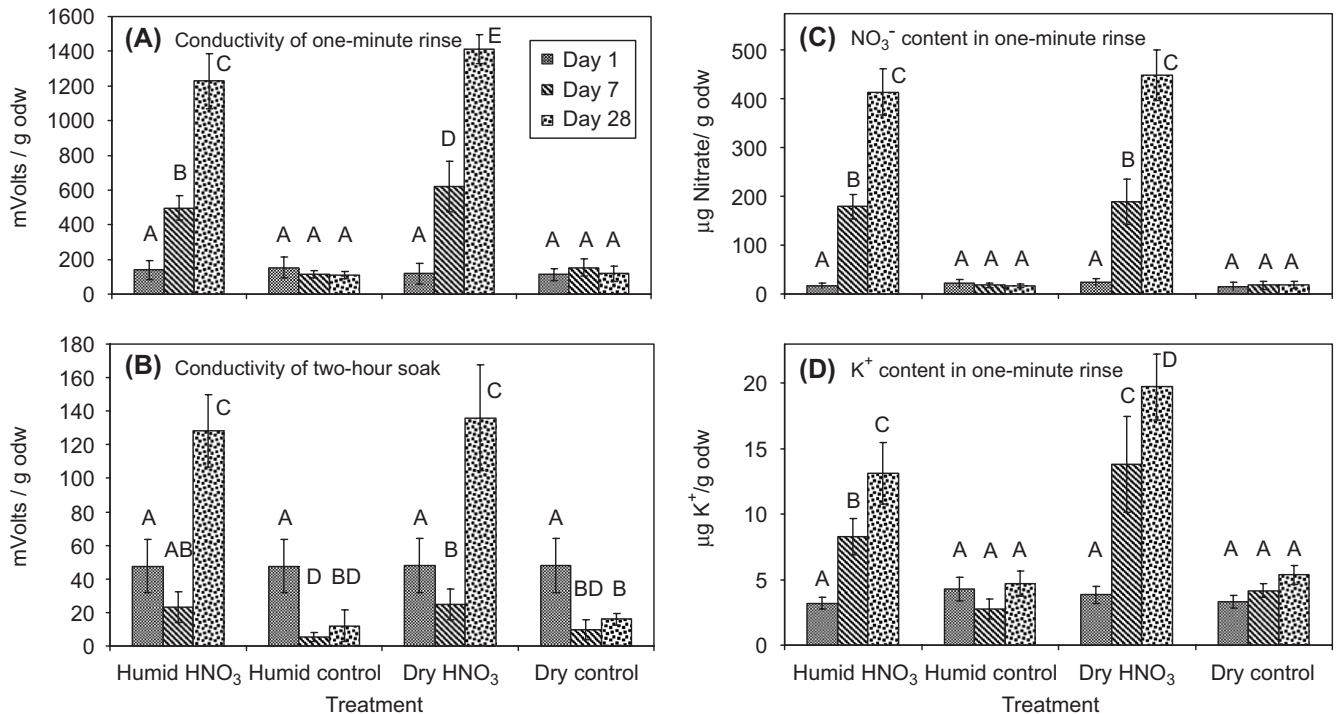


Fig. 1. Experiment 1. Conductivity and ion contents of 1-min rinse and 2-h soak solutions, reported per oven dry weight of sample (ODW). All day 1 rinse and soak data are means of untreated thalli ($n = 36$), days 7 and 28 means ($n = 10$) are plotted for each treatment by time combination due to the occurrence of a significant interaction. Rinse conductivity (A) means are affected by all factors ($p = 0.039$, $F = 3.1$, humidity \times time; $p < 0.0001$, $F = 242.56$, HNO₃ \times time; $p = 0.0416$, $F = 4.25$, HNO₃ \times humidity), as are soak conductivity (B) means ($p < 0.001$, $F = 87.55$, for the three-way interaction). Nitrate content (C) in rinses changed significantly over time in lichens treated with HNO₃ ($p < 0.0001$, $F = 236.35$). Potassium content of rinse solutions (D) is significantly affected by all factors ($p < 0.0001$, $F = 10.39$, humidity \times time; $p < 0.0001$, $F = 81.19$, HNO₃ \times time). Identical letters above means indicate that means are not significantly different from each other (Tukey's multiple comparison test, i.e. HSDs). Error bars represent two standard errors from the mean.

solutions had significantly higher conductivity than control chambers over time (Fig. 1A). Conductivity was an order of magnitude lower in the 2-h soak solutions than the initial 1-min rinse solutions (Fig. 1B), demonstrating that the 1-min rinse was sufficient to remove the majority of ions. While humidity itself did not significantly alter the conductivity, the interactions between HNO₃ and humidity, HNO₃ and time, and between humidity and time all had significant effects ($p = 0.0416$, $p < 0.0001$, $p = 0.049$, respectively). The interaction of all three factors was not significant ($p = 0.3781$).

Nitrate content of the rinse solutions increased significantly (two-way ANOVA) over time in all HNO₃ treatments, but did not change in controls ($p < 0.0001$, Fig. 1C). Potassium leakage increased in both humid and dry HNO₃ treatments, and only slightly in control treatments, roughly doubling every 2 weeks in HNO₃ treatments (Fig. 1D). There was a significant interaction between exposure time, and both humidity and HNO₃ treatments, as well as between HNO₃ and humidity ($p < 0.0001$ each). Data for day 1 for all conductivity and ion losses are from second fumigation baseline data.

Net photosynthesis and respiration

Net photosynthesis (NPS) and respiration responses were significantly different among treatments, although both declined to near zero in humid and dry HNO₃ fumigations by day 28 (Fig. 2). The humid HNO₃ treatment exhibited a significant initial increase in NPS in both fumigated and control samples, but the fumigated sample declined to nearly zero by day 28 as had the dark respiratory rate. The dry HNO₃ treatments exhibited an immediate decline in net photosynthetic capacity, which became essentially zero by day 28. Controls varied; the humid control maintained high levels of NPS and respiration; dry control had a nearly significant decrease in NPS by day 28, but maintained unchanged dark respiration levels.

Chlorophyll content and acidification

Control treatments did not significantly change in chlorophyll content over the course of the experiment, and 0% of the chlorophyll pigments were converted to phaeophytins by day 28; whereas, both HNO₃ treatments experienced substantial declines in chlorophyll content (Fig. 3A), and had significant increases in

phaeophytinization (Fig. 3B). HNO₃, humidity and time all had significant effects on chlorophyll content in tissues and percent of pigments converted to phaeophytins (Figs. 3A and B). There was also a significant difference between HNO₃ treatments. Chlorophyll decreased significantly by each sample date in dry HNO₃ treatments, and by day 28 was at ~34% (actually 0%, given the next statement) of the original concentration; percent phaeophytins increased to 100% by day 28 (Fig. 3B). In the humid HNO₃ treatment, chlorophyll content stayed at baseline concentrations until day 14, but by day 28 had also decreased to ~65% of the

baseline concentrations. Phaeophytins increased more rapidly in the dry HNO₃ treatment than in the humid one.

Experiment 2: varying levels of HNO₃

Although the levels of HNO₃ in the chambers were higher than in the first experiment, the results were similar, showing qualitative and quantitative physiological decline when treated with HNO₃. Conductivity of the 1-min rinse solutions increased significantly (two-way ANOVA) with time and HNO₃ dosage levels (Fig. 4), but controls remained unchanged. Chlorophyll

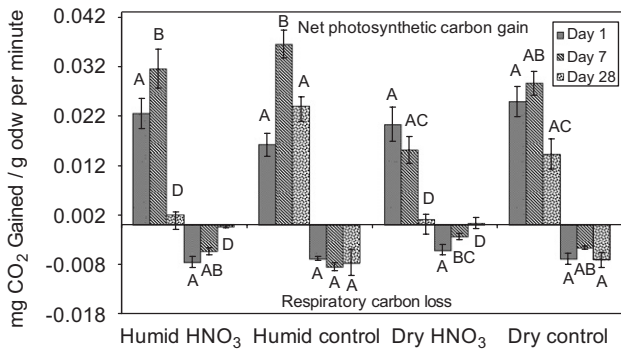


Fig. 2. Experiment 1. Net photosynthetic (NPS) carbon exchange during photosynthesis and respiration in humid and dry chambers. While carbon exchange is variable in control dry treatments, it declines to zero in both HNO₃ treatments by day 28. Means ($n = 10$) are plotted for each treatment by time combination due to the occurrence of a significant interaction ($p < 0.0001$, $F = 24.29$, for NPS, $p = 0.0005$, $F = 12.30$, for respiration, ANOVA). Identical letters above means indicate that means are *not* significantly different from each other (Tukey's multiple comparison test, i.e. HSDs). Error bars represent two standard errors from the mean.

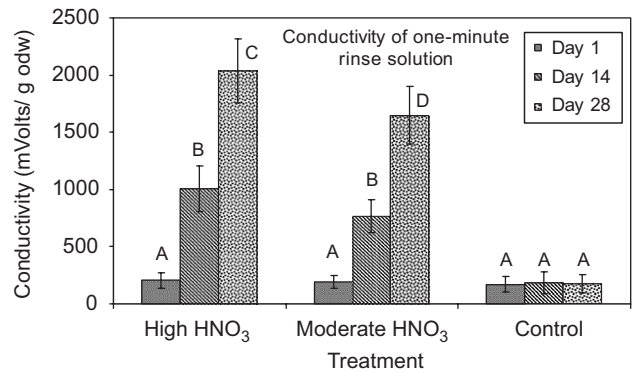


Fig. 4. Experiment 2. Conductivity of 1-min rinse solution from thalli treated with varying levels of HNO₃. To meet the normality assumption, the data were log transformed before running the 2 × 2 ANOVA. Means ($n = 12$) are plotted for each treatment by time combination due to the occurrence of a significant interaction ($p < 0.0001$, $F = 36.92$). Letters above means that are the same indicate that means are *not* significantly different from each other (Tukey's multiple comparison test, i.e. HSDs). Error bars represent two standard errors from the mean.

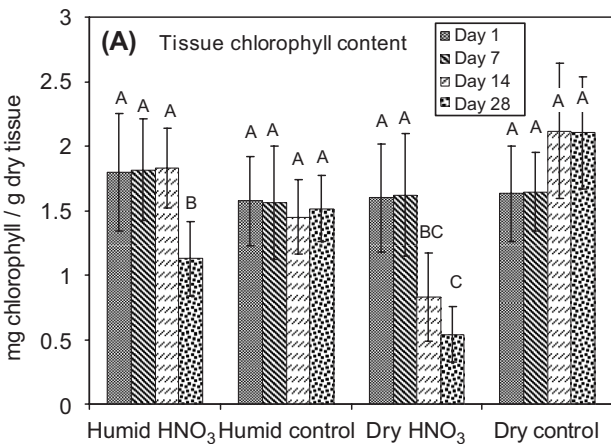


Fig. 3. Experiment 1. Chlorophyll content (A) and percent pigments converted to phaeophytins (B). Means ($n = 10$) are plotted for each treatment by time combination due to the occurrence of a significant interaction ($p < 0.0191$, $F = 3.42$, chlorophyll; $p < 0.0001$, $F = 7.64$, phaeophytins). Letters above means that are the same are *not* significantly different from each other (Tukey's multiple comparison test, i.e. HSDs). Error bars represent two standard errors from the mean.

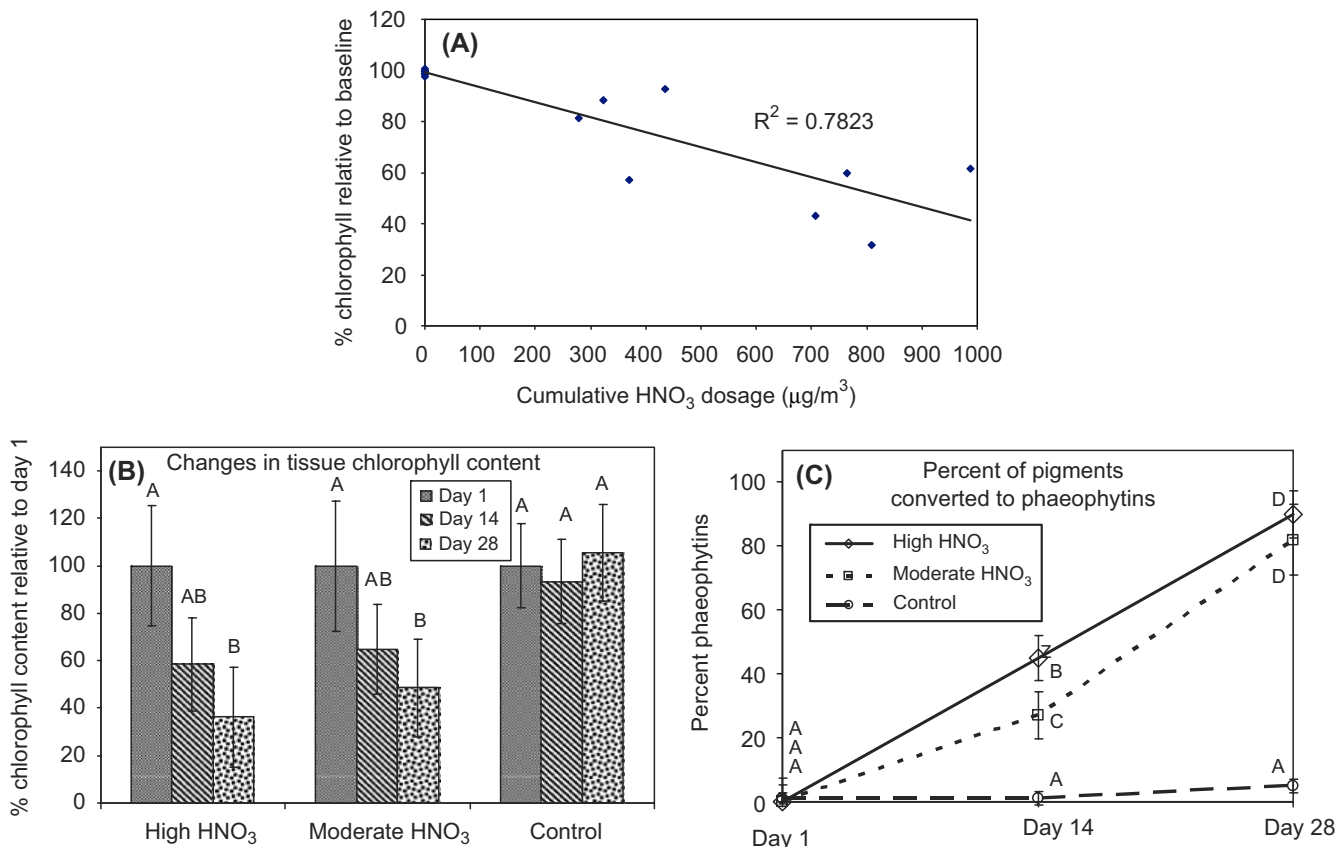


Fig. 5. Experiment 2. Chlorophyll and phaeophytin content of thalli treated at various levels of HNO₃. A regression of the chlorophyll loss relative (A) shows a correlation to the baseline tissue content ($r^2 = 0.7823$, $n = 6$ for each point). The interaction between time and HNO₃ treatment significantly affected (B) the changes in chlorophyll content ($p = 0.0008$, $F = 5.18$), as well as (C) the percent of pigments converted to phaeophytins ($p < 0.0001$, $F = 14.21$). Letters above means that are the same indicate that means are *not* significantly different from each other (Tukey's multiple comparison test, i.e. HSDs). Error bars represent two standard errors from the mean; means ($n = 12$) are plotted for each treatment by time combination due to the occurrence of a significant interaction.

content in thallus tissue decreased in both high and moderate HNO₃ treatments (Fig. 5B). Both treatments had less than half of their original chlorophyll levels (two-way ANOVA) by day 28. Chlorophyll loss correlated with the dosage (Figs. 5A and C). Chlorophyll content remained close to baseline levels in control treatments and declined in all HNO₃ treatments (Figs. 5B and C). Phaeophytization in HNO₃-treated specimens increased to ca. 80–90% by day 28 (Fig. 5C).

Discussion

Our results show unequivocally that HNO₃ is toxic to *R. menziesii*, albeit at somewhat higher levels than typically encountered in the LA air basin. While HNO₃ concentration levels varied among chambers and treatments from levels commonly encountered in summer months to levels only found in extreme cases, ultimately all thalli treated with gaseous HNO₃ declined physiolo-

gically. Decreasing respiration and photosynthesis indicate that both algal and fungal cells sustained damage. This damage may occur through several mechanisms, including acidification of pigments and cell membrane damage.

The increased percentage of phaeophytin pigments in fumigated thalli suggests that the decline of photosynthesis may, in part, be due to acidification of pigments, as occurs with exposure to SO₂ (Nash and Gries, 2002). Acidification removes the central magnesium ion from the chlorophyll molecule, creating phaeophytins, which are non-photosynthetic pigments (Moss, 1967). This loss of photosynthetic pigments has the obvious result that the lichen loses its ability to fix carbon into energy storing sugars, limiting the ability of both symbiotic partners to perform basic metabolic processes, including cell repair, growth and reproduction.

Cell membranes become "leaky" when damaged (Pearson, 1985), experiencing uncontrolled ion loss. Potassium is an especially good indicator of this type of cell damage, as cell membranes regulate the passage of

K^+ , and accumulate rather than release this essential ion. Solutions from both 1-min rinses and 2-h soaks of our fumigated material contained significantly higher levels of K^+ than did our controls. Increased losses of K^+ occurred with greater time and dosage of exposure. Lichens in humidified chambers lost less K^+ than those kept in dry chambers, indicating that humidity may have a mitigating effect on the lichen's ability to tolerate the pollutant, allowing the lichen to repair some of the damage during the treatment period. Interestingly, as NO_3^- levels in rinse solutions in both treatments were not significantly different, we can assume that the gaseous nitrogen was not incorporated into the lichen cells.

In a 1985–1986 ambient transplant experiment, Boonpragob et al. (1989) found correspondingly high levels of NO_3^- rinsing from the surface of *R. menziesii* thalli in the summer months, and low levels in the winter when precipitation is higher, and dry deposition lower. Thus, we feel confident that we had mimicked ambient environmental conditions, at least at the higher end of the deposition range. Transplanted thalli in these same experiments were physiological dead within 8–10 weeks, surviving 4–6 weeks longer than under our fumigations (Boonpragob and Nash, 1991).

We can conclude that it is very likely that HNO_3 has contributed to the disappearance of this sensitive lichen species from the LA air basin, as well as in other arid locations with high dry deposition loads. Prior researchers reported that ca. 50% of the lichen flora had disappeared in this region (Sigal and Nash, 1983; Ross, 1982), and they inferred that the high levels of O_3 were responsible for the decline in abundance, morphological variability, and diversity of lichen species in the LA air basin. Since we have not yet investigated effects of O_3 , we cannot rule out the possibility of a deleterious or synergistic effect between HNO_3 and O_3 . In future experiments, we will address this question with a 2×2 factorial design, involving fumigations of HNO_3 and O_3 both together and separately. Our future research will also seek to address the questions of toxicity thresholds for HNO_3 , as well as working with other, potentially more tolerant species to allow a more complete understanding of how this pollutant affects lichen community composition and distribution.

Since lichens in humidified chambers had slower declines in both carbon exchange capacity (NPS and dark respiration) and chlorophyll content, and smaller losses of K^+ , climate may be a significant factor in the survival of *R. menziesii* in the LA air basin. Clearly, pollution has had a significant effect on the survival of the species, but habitat loss, lower humidity levels in the inland areas with urbanization, and increased fire incidence may all play important roles in the disappearance of this and other lichen species.

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