Environmental Pollution 170 (2012) 202–210

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Physiological responses of lichens to factorial fumigations with nitric acid and ozone

J. Riddell [a,b], P.E. Padgett [b,*], T.H. Nash III [a]

[a] Arizona State University, School of Life Sciences, PO Box 874501, Tempe, AZ 85287, USA
[b] USDA Forest Service, Pacific SW Research Station, 4955 Canyon Crest Drive, Riverside, CA 92507, USA

ARTICLE INFO

Article history:
Received 23 January 2012
Received in revised form
4 June 2012
Accepted 6 June 2012

Keywords:
Lichens
Air pollution
HNO_3
O_3
Biomonitoring

ABSTRACT

This paper addresses the effects of gaseous nitric acid (HNO_3) and ozone (O_3), two important air pollutants, on six lichen species with different morphological, ecological, and biological characteristics. The treatment chambers were set up in a factorial design consisting of control chambers, chambers fumigated with HNO_3, with O_3, and with HNO_3 and O_3, together. Each species showed a different sensitivity to the fumigations, reflecting the physiological variation among species. Our results clearly indicate that HNO_3 is a strong phytotoxin to many lichens, and that O_3 alone has little effect on the measured parameters. The combined fumigation effects of HNO_3 and O_3 were not significantly different from HNO_3 alone.

Published by Elsevier Ltd.

1. Introduction

Our work investigates the physiological effects of two important urban pollutants, gaseous nitric acid (HNO_3) and tropospheric ozone (O_3) on lichens. Urban areas within arid, Mediterranean climates often experience high levels of HNO_3 and O_3 pollution. The Los Angeles basin in southern California has some of the worst air quality in the United States, and is frequently in non-compliance with both state and federal Environmental Protection Agency O_3 and NO_x standards (Cox et al., 2009). One method of identifying the distribution of air pollutants in rural and forested areas surrounding the L.A. basin is to look at lichen community composition patterns. However, in order for this method of monitoring air quality to succeed, it is important to have an in-depth understanding of how individual lichen species respond to individual pollutants.

Epiphytic lichens subsist primarily on atmospheric moisture and nutrients, and are therefore highly responsive to changes in air quality. Individual species of lichens, with different morphological and physiological characteristics, are differentially responsive to pollutants, making them useful in identifying shifts in air quality by observing changing lichen community composition (Nash, 2008).

In previous fumigation experiments with the lichen species *Ramalina menziesii* Tayl., we demonstrated that *R. menziesii* is highly sensitive to even moderate levels of HNO_3 typical of the LA basin (Riddell et al., 2008), but not responsive to high levels (120 ppb) of O_3 (Riddell et al., 2010). Earlier O_3 fumigation studies have shown equivocal results, demonstrating declines in the vitality of some species, but not others (Ross and Nash, 1983; Scheidegger and Schroeter, 1995; Tarhanen et al., 1997). In contrast to earlier O_3 fumigation studies, community studies conducted in areas subjected to both O_3 and N pollution have found much stronger correlations between lichen community changes and N pollution (Larsen et al., 2007) than with O_3 (Bates et al., 1996; Gombert et al., 2006). Frati et al. (2007) demonstrated strong correlations between N pollution (NH_4^+ and NH_3) and shifts in lichen community composition, and changes in lichen chemistry and symbiotic relations were shown by Ra et al. (2004) and; Gaio-Oliveira et al. (2005). Several studies have demonstrated an effect of total oxidized N (NO_x) on lichens (e.g. Lorenzini et al., 2003), but because of the difficulty in monitoring HNO_3 apart from all other NO_y species, little work has been conducted with HNO_3 alone. The aim of this study was to tease apart the effects of gaseous HNO_3 and O_3, in factorial fumigation treatments, on six lichen species collected near the LA basin: *Ramalina menziesii* Tayl., *Hypogymnia imshaugii* Krog., *Flavopunctelia flaventior* (Stirt.) Hale, *Pseudocyphellaria anthraspis* (Ach.) H. Magn., *Usnea hirta* (L.) F.H. Wigg. and two species of *Physconia*, *P. enteroxantha* (Nyl.) Poelt and *P. isidiigera* (Zahlbr.) Essl., which occurred in mixed populations. Each species differs from the others in shape, surface area to mass ratios, algal

* Corresponding author.
E-mail address: ppadgett@fs.fed.us (P.E. Padgett).

0269-7491/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.envpol.2012.06.014

and/or cyanobacteria species, and secondary chemical composition of the thallus. By choosing these six morphologically and physiologically different lichens, we hope to improve our ability to predict air quality using lichen "functional groups" that can be classified as HNO_3 or O_3 sensitive.

2. Methods

2.1. Fumigation chambers (constantly stirred tank reactors)

The fumigation chambers (constantly stirred tank reactors, CSTRs) are 1.35 × 1.35 m clear, cylindrical, enclosed Teflon film chambers housed in a climate-controlled greenhouse with particulate and charcoal air filtration systems, on the University of California Riverside campus in Riverside, CA (a detailed description can be found in Padgett et al., 2004). To mimic light conditions under tree canopies, the CSTRs were covered with shade cloth so that afternoon light levels are between 200 and 400 μmol m^{-2} s^{-1} PPFD. Because the highest levels of pollution occur in the arid, hot summer months in southern California, we did not humidify our chambers or spray water on the lichens, lichens were exposed to ambient moisture in the chambers. Relative humidity during the day was very low ranging from 15% to 20%, and elevated overnight to roughly 30%–40% as is typical for the ambient conditions in Riverside, CA.

Ozone was generated by injecting oxygen into an O_3 generator (Superior Electric Co., Bristol, CT, USA) and 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. To simulate real world conditions, we generated O_3 from 10:00 to 16:00 daily. Ozone concentrations were measured using a Dasibi Ozone monitor (Dasibi Environmental Corp Model 1003-AH, Glendale, CA).

The HNO_3 fumigation (see Padgett et al., 2004) introduces HNO_3 in super dried air via Teflon tubes to the fumigation chambers. Nitric acid was generated between 09:30 and 16:00 daily. Concentrations started low, and ramped up to an afternoon peak, mimicking patterns in the Los Angeles air basin.

2.2. Species, treatments, and measurements

Chambers were established in a factorial design: two control chambers, two chambers fumigated with gaseous HNO_3 (daily peak levels near 50 ppb HNO_3), two chambers with 80 ppb O_3, and two chambers with approximately the same concentrations of HNO_3 and O_3, together. The combination of O_3 and HNO_3 tended to suppress total HNO_3 concentrations. Cumulative doses of HNO_3 were calculated for each species in each treatment, for each sample date (Table 1). We fumigated *R. menziesii, H. imshaugii, U. hirta, F. flaventior, P. anthraspis*, and two *Physconia* species (consisting of mixed populations of *P. enteroxantha* and *P. isidiigera*, hereafter referred to as *P. enteroxantha* s.lat.). McCune and Geiser's (2009) index of macrolichen sensitivity to pollutants lists *R. menziesii*, *U. hirta, P. anthraspis*, and *H. imshaugii* as N oligotrophs, meaning that they are sensitive to N pollution. *Physconia enteroxantha* is listed as a mesotroph (moderately N tolerant), and *P. isidiigera* as eutrophic or nitrophilic (highly tolerant of N deposition). Because the two *Physconia* species occur in highly intermixed populations in southern California, separating each thallus for treatment would have damaged the specimens and created samples too small to measure physiological responses. Although *F. flaventior* is not listed in the index, transplant experiments with a closely related, morphologically similar species, *Flavoparmelia caperata,* demonstrated a negative correlation of photosynthetic vitality with increasing NO_2 concentrations (Tretiach et al., 2007). *Ramalina menziesii* and *U. hirta*, to our knowledge, are extirpated from the LA basin, although they were formerly found in abundance and are well represented with herbaria specimens collected in the basin. *Pseudocyphellaria anthraspis* has a cyanobacterial photobiont, which are thought to be more susceptible to N deposition (Geiser and Neitlich, 2007). *Hypogymnia imshaugii* and *P. enteroxantha* s.lat. species are still found at sites with high pollution loads within southern California. *Physconia enteroxantha* s.lat. is considered to be nitrophilic and the species are very abundant at sites with high N deposition throughout California (Jovan and McCune, 2005; Jovan, 2008; Jovan et al., in press).

Each species was placed in the fumigation chambers by attaching the thalli to oak or fir branches (corresponding with their respective natural substrates) using nylon fishing line. Lichens were monitored at regular intervals until thalli showed signs of severe physiological distress. The different morphologies of each species determined the frequency and type of assays we were able to perform. Because *R. menziesii* thalli are large, they were each sampled repeatedly, and are treated as repeated measures in statistical analyses. Samples were collected from the fresh growing tips of the fumigated thalli. All other species were sampled as whole individuals at each sample date. *Ramalina menziesii, H. imshaugii, U. hirta,* and *P. anthraspis* were sampled for chlorophyll content, gross photosynthetic (GP) and dark respiration (DR) CO_2 exchange capacity, chlorophyll fluorescence (F_v/F_m), and cell membrane integrity. *Flavopunctelia flaventior* and *P. enteroxantha* s.lat. are both strongly attached to their substrate, so that removal results in extensive damage to the thallus. Therefore, they were sampled for chlorophyll content, phaeophytin *a* and chlorophyll fluorescence, which can be done without removing them from their substrate. *F. flaventior* was tested for CO_2 exchange capacity on the last day of treatment (*P. enteroxantha* s.lat. samples were too small for this test).

2.3. Chlorophylls and phaeophytin a

We measured chlorophyll *a* and *b* concentrations from 20 mg of material per sample by extraction in 5 ml dimethyl sulfoxide (DMSO) following Ronen and Galun (1984). The samples were incubated in test tubes for 45 min at 60 °C, removed from the incubating oven, and diluted with an additional 5 ml DMSO. Optical density (OD) was measured in a spectrophotometer at 415, 433, 645, 665, and 750 nm. Because *Pseudocyphellaria anthraspis* contains a cyanobacterial photobiont, measurements represent only chlorophyll *a* content for that species.

Acidification of the chlorophyll *a* molecule causes the removal of the central magnesium atom, converting the pigment to phaeophytin *a*, a brown pigment utilized in the electron chain transport system of photosynthesis. This conversion reduces the photosynthetic capacity of the lichen thallus. The conversion can be measured using the ratio of OD 433:415 nm. An OD_{433}:OD_{415} curve developed by Ronen and Galun (1984) allowed us to calculate the percent phaeophytin *a*:chlorophyll *a* ratio using the equation: % phaeophytin $a = -104.8 \times \ln(OD_{433}{:}OD_{415}) + 39.715$.

2.4. CO_2 exchange capacity

We measured CO_2 exchange capacity using methods developed by Larson and Kershaw (1975) under standardized conditions. Sample material (0.3–1.5 g, depending on the species) were soaked for two hours in deionized (DI) water, and patted dry prior to initiating CO_2 exchange measurements. Measurements took place in sealed clear (net photosynthesis) and blackened (dark respiration) glass cuvettes, placed in a temperature-controlled chamber (17 °C) under metal halide lights (light levels 250 μmol photons m^{-2} s^{-1}). The CO_2 concentration inside the cuvettes was measured immediately before the cuvettes were sealed and 10 min after by injecting two ml of the sample air into a constantly flowing stream of pure N_2 feeding into a LI-COR 6200 Infrared Gas Analyzer (Riddell et al., 2008). Net photosynthetic CO_2 absorption (NP) and dark respiratory CO_2 release (DR) were measured three times for each sample, and are reported as μg CO_2 exchanged per gram oven-dry weight per minute. Gross photosynthesis is given as net photosynthesis, plus dark respiration (GP = NP + |DR|).

Table 1
Cumulative doses of HNO_3 in ppb × hour, for each species by treatment by sample date.

Species	Treatment	Sample day							
		16	18	20	35	42	50	71	78
Flavopunctelia flaventior	HNO_3	131			178		271	609	
	$HNO_3 + O_3$	83			110		198	467	
Hypogymnia imshaugii	HNO_3			118			616		1166
	$HNO_3 + O_3$			103			533		1000
P. enteroxantha s.lat.	HNO_3							609	
	$HNO_3 + O_3$							467	
Pseudocyphellaria anthraspis	HNO_3				710	1081			
	$HNO_3 + O_3$				627	1001			
Ramalina menziesii	HNO_3		190		225				
	$HNO_3 + O_3$		171		194				
Usnea hirta	HNO_3			190		245			
	$HNO_3 + O_3$			171		206			

2.5. Chlorophyll a fluorescence

The integrity of photosystem II (PS II) and the electron chain transport system was assessed by means of chlorophyll *a* fluorescence measurements (Bilger et al., 1995). 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 soaked with DI water, dark adapted for 15 min, and patted dry in a dark chamber at 22 °C with clean paper towels. The lobe tips of the thalli were exposed to modulated light to obtain the minimal fluorescent yield (F_o), and then to a saturating light pulse for 0.6 s to obtain maximum fluorescent yield (F_m). The samples were then kept in the dark for 40 s before being exposed to saturating light pulses at 20-s 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. Ten samples per species were measured ($n = 10$) per sapling period. Mean F_v/F_m values of three measurements per sample were used for analyses.

2.6. Cell membrane integrity and ion leakage

Damaged cell membranes can be detected as uncontrolled ion loss from cells to a bathing solution. Potassium is considered to be a good indicator of cell membrane integrity (Pearson, 1985). To estimate cell membrane damage, we soaked all samples in DI water for one minute (a quick rinse) and for two hours. The two solutions were analyzed for K^+ and NO_3^- concentrations using an ion chromatograph (Dionex Corp., Sunnyvale, CA) and conductivity of the solutions using an Accumet Basic AB30 (Fisher Scientific) conductivity meter calibrated with standard solutions. Higher levels of conductivity indicate greater ion leakage from damaged cell membranes. We used both techniques to validate the use of conductivity alone as a measure of cell leakage, recognizing that not all investigators have access to ion chromatographs.

2.7. Nitrate deposition

Nitric acid accumulates on surfaces with increased exposure. Rinse solutions were analyzed for NO_3^- concentrations to confirm exposure and to estimate deposition rates to the different lichen species. Because of the large differences in surface characteristics and morphology among the lichens, calculations of deposition rates were not informative.

2.8. Statistical analyses

All results were analyzed with two-way mixed model ANOVA in SAS (SAS, 2002). Adjusted Tukey multiple comparisons were used to compare least square means between sample dates for each species across all treatments (Steel and Torrie, 1980). Non-normally distributed or non-heterogeneous data were log transformed. Repeated measure analyses were used for *R. menziesii*, as each thallus was sampled multiple times. New individual thalli were sampled on each sample date for all other species.

3. Results

3.1. Chlorophylls and percent phaeophytin a

Chlorophyll content of O_3 fumigated thalli was not significantly different than the control treatments in any of the treated species (Fig. 1). *Flavopunctelia flaventior* and *P. enteroxantha* s.lat. experienced no significant losses of chlorophyll in any treatments relative to controls, and in *P. anthraspis*, chlorophyll increased slightly in all treatments, although the increase was only significant in the O_3 and

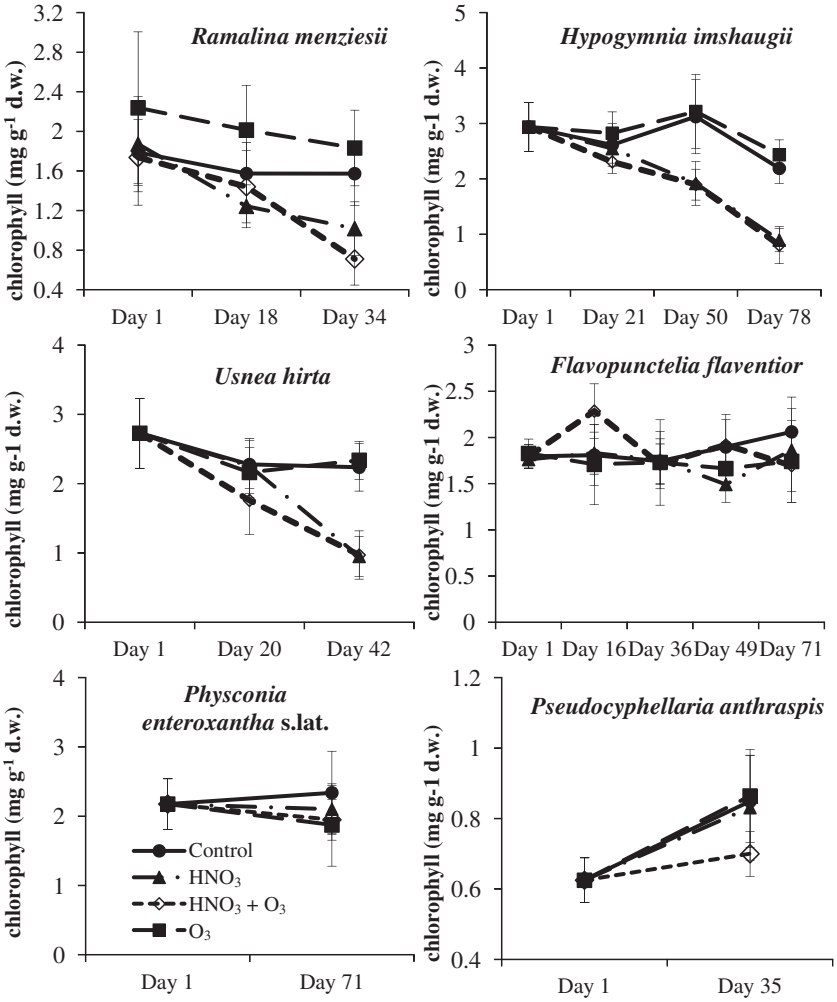

Fig. 1. Chlorophyll content of lichens fumigated with HNO_3 and O_3 in factorial combinations, by species, in mg of chlorophyll per gram air-dried material. Error bars represent two standard errors above and below the mean.

control treatments ($p < 0.05$ for both). However, chlorophyll concentrations of *R. menziesii* decreased significantly in the HNO_3 and $HNO_3 + O_3$ treatments by day 34 compared to day one, control, and O_3 samples ($p < 0.05$, all hsd comparisons); compared to control and O_3 treatments by day 50 for *H. imshaugii* ($p < 0.01$), and by day 42 for *U. hirta* thalli ($p < 0.01$, all hsd comparisons). The key difference between the three affected species was the amount of time before the decline was detected.

The percent of chlorophyll *a* degraded into phaeophytin *a* through acidification (Fig. 2) increased significantly by the first sample date in the HNO_3 and $HNO_3 + O_3$ treatments compared to control and O_3 treatments in *R. menziesii* ($p < 0.01$), *H. imshaugii* ($p < 0.01$), *P. anthraspis* ($p < 0.01$, all hsd comparisons) and *U. hirta* ($p < 0.01$) thalli. The significant increase occurred after the first sample date for all three lichen species, and the degradation of chlorophyll *a* into phaeophytin *a* continued to increase in each species over time. There was a significant increase in phaeophytin *a* in $HNO_3 + O_3$ fumigated *F. flaventior* thalli relative to all other treatments between days 49 and 71 ($p < 0.05$), but no significant change in the percent of phaeophytin *a* in the fumigated *P. enteroxantha* s.lat. after 71 days of treatment.

3.2. CO_2 exchange capacity

Carbon dioxide exchange capacity (GP and DR, Fig. 3) decreased significantly in HNO_3 and $HNO_3 + O_3$ treatments relative to controls in *R. menziesii* ($p < 0.01$ for GP, and $p < 0.01$ day 18 DR) and *H. imshaugii* ($p < 0.01$ for GP by day 50, and $p < 0.05$ for DR by day 78) thalli, in *U. hirta* on day 20 ($p < 0.01$ (GP), $p < 0.05$ (DR)), but by day 42, GP and DR were the same for all *U. hirta* treatments. Gross photosynthesis of *P. anthraspis* was significantly lower in HNO_3 and $HNO_3 + O_3$ treatments relative to O_3 and control treatments ($p < 0.05$, all hsd comparisons), but there was no difference in DR. Gross photosynthesis decreased significantly in all treatments relative to controls in *F. flaventior* thalli ($p < 0.05$, all hsd comparisons), but there were no significant changes in DR. Again, the key difference between responses of species was the amount of time that passed before the treated thalli began to demonstrate a significant decline. *Ramalina menziesii* and *U. hirta* GP and DR declined immediately after the treatments began, whereas for *H. imshaugii*, GP and DR began to decline significantly after 50 days. Because GP and DR were only sampled twice for *F. flaventior* and *P. anthraspis*, we do not have an accurate time frame for the initial declines, or if there was a point at which treatments were different from each other.

3.3. Chlorophyll fluorescence

The potential quantum yield of photosystem II (F_v/F_m, Fig. 4) of all species declined significantly in all treatments ($p < 0.01$, all hsd comparisons with day one and subsequent sample days), indicating a negative "chamber effects" from the non-ideal conditions for all

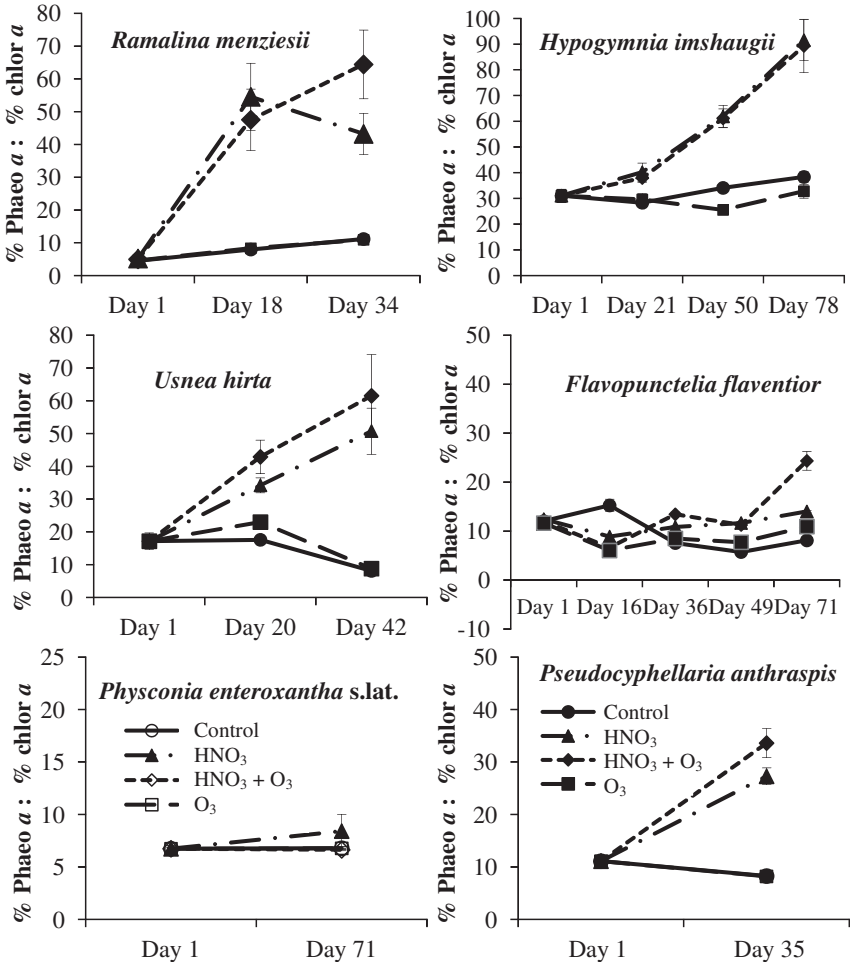

Fig. 2. The ratio of percent phaeophytin *a* to chlorophyll *a* in lichens fumigated with HNO_3 and O_3 in factorial combinations, by species. Error bars represent two standard errors above and below the mean. Ozone and control treatment results were identical for both *R. menziesii* and *P. anthraspis*, and therefore overlapping.

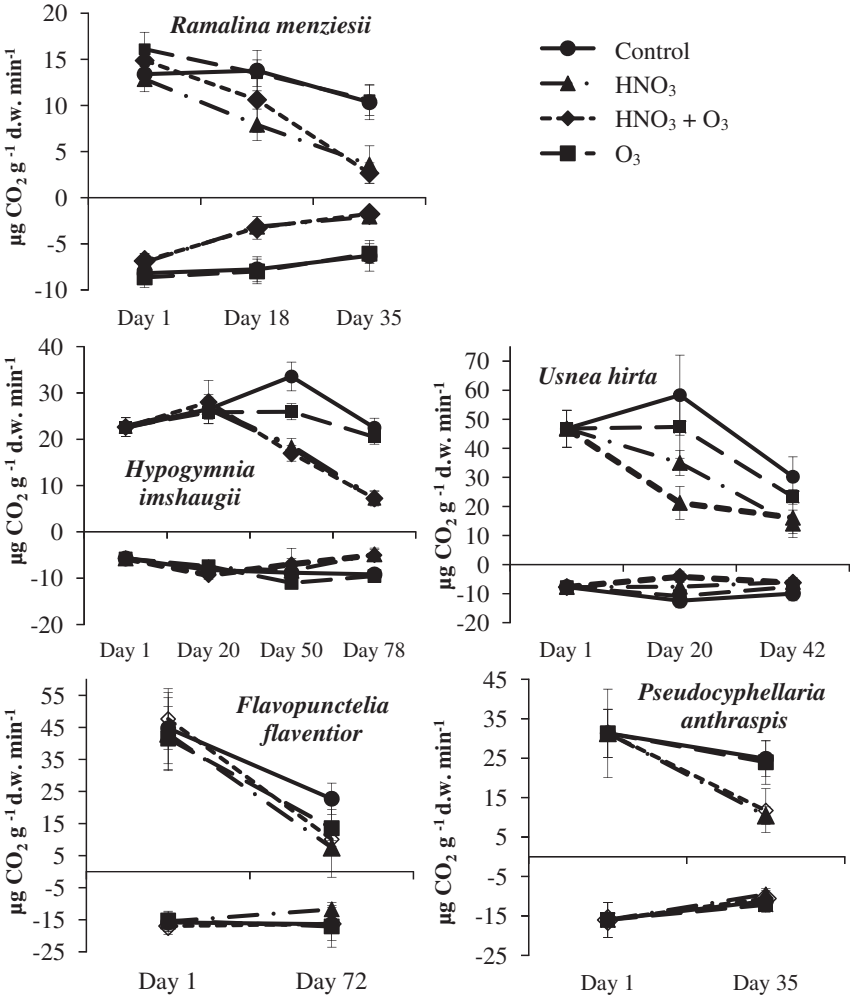

Fig. 3. Dark respiration (negative values) and gross photosynthesis (positive values) of lichen thalli fumigated with factorial combinations of HNO_3 and O_3. Gross photosynthesis represents the gross carbon gained by the fumigated thalli per minute, and dark respiration on each graph represents the carbon released by sample thalli. Error bars represent two standard errors above and below the mean.

individuals. Never the less, there were differences in F_v/F_m among treatments and between species beyond those due to the chambers. The decrease in F_v/F_m was significantly greater in HNO_3 and $HNO_3 + O_3$ treated thalli than control and O_3 treated samples on day 35 in *R. menziesii* samples ($p < 0.01$), on day 20 (but not 42) in *U. hirta* samples ($p < 0.01$, day 20), and *P. anthraspis* on day 35 ($p < 0.01$, all hsd comparisons). For *H. imshaugii*, the decline in F_v/F_m was significantly greater in the control, HNO_3 and $HNO_3 + O_3$ treatments than in the O_3 treatment ($p < 0.01$ for O_3 vs. all other treatments, $p < 0.05$ for HNO_3 vs. control day 78). F_v/F_m was significantly higher ($p = 0.03$) in O_3 than in all other treatments for *F. flaventior* on day 72, but otherwise there were no differences among treatments. There was no significant difference between treatments for the *P. enteroxantha* s.lat.

3.4. Cell membrane integrity and ion leakage

We sampled the leachate conductivity and ion leakage of *R. menziesii, H. imshaugii, U. hirta,* and *P. anthraspis* (Fig. 5). One-minute rinse solution data were log transformed to meet the equal variance criteria for the two-way ANOVA. All of the sampled species had significantly greater conductivity in one-minute rinse solutions in the HNO_3 and $HNO_3 + O_3$ treatments than in the control or O_3 treatments by the first sample date for each species ($p < 0.01$, all hsd comparisons). The combined $HNO_3 + O_3$ treatment had greater conductivity than the HNO_3 treatment by day 35 for both *R. menziesii* and *P. anthraspis* ($p < 0.01$ for both).

Potassium leakage (Fig. 6) increased significantly over time for all species in HNO_3 and $HNO_3 + O_3$ fumigation treatments relative to control and O_3 fumigation treatments ($p < 0.02$ by the second sample date for all species comparisons). Again, K^+ leakage increased more rapidly in *R. menziesii* and *U. hirta* than in *H. imshaugii* or *P. anthraspis*, directly corresponding with the measured amounts of NO_3^- rinsed from the surface of the lichen thalli. Leakage was slightly greater in $HNO_3 + O_3$ treatments than in HNO_3 treatments at some sample dates, but the differences were only significant for *H. imshaugii* on day 20 ($p < 0.05$).

3.5. Nitrate content in rinse solutions

Nitrate rinsed from the surface of the lichen thalli (Fig. 7) was significantly greater in HNO_3 and $HNO_3 + O_3$ treatments of all species after the first sample date ($p < 0.01$, *R. menziesii*, $p < 0.05$, *H. imshaugii* day 20, $p < 0.01$, *U. hirta*, and $p < 0.01$, *P. anthraspis*). However, NO_3^- accumulated at a much greater rate on *R. menziesii* and *U. hirta* than on *H. imshaugii* and *P. anthraspis*, perhaps reflecting the greater surface area to volume ratios of fruticose (shrubby) lichens.

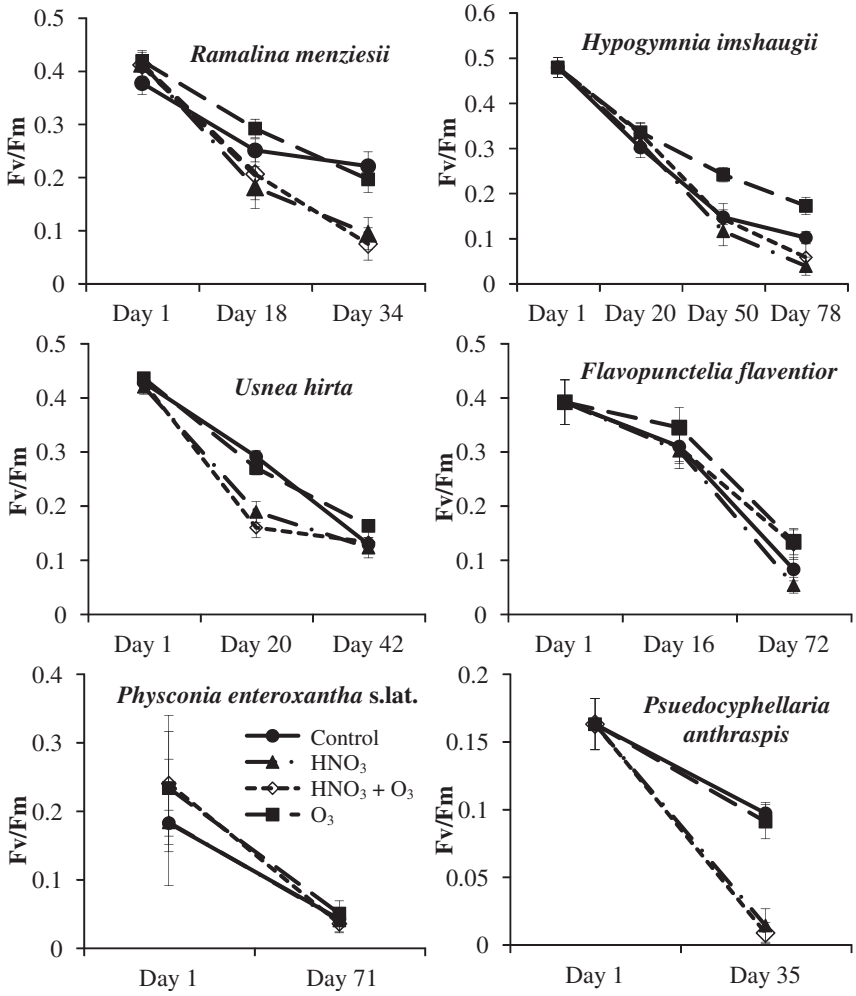

Fig. 4. Potential quantum yield of photosystem II (F_v/F_m) in lichen species fumigated with factorial combinations of HNO_3 and O_3. Error bars represent two standard errors above and below the mean.

4. Discussion

Ozone appeared to have no major negative impact on fumigated thalli, even on species fumigated with 80 ppb, six hours per day, for more than two months. Lichens fumigated with HNO_3 alone appeared to have similar physiological responses to lichens fumigated with HNO_3 and O_3 together, although there were some responses that were greater in the $HNO_3 + O_3$ treatments. The increased damage in some of the $HNO_3 + O_3$ treatment suggest a possible synergistic response to both pollutants. However, because only some responses were different between HNO_3 and $HNO_3 + O_3$ treatments, and those differed among species, it is difficult to define a standard synergistic response. The different rates of decline are likely related to the morphology of the lichen thallus (Johansson et al., 2010), and possibly to secondary compounds produced by the lichen species (Gaio-Oliveira et al., 2005; Hauck and Jurgens, 2008). The net-like structure of *R. menziesii*, and the finely branched structure of *U. hirta* create a large surface area in a complex network that collects nutrients and moisture, including pollutants. *Ramalina menziesii* was the first to show signs of distress in all HNO_3 treatments, becoming brittle and brown after 35 days of treatment. *Usnea hirta* responses to all HNO_3 treatments were similarly rapid, but the physiological decline in all treatments by day 42 indicates that the chamber effect was more important after that period of time than the treatments. *Pseudocyphellaria anthraspis* treatment responses were also rapid, with strong negative correlations to both HNO_3 treatments, although the sample thalli were only tested after 35 days. *Hypogymnia imshaugii* had an intermediate response, showing signs of physiological decline by day 50.

In contrast, *H. imshaugii* transplanted to sites with the highest recorded N throughfall measurements in the Los Angeles basin (Fenn and Bytnerowicz, 1997), as well as historically high O_3 levels (Lee et al., 2003) required as long as four months to show significant physiological decline, even though they had very high levels of NO_3^- collecting on their surfaces (Riddell, unpublished data). There are several possible explanations for the faster response of thalli under fumigation as compared to field transplants, including chamber effects such as drier conditions (Riddell et al., 2008), and the consistency of the pollutant doses in the chambers. The fumigation chambers produce realistic diurnal patterns reflective of ambient pollution loads, and concentrations even vary to some extent with the weather, tending to maintain higher levels of pollution on hotter, drier days. But the variation is modest compared to ambient conditions, which can vary widely based on traffic and weather patterns, even in higher pollution seasons. While we shaded the chambers, and conducted our experiments in the winter months when the greenhouse temperatures were lower, all fumigated lichens, including controls, had significant declines in F_v/F_m and GP with time. This suggests that lichen physiological

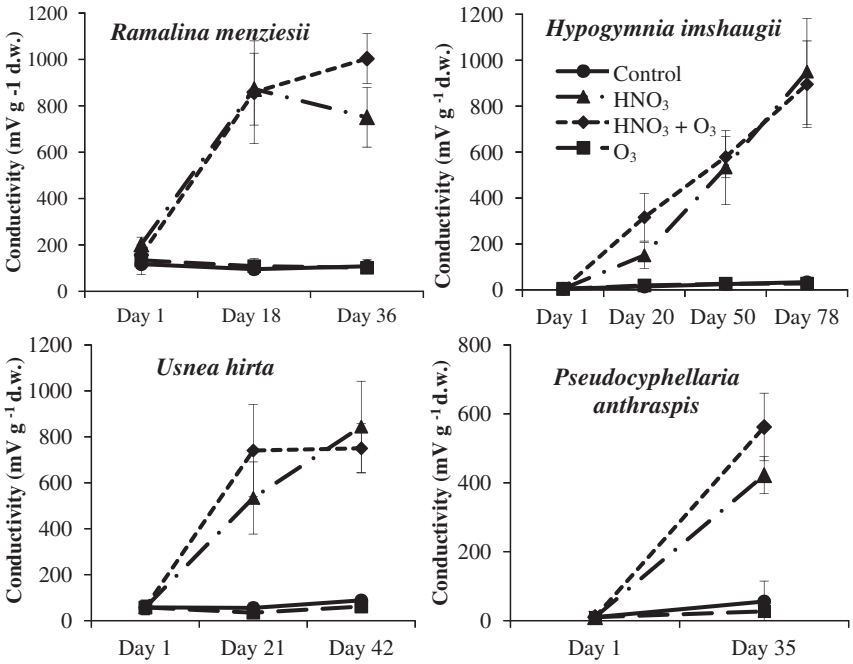

Fig. 5. Conductivity of solutions of lichen material rinsed for one minute, indicating the amount of ions rinsing off of the thallus surface and from membrane damaged cells. For all species, conductivity increased significantly in lichens fumigated with HNO_3 and $HNO_3 \times O_3$ ($p < 0.01$), but not in control or O_3 treatments. Error bars represent two standard errors above and below the mean.

parameters may be affected more quickly in the chambers than under real world conditions.

Our results compare well with our previous HNO_3 (Riddell et al., 2008) and O_3 (Riddell et al., 2010) fumigations. In these experiments, HNO_3 fumigations had dramatic negative effects on *R. menziesii*, whereas O_3 had little to no effect. However, our results in this paper differ from other experiments where differential responses between species to O_3 fumigations (albeit often very high concentrations) were observed. Nash and Sigal (1979) saw photosynthetic declines in *Parmelia sulcata* Tayl. but not *Hypogymnia imshaugii* (referred to as *H. enteromorpha* in publication.) after 3 and 12 h O_3 fumigations at 500 and 800 ppb; Ross and Nash

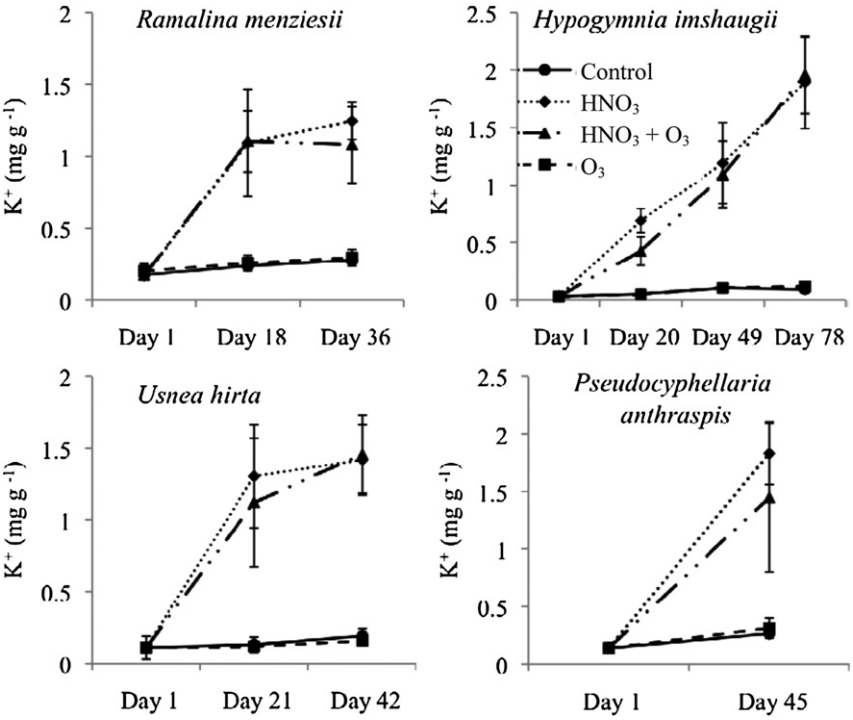

Fig. 6. Potassium leached per gram in one-minute rinse solutions of oven-dried material in lichen thalli fumigated with factorial combinations of HNO_3 and O_3. Error bars represent two standard errors above and below the mean.

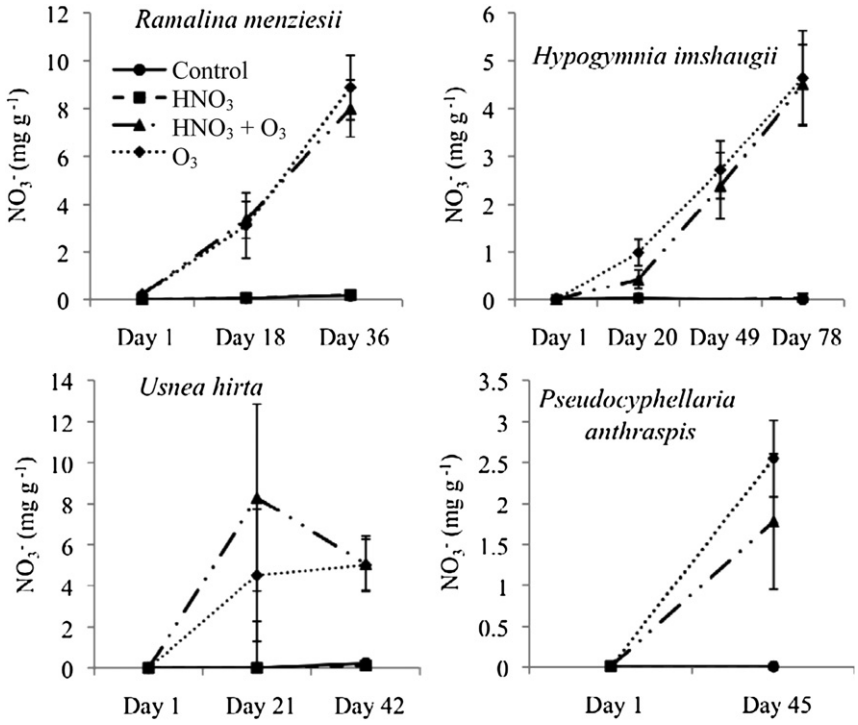

Fig. 7. Nitrate (NO_3^-) rinsed from the surface of fumigated lichen thalli per gram of oven-dried material. These values indicate not only amounts of HNO_3 deposited to fumigated lichen surfaces, but give an idea of how the different surface area to mass ratios influence deposition rates to individual lichen thalli. Error bars represent two standard errors above and below the mean.

(1983) showed that *Psuedoparmelia caperata* (L.) Hale had a negative photosynthetic response to 12 h fumigations at 100–500 ppb O_3, while *Ramalina menziesii* did not; Scheidegger and Schroeter (1995) found significant declines in F_v/F_m in 5 of 7 species fumigated for 80 days at 90 ppb O_3 (daytime) and 40 ppb (night time); Tarhanen et al. (1997) found ultrastructural changes in 3 of 4 species fumigated for 2–4 weeks at 150 and 300 ppb O_3, and, interestingly, a decrease in K^+ leakage. While it is interesting to note that some species had clear responses to O_3, the lack of standardized conditions between the experiments makes it difficult to generalize about O_3 effects on lichens.

The idea that lichen physiological responses, and therefore species distribution, may be more influenced by N pollutants than by O_3 is supported by both physiological and lichen community studies. Gombert et al. (2006), in a comparative study of lichen distribution patterns and tobacco sensitivity, found no correlation between O_3 and lichen species distribution, but a strong correlation with N pollution. Similarly, Bates et al. (1996) found no correlation between O_3 and lichen colonization in factorial fumigation with O_3 and SO_2. There have been many studies linking lichen distribution patterns with NH_3 and NH_4^+ deposition (for example, van Herk, 1999; Jovan and McCune, 2004; Sparrius, 2007). These studies, combined with studies linking NO_x and NO_2 distribution with lichen physiological decline and species distribution (e.g. Davies et al., 2007; Mayer et al., 2009), lead us to conclude that N deposition may be more important than O_3 in affecting lichen species distribution in southern California. The presence of several antioxidant compounds in lichens, and the up-regulation of antioxidant production under high O_3 conditions, (Valencia-Islas et al., 2007) is another indication that at least some lichens may be more resilient to O_3 than N pollutants. The resilience of *Physconia enteroxantha* s.lat. species in our experiments supports the concept that eutrophic lichens readily absorb NO_x species, resulting in increased eutroph abundance in high NO_x pollution (e.g. Davies et al., 2007).

Studies investigating the effects of different N pollutants on lichens have demonstrated differential N uptake capabilities. Some nitrophilic species have low cation exchange capacities, as well as a capacity to incorporate excess N into carbohydrate skeletons (Gaio-Oliveira et al., 2005). It has been suggested that oligotrophic species may absorb excess N because they lack the capacity to down-regulate N absorption, or to utilize the excess absorbed N (Johansson et al., 2010). In our study, the species with high surface area to mass ratios (*R. menziesii* and *U. hirta*) or with photobionts sensitive to excess N in the environment (*P. anthraspis*) responded most rapidly to the fumigations. *Hypogymnia imshaugii*, considered N sensitive by McCune and Geiser (2009), may survive, albeit in degraded condition, in the mountains surrounding the LA basin (Sigal and Nash, 1983) because of a relatively low surface area, a low cation exchange capacity, or other characteristics that we have not considered.

Although the response of *F. flaventior* was very slow in comparison with our expectations, we suspect that the species is sensitive to N pollutants over the long-term. The lack of response on the part of fumigated *P. enteroxantha* s.lat. was expected, as these species are dominant in the areas of N high pollution. These results enhance the use of lichen community surveys to indicate relative air quality changes over large areas. Because NO_x pollutants include HNO_3, it may be that correlations between community composition and NO_x concentrations found by Tretiach et al. (2007) and Gadsdon et al. (2010) are, in part, a response to HNO_3. It is as yet unknown whether HNO_3 affects lichens differently than other N pollutants.

5. Conclusions

This work demonstrates that HNO_3 can be highly toxic to sensitive lichen species through several mechanisms. As doses increased, the physiological health of sensitive lichens declined

through loss of total chlorophyll, chlorophyll degradation, decreased photosynthesis, and respiration, and cellular integrity as indicated by loss of K^+, (and Mg^{++} and Ca^{++}). In comparison, O_3 was relatively non-toxic to fumigated lichens. The strong effects of HNO_3, and equivocal synergistic effects of O_3 combined with HNO_3, support work conducted in other studies that have demonstrated a strong relationship with lichen community composition and N pollution loads. We can define the relative sensitivity of the six species fumigated to relative HNO_3, in order of sensitivity, with the most sensitive listed first, as: *R. menziesii, P. anthraspis* and *U. hirta* > *H. imshaugii* > *F. flaventior* >> *P. enteroxantha* s.lat. The relative responses of each species give us the ability to define the characteristics of N pollution sensitive versus tolerant species.

Acknowledgments

We would like to thank David Jones for assistance with laboratory analysis, Dr. Robert Heath for intellectual guidance, and Dr. Sarah Jovan for manuscript reviews. This research was supported through an Environmental Protection Agency STAR Fellowship, and through a Dissertation Completion Fellowship from Arizona State University's School of Life Sciences.

References

Bates, J.W., McNee, P.J., McLeod, A.R., 1996. Effects of sulphur dioxide and ozone on lichen colonization of conifers in the Liphook forest fumigation project. New Phytologist 132, 653–660.

Bilger, W., Schreiber, U., Bock, M., 1995. Determination of the quantum efficiency of photosystem II, and of nonphotochemical quenching of chlorophyll in fluorescence in the field. Oecologia 102, 425–432.

Cox, P., Delao, A., Komorniczak, A., Weller, R., 2009. The California Almanac of Emissions and Air Quality – 2009 Edition. California Air Resources Board, Sacramento, CA.

Davies, L., Bates, J.W., Bell, J.N.B., James, P.W., Purvis, O.W., 2007. Diversity and sensitivity of epiphytes to oxides of nitrogen in London. Environmental Pollution 146, 299–310.

Fenn, M.E., Bytnerowicz, A., 1997. Summer throughfall and winter deposition in the San Bernardino Mountains in Southern California. Atmospheric Environment 31, 673–683.

Frati, L., Santoni, S., Nicolardi, V., Gaggi, C., Brunialti, G., Guttova, A., Gaudino, S., Pati, A., Pirintsos, S.A., Loppi, S., 2007. Lichen biomonitoring of ammonia emission and nitrogen deposition around a pig stockfarm. Environmental Pollution 146, 311–316.

Gadsdon, S.R., Dagley, J.R., Wolseley, P.A., Power, S.A., 2010. Relationships between lichen community composition and concentrations of NO_2 and NH_3. Environmental Pollution 158, 2553–2560.

Gaio-Oliveira, G., Dahlman, L., Palmqvist, K., Martins-Loucao, M., Maguas, C., 2005. Nitrogen uptake in relation to excess supply and its effects on the lichens *Evernia prunastri* (L.) Ach and *Xanthoria parietina* (L.) Th. Fr. Planta 220, 794–803.

Geiser, L.H., Neitlich, P.N., 2007. Pollution and climate gradients in western Oregon and Washington indicated by epiphytic macrolichens. Environmental Pollution 145, 203–218.

Gombert, S., Asta, J., Seaward, M.R.D., 2006. Lichens and tobacco plants as complementary biomonitors of air pollution in the Grenoble area (Isere, southeast France). Ecological Indicators 6, 429–443.

Hauck, M., Jurgens, S.R., 2008. Usnic acid controls the acidity tolerance of lichens. Environmental Pollution 156, 115–122.

Johansson, O., Nordin, A., Olofsson, J., Palmqvist, K., 2010. Responses of epiphytic lichens to an experimental whole-tree nitrogen-deposition gradient. New Phytologist 188, 1075–1084.

Jovan, S., 2008. Lichen Bioindication of Biodiversity, Air Quality, and Climate: Baseline Results from Monitoring in Washington, Oregon, and California. U.S.D.A. Forest Service, Pacific Northwest Research Station, General Technical Report PNW-GTR-737, 115 pp.

Jovan, S., McCune, B., 2004. Regional variation in epiphytic macrolichen communities in northern and central California forests. Bryologist 107, 328–339.

Jovan, S., McCune, B., 2005. Air-quality bioindication in the greater central valley of California, with epiphytic macrolichen communities. Ecological Applications 15, 1712–1726.

Jovan, S., Riddell, J., Padgett, P., Nash, T.H. Eutrophic lichens respond to multiple forms of N: implications for critical loads and critical loads research. Ecological Applications, in press.

Larsen, R.S., Bell, J.N.B., James, P.W., Chimonides, P.J., Rumsey, F.J., Tremper, A., Purvis, O.W., 2007. Lichen and Bryophyte Distribution on Oak in London in Relation to Air Pollution and Bark Acidity, pp. 332–340.

Larson, D.W., Kershaw, K.A., 1975. Measurement of CO_2 exchange in lichens – new method. Canadian Journal of Botany-Revue Canadienne De Botanique 53, 1535–1541.

Lee, E.H., Tingey, D.T., Hogsett, W.E., Laurence, J.A., 2003. History of tropospheric ozone for the San Bernardino Mountains of Southern California, 1963–1999. Atmospheric Environment 37, 2705–2717.

Lorenzini, G., Landi, U., Loppi, S., Nali, C., 2003. Lichen distribution and bioindicator tobacco plants give discordant response: a case study from Italy. Environmental Monitoring and Assessment 82, 243–264.

Mayer, A.L., Vihermaa, L., Nieminen, N., Luomi, A., Posch, M., 2009. Epiphytic macrolichen community correlates with modeled air pollutants and forest conditions. Ecological Indicators 9, 992–1000.

McCune, B., Geiser, L., 2009. Macrolichens of the Pacific Northwest, second ed. Oregon State University Press, Corvallis, OR.

Nash, T.H., 2008. Lichen sensitivity to air pollution. In: Nash, T.H.I. (Ed.), Lichen Biology, second ed. Cambridge University Press, Cambridge, UK, p. 486.

Nash, T.H., Sigal, L.L., 1979. Gross photosynthetic response of lichens to short term ozone fumigations. Bryologist 82, 280–285.

Padgett, P.E., Bytnerowicz, A., Dawson, P.J., Riechers, G.H., Fitz, D.R., 2004. Design, evaluation and application of a continuously stirred tank reactor system for use in nitric acid air pollutant studies. Water Air and Soil Pollution 151, 35–51.

Pearson, L.C., 1985. Air-pollution damage to cell-membranes in lichens. 1. Development of a simple monitoring test. Atmospheric Environment 19, 209–212.

Ra, H.S.Y., Rubin, L., Crang, R.F.E., 2004. Structural impacts on thallus and algal cell components of two lichen species in response to low-level air pollution in Pacific Northwest forests. Microscopy and Microanalysis 10, 270–279.

Riddell, J., Nash, T.H., Padgett, P., 2008. The effect of HNO_3 gas on the lichen *Ramalina menziesii*. Flora 204, 47–54.

Riddell, J., Padgett, P., Nash, T.H., 2010. Responses of the lichen *Ramalina menziesii* Tayl. to ozone fumigations. Bibliotheca Lichenologica 105, 113–123.

Ronen, R., Galun, M., 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. Environmental and Experimental Botany 24, 239–245.

Ross, L.J., Nash, T.H., 1983. Effect of ozone on gross photosynthesis of lichens. Environmental and Experimental Botany 23, 71–77.

SAS, 2002. SAS Institute Inc. Version 9.1, Cary, NC, USA.

Scheidegger, C., Schroeter, B., 1995. Effects of ozone fumigation on epiphytic macrolichens: ultrastructure, CO_2 gas exchange and chlorophyll fluorescence. Environmental Pollution 88, 345–354.

Sigal, L.L., Nash, T.H., 1983. Lichen communities on conifers in southern California mountains: an ecological survey relative to oxidant air pollution. Ecology 64, 1343–1354.

Sparrius, L.B., 2007. Response of epiphytic lichen communities to decreasing ammonia air concentrations in a moderately polluted area of The Netherlands. Environmental Pollution 146, 375–379.

Steel, R.G.D., Torrie, J.H., 1980. Principles and Procedures of Statistics. A Biometric Approach, second ed. McGraw-Hill, New York.

Tarhanen, S., Holopainen, T., Oksanen, J., 1997. Ultrastructural changes and electrolyte leakage from ozone fumigated epiphytic lichens. Annals of Botany 80, 611–621.

Tretiach, M., Piccotto, M., Baruffo, L., 2007. Effects of ambient NO_x on chlorophyll a fluorescence in transplanted *Flavoparmelia caperata* (Lichen). Environmental Science and Technology 41, 2978–2984.

Valencia-Islas, N., Zambrano, A., Rojas, J.L., 2007. Ozone reactivity and free radical scavenging behavior of phenolic secondary metabolites in lichens exposed to chronic oxidant air pollution from Mexico City. Journal of Chemical Ecology 33, 1619–1634.

van Herk, C.M., 1999. Mapping of ammonia pollution with epiphytic lichens in the Netherlands. The Lichenologist 31, 9–20.