Predictability of the combined effects of sulphur dioxide and nitrate on the green-algal lichen *Ramalina farinacea*

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Abstract: The interactive effects of SO_2 and NO_3^- on the green-algal lichen *Ramalina farinacea* (L.) Ach. were investigated using the degree of chlorophyll pheophytinization and ultrastructural damage to the *Trebouxia* photobiont. Thalli were submitted to several factorial design experiments in which they were exposed to mean SO_2 concentrations ranging from 118 to 140 $\mu\text{mol} \cdot \text{mol}^{-1}$ plus or minus aqueous solutions of NO_3^- at either 50 or 1000 $\mu\text{mol} \cdot \text{L}^{-1}$, for 6–14 days. Interactive effects of SO_2 and NO_3^- were synergistic when the effects of each individual pollutant were slight. However, this pattern reverted to additive effects, and sequentially to antagonistic effects as the detrimental impact of SO_2 alone became more severe. Despite this transition, neutralization of the damaging impact of SO_2 by simultaneous treatment with NO_3^- was not observed. The results show that interactions between SO_2 and NO_3^- were variable, but predictable on the basis of the expected effects following exposure to each pollutant alone. The coincidence of this empirical pattern with those observed in previous studies suggests involvement of nonspecific causes. The wider ecological significance of interactions between SO_2 and NO_3^- is discussed in the light of other findings with respect to the effects of air pollution on lichens in the field.

Key words: air pollution, sulphur dioxide, nitrate, interactive effects, lichens, pigments, ultrastructure.

Résumé : Les auteurs ont examiné les effets interactifs du SO_2 et du NO_3^- sur un lichen à algue verte, le *Ramalina farinacea* (L.) Ach., en utilisant le degré de phaeophytinisation de la chlorophylle et les dommages ultrastructuraux chez le photobionte *Trebouxia*. Ils ont soumis les thalles à plusieurs expériences en disposition factorielle, en les exposant à des teneurs moyennes en SO_2 allant de 118 à 140 $\mu\text{mol} \cdot \text{mol}^{-1}$, avec ou sans solutions aqueuses de NO_3^- à 50 ou 1000 $\mu\text{mol} \cdot \text{L}^{-1}$, pendant 6 à 14 jours. Les effets interactifs du SO_2 et du NO_3^- sont synergiques quand les effets de chaque polluant individuel sont légers. Cependant, ce patron retourne aux effets additifs, et séquentiellement aux effets antagonistes, à mesure que l'effet nocif du SO_2 seul devient plus sévère. En dépit de cette transition, la neutralisation de l'impact destructeur du SO_2 par un traitement simultané avec du NO_3^- ne se présente pas. Les résultats montrent que les interactions entre le SO_2 et le NO_3^- sont variables, mais prévisibles sur la base des effets attendus suite à l'exposition à chaque polluant pris séparément. La coïncidence de ce patron empirique avec ceux observés dans des études précédentes suggère l'implication de causes non-spécifiques. Les auteurs discutent la signification écologique étendue des interactions entre le SO_2 et le NO_3^- à la lumière d'autres constatactions relativement aux effets de la pollution atmosphérique sur les lichens en conditions naturelles.

Mots clés : pollution de l'air, bioxyde de soufre, nitrate, effets interactifs, lichens, pigments, ultrastructure.
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Introduction

Long-range transport of air pollutants has heightened international concern and prompted the development of strategies and methods for reducing emissions and assessing potential impact on natural ecosystems (Bull 1991). Proposed emission-control policies are currently based on present knowledge of

the critical loads or levels above which direct adverse effects on receptors, such as vegetation, may occur (United Nations Economic Commission for Europe 1988). However, as has been demonstrated in studies on vascular plants, the values of toxicity thresholds for each individual pollutant are strongly dependent on the joint action of different chemicals which coexist and interact in polluted atmospheres (Lefohn and Ormrod 1984). Even though there is a wealth of knowledge on the effects of individual gaseous pollutants, few studies relate to the impact of mixtures of pollutants (Bull 1991).

The simultaneous occurrence of pollutants such as sulphur dioxide (SO_2) and nitrogen oxides (NO_2 , NO) which share the same combustion sources is to be expected. Furthermore, in polluted atmospheres, exposure to SO_2 is shown to enhance nitrogen deposition to the surface of vegetation, through reciprocal increases in their solubilities (McLeod

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et al. 1990). This SO_2 effect on nitrogen deposition may be of particular relevance to poikilohydric organisms such as lichens (Bates et al. 1996).

Considering the heterogeneous group of nitrogenous pollutants, current studies on their effect in combination with either SO_2 or SO_4^{2-} on lichens are not only scarce, but have also yielded contrasting results (Hyvärinen et al. 1993). It has been reported that nitrogenous pollutants ameliorate (Kytöviita and Crittenden 1994) or neutralize (Kauppi 1980; Kauppi and Mikkonen 1980; Kauppi and Halonen 1992) the damaging effect of SO_4^{2-} , or even maintain their fertilizing effect in combination with this pollutant (Scott et al. 1989). Such a fertilizing effect is consistent with the fact that NO_3^- and NH_4^+ are probably the main sources of nitrogen for most lichens (Nash 1996). However, it has been also reported that the damaging effect of H_2SO_4 was either not ameliorated by simultaneous NO_3^- addition, or even exacerbated in the presence of NH_4^+ (Hallingbäck and Kellner 1992). Exposure to SO_2 interferes with the metabolic pathway of NO_3^- assimilation and consequently the mixture SO_2 and NO_3^- or NO_2^- may exhibit synergistic deleterious effects on autotrophs (Wellburn et al. 1981). In this context, the role of NO_3^- in green-algal lichens could change dramatically from a nutrient to a phytotoxic agent in the presence of SO_2 (Balaguer and Manrique 1991). Evidence of synergistic effects of SO_2 together with NO_3^- (Balaguer and Manrique 1991) completes a full range of potential interactions between nitrogenous pollutants and SO_2 on lichens.

In view of the lack of a general agreement among studies on interactive effects of SO_2 or SO_4^{2-} and nitrogenous pollutants on lichens, it is relevant to consider that vascular plants also display variable effects ranging from antagonistic to synergistic responses to the same pollutant mixtures (Heagle and Johnston 1979). It has been suggested that the direction and intensity of the interaction depend on the dose and toxicity threshold of each single pollutant (Lefohn and Ormrod 1984). Consequently, lichen response to SO_2 exposure, which has been widely used as a reliable indicator of SO_2 toxicity in field studies, may actually be significantly altered by simultaneous exposure to NO_3^- and, as observed in vascular plants, the direction and extent of this interaction could vary from an amelioration to an enhancement of the damaging effects of SO_2 . In agreement with such a variable pattern of response, Balaguer and Manrique (1995) recently indicated that the action of NO_3^- on SO_2 toxicity on lichens was not explained by a linear model.

In the present study, we argue that even when simultaneous NO_3^- supply can either ameliorate or promote the deleterious effects of SO_2 , the divergence from an additive pattern can be predicted from the impacts produced by exposure to each individual pollutant. The response of *Ramalina farinacea* (L.) Ach. to chronic exposure to SO_2 singly or in combination with NO_3^- during several factorial design experiments was assessed by determining the phaeophytinization quotient of chlorophyll *a* (PQa), a quantitative indicator of chlorophyll degradation (Ronen and Galun 1984; Manrique et al. 1989). The extent to which this estimate of injury was accompanied by changes in photobiont ultrastructure was also examined.

Materials and methods

Samples of *R. farinacea* were collected in natural forests of *Quercus faginea* and *Quercus pyrenaica* in Selas (Guadalajara) and San Pablos de los Montes (Toledo), situated in "clean air" regions in central Spain.

Thalli were maintained in separate chambers in a glasshouse illuminated by natural daylight in Audorra, Teruel, Spain, a region in which *R. farinacea* flourishes. Relative humidity and temperature conditions in the chambers tracked those registered externally, and mean values ranged from 60 to 100% and from 7 to 20°C, respectively (for a more detailed description of ambient conditions see Balaguer and Manrique 1995). During six independent experiments, thalli were exposed in factorial design experiments to two SO_2 treatments (charcoal-filtered air and elevated SO_2 ; SO_2 levels were maintained at values considered to be intermediate between good and poor air quality (125 nmol · mol⁻¹ hourly average concentration; Quality of Urban Air Review Group 1993) 24 h · day⁻¹) and two NO_3^- regimes (spray of distilled water, and NO_3^- enriched; NaNO_3 was supplied to the water spray (final pH = 5.8–6.1) at 50 µmol · L⁻¹ NO_3^- concentration observed in rainfall from polluted areas; United Kingdom Review Group on Acid Rain 1987). In all experiments thalli were wetted once a day, early in the morning. In one of the experiments, in addition to realistic NO_3^- levels, a high- NO_3^- treatment (1000 µmol · L⁻¹) was also included. Those thalli which were daily sprayed with distilled water and maintained in charcoal-filtered air were taken as controls. The length of chronic SO_2 fumigations ranged from 6 to 14 days to assay different doses of pollutant, but treatment duration did not exceed 15 days to avoid a significant decline in control thalli. Concentrations of SO_2 in the centre of each chamber were monitored, logged, and adjusted as described elsewhere (Balaguer and Manrique 1991). Experimental treatments and duration of individual experiments are shown in Table 1.

Chlorophyll phaeophytinization was estimated as previously described (Barnes et al. 1992). Briefly, thallus fragments were washed in carbonate-saturated acetone and extracted with dimethylsulphoxide, and the optical densities of the extracts were measured at 415 and 435 nm. The ratio between these values provides an estimate of the phaeophytinization quotient (PQa), i.e., the ratio between chlorophyll *a* and phaeophytin *a* (Ronen and Galun 1984; Manrique et al. 1989). Pollutant effects were assessed using the percent decrease in PQa with respect to the corresponding mean control values.

Samples selected for the ultrastructural study of the photobiont were fixed, dehydrated, and embedded in Spurr's resin as described by Ascaso et al. (1988), within 2 days after termination of the experimental treatment. Ultrathin sections were stained with lead citrate and observed using a Phillips EM 300 electron microscope.

Data relating to the decline in PQa were subjected to a two-way analysis of variance (ANOVA) to determine the significance of the main factors (SO_2 and NO_3^-) and their interaction. Assumptions of normality and homoscedasticity were tested using the Kolmogorov–Smirnov test and Cochran's C test, respectively. Where necessary, data were logarithm transformed prior to ANOVA. Pairwise comparisons were performed using the least significant difference (LSD) calculated at $p = 0.05$.

Reciprocal effects of NO_3^- and SO_2 were summarized in a response-surface model, as previously proposed for the interaction between SO_2 and HF (McCune 1984). In the present study, a nonadditive effect was defined as the divergence from an additive pattern and was calculated by subtracting the effects observed following single exposures from the effect produced by both pollutants applied in combination. A response-surface model was generated by plotting the nonadditive effect of the pollutant mixture as a function of the decrease in PQa resulting from single exposure to SO_2 or

Fig. 1. Electron micrographs of *Trebouxia* from *Ramalina farinacea*. (A) Photobiont cell submitted to control treatment for 14 days. (B) Close occurrence of algal cells displaying contrasting degrees of injury after fumigation with $122 \text{ nmol} \cdot \text{mol}^{-1} \text{ SO}_2$ and daily spray of distilled water. (C, D) Photobiont cell showing moderate symptoms of SO_2 -induced damage with severely affected mitochondria (arrowheads; $122 \text{ nmol} \cdot \text{mol}^{-1} \text{ SO}_2$ and daily spray of distilled water). (E) Plasmolyzed cells in thalli exposed to $122 \text{ nmol} \cdot \text{mol}^{-1} \text{ SO}_2$ and daily spray of $50 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ aqueous NO_3^- . (F) Appearance of electron-opaque bodies (arrowheads) after treatment with $50 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ aqueous NO_3^- for 14 days in charcoal-filtered air. Scale bars = $1 \text{ } \mu\text{m}$.

Table 1. Effect of single and simultaneous exposure to SO_2 and NO_3^- on chlorophyll phaeophytinization in thalli of *Ramalina farinacea* (L.) Ach.

Pollutant concentration		Exposure duration (days)	Percentage of PQa decrease ^a			Nonadditive effect ^b
SO_2 ($\text{nmol} \cdot \text{mol}^{-1}$)	NO_3^- ($\mu\text{mol} \cdot \text{L}^{-1}$)		SO_2	NO_3^-	$\text{SO}_2 + \text{NO}_3^-$	
122	50	14	3.4	-6.0	17.9*	20.5 [†]
141	50	6	6.3	-1.6	3.9	-0.8
140	50	10	18.2*	1.4	14.7*	-4.9
118	50	7	17.3*	3.9	23.6*	2.4
124	50	14	40.4*	-7.9	21.1*	-11.4 [†]
124	1000	14	40.4*	-4.4	30.7*	-5.3

^aValues represent the mean quotient for 5–10 independent measurements of separate thalli. Asterisks indicate a significant difference from the controls (one-way ANOVA followed by LSD, $p < 0.05$).

^bValues were obtained by subtracting the addition of PQa decreases caused by single exposures from those observed following simultaneous exposure to both pollutants. More or less than additive effects correspond to positive or negative values, respectively. Daggers indicate a significant interaction between SO_2 and NO_3^- (either synergism or antagonism) according to a two-way ANOVA ($p < 0.05$).

NO_3^- , considered as independent variables. This response surface was interpolated and then depicted as a contour map using the mapping software package Surfer (Golden Software Inc. 1990).

Results

Control thalli, wetted with distilled water and maintained in charcoal-filtered air, displayed a decline of mean PQa values over the period of the experiments which was always less than 15% and not statistically significant.

Exposure to elevated SO_2 always resulted in mean values of PQa lower than controls (Table 1), and effects on PQa were paralleled by severe alterations in normal photobiont ultrastructure (Fig. 1A). Early stages of damage (PQa decrease from control values $< 5\%$) were characterized by the close occurrence of algal cells with contrasting degrees of injury (Fig. 1B). In initial stages of damage, swollen and everted thylakoids resulted in wider interthylakoidal spaces and led to a granulated appearance of the stroma in the absence of any apparent change in the shape of the chloroplasts (Fig. 1C). At this stage, mitochondria presented severe symptoms of injury such as degeneration of cristae and mitochondrial matrix as well as the appearance of concentric, myelin-like figures (Fig. 1D).

When the percent PQa decrease was greater than 17% most of the photobiont cells appeared plasmolyzed. There was a complete loss of organelle integrity, and photobiont cells appeared to consist of amorphous matrices in which only lipid bodies and starch granules were recognizable (Fig. 1E).

Daily supply of NO_3^- did not produce any significant variation in PQa at any of the tested doses (Table 1). Consistent with the lack of change in PQa, no qualitative changes were observed in the ultrastructure of the algal cells (Fig. 1F).

However, electron-opaque bodies occasionally appeared in the cytosol of thalli treated with NO_3^- applied singly or in combination with SO_2 (Fig. 1F).

Simultaneous treatment with NO_3^- and SO_2 resulted in a variable response which ranged from more than additive to less than additive effects. Nonadditive effects of the mixture varied from approximately +20%, a significant synergism ($p < 0.05$), to approximately -10%, a significant antagonism ($p < 0.05$; Table 1). These values were more highly correlated with the degree of injury caused by SO_2 singly than to the pollutant average concentration or the dose ($\log(\text{SO}_2 \text{ effect})$, $r = -0.85$, $p < 0.03$; $\log(\text{SO}_2 \text{ concentration})$, $r = -0.28$, $p < 0.59$; $\log(\text{SO}_2 \text{ dose})$, $r = -0.02$, $p < 0.98$). Nonadditive effects were not significantly correlated with the concentration, dose, or effect on PQa of NO_3^- individually ($p > 0.60$).

A response surface was interpolated to the distances from an additive pattern using the percent PQa decrease induced by exposure to each pollutant alone as independent variables in an effort to search for a spatial pattern in the variation of the interaction between both pollutants (Fig. 2). This response-surface model revealed a region where NO_3^- levels that would have produced a slight PQa increase if supplied alone paradoxically promoted the damaging effect of slightly toxic SO_2 levels. Conversely, antagonistic or less than additive effects were observed when injury was severe following single exposure to SO_2 . It is relevant that despite this ameliorating effect of NO_3^- in the mixture it was never sufficient to neutralize the damaging effect of SO_2 at any of the doses of pollutants tested. Nonadditive effects followed a clear-cut continuous gradient from synergism to antagonism, with a boundary line roughly positioned between 13 and 21% PQa decrease after exposure to SO_2 singly

Fig. 2. Divergence from an additive pattern is shown with reference to the effect of exposure to SO_2 on the apparent effect of NO_3^- , measured in both cases as percent PQa decrease from control values. In this response surface, regions of exposure in which joint action of both pollutants resulted in greater than additive, additive, or less than additive effects are denoted by positive, zero, or negative values, respectively. Each data point (■) represents the mean for 5–10 independent measurements of separate thalli per treatment.

(Fig. 2). Ultrastructural symptoms produced by simultaneous exposure to both pollutants did not differ qualitatively from those described above, induced by single treatments.

Discussion

The close relationship observed in the present study between qualitative changes in cell ultrastructure of the photobiont and chlorophyll degradation supports the use of variations in PQa as a quantitative estimation of SO_2 -induced damage. At a slight degree of chlorophyll phaeophytinization, algal cells already displayed marked ultrastructural changes, which were particularly evident in the case of mitochondria. The sequence of symptoms of ultrastructural injury following exposure to SO_2 or NO_3^- was consistent with that previously described by Holopainen (1986).

The results of the present investigation indicate that the mere knowledge that NO_3^- deposition and SO_2 coexist in polluted areas is not sufficient to predict the pattern of their interactive effect (i.e., synergistic, antagonistic, or additive). The response of *R. farinacea* after simultaneous exposure to mixtures of NO_3^- and SO_2 ranged from synergistic to antagonistic. The variation in the pattern of response has been described as a variable response in previous studies on the interaction of SO_2 with other pollutants (Heagle and Johnston 1979). However, the present study indicates that response does not vary erratically. Its divergence from additive effects is related to the degree of injury that SO_2 would

have caused if supplied alone, in the absence of any other pollutant. Thus, when slight damage was produced by single exposure to SO_2 the corresponding effect of the same dose of this pollutant in combination with added NO_3^- resulted in greater than additive effects. On the other hand, antagonistic interactions were observed when NO_3^- was supplied with SO_2 doses that, alone, induced severe degrees of injury.

In contrast to what has been hypothesized elsewhere (Kauppi 1980), slight SO_2 effects were not nullified but enhanced by simultaneous exposure to NO_3^- , and they were ameliorated but not neutralized only when severe effects induced by SO_2 were expected.

The observed response pattern is consistent with the general model proposed by Lefohn and Ormrod (1984) for interactive effects of SO_2 , NO_2 , and O_3 on crops. These authors indicated that synergistic effects of gas mixtures are observed when pollutant doses are below or at the threshold for individual injury responses. As doses increase, damage may be additive, and finally, at relatively high doses antagonistic effects develop. The simplest and most general model to summarize and interpret this variable response is a two-dimensional response surface, previously proposed by McCune (1984) for the joint action of SO_2 and HF on vascular plants. It is striking that the response surface which depicted interactions between SO_2 and HF (McCune 1984) broadly overlaps with that interpolated in the present study for the response of *R. farinacea* to the combination of SO_2 and NO_3^- (Fig. 2).

In contrast to the model described by Lefohn and Ormrod (1984), the response-surface model described in the present study considers the effects induced by each pollutant individually as independent variables, instead of pollutant doses. Although a highly significant relationship between SO_2 dose and effect on PQa has been previously observed (Balaguer and Manrique 1995), the variation in the dependent variable not explained by the SO_2 dose annuls its suitability for describing the divergence of the lichen response from an additive effect of the pollutant mixture. Even when environmental conditions in the chamber or thallus status would partly explain large differences in the lichen response to similar treatments (Balaguer and Manrique 1995), direct effects of these variables on the direction of the pollutant interaction seem unlikely to occur considering the high correlation between the effect induced by SO_2 alone and the nonadditive effect of the mixture.

The results of the present study suggest that, although lichen responses to pollutant mixtures may differ from those caused by each pollutant individually, interactive effects can be estimated from the damaging effect of each pollutant alone. Thus, the values predicted by models based on effects on lichens caused by exposure to either SO_2 alone (Grace et al. 1985) or NO_3^- alone (Kauppi 1980; Blum et al. 1989) would be suitable inputs for a response surface designed to estimate the expected interactive effects of simultaneous exposure to both pollutants. From this alternative point of view, apparently contradictory observations such as the potential role of nitrogen-rich pollution as mitigator (Scott et al. 1989; Kauppi and Halonen 1992; Kytöviita and Crittenden 1994) versus enhancer (Balaguer and Manrique 1991) of the damaging effect of SO_2 could be interpreted as extreme phenomena of the same underlying pattern of interaction.

Fig. 3. Nonadditive effect of a varied set of pollutant mixtures was significantly correlated ($r = -0.72$, $p < 0.00001$) with the effect induced by the most damaging pollutant of the mixture when applied alone, regardless of the species of autotroph studied or the physiological indicator measured. The effect of the most damaging pollutant alone was calculated as percent reduction from mean control values. In addition to the data of the present study (■), the figure shows data derived from previous studies on the interactive effects of SO_2 and NO_2 on growth of several grasses (×; Ashenden and Mansfield 1978); SO_2 and O_3 on either photosynthetic rates of green-algal lichens (□; Eversman and Sigal 1987) or foliar injury in soybean plants (▽; Heagle and Johnston 1979); H_2SO_4 and HNO_3 on nitrogenase activity of lichenized cyanobacteria (◇; Kytöviita and Crittenden 1994); heavy metals on nitrate uptake of free-living cyanobacteria (○; Rai and Raizada 1989); and SO_2 and NO_2 or O_3 on photosystem performance in chloroplasts of spinach (△; Sugahara 1984).

As mentioned earlier, the variable response of *R. farinacea* to SO_2 and NO_3^- observed in the present study displays the same pattern as those previously described for the interactive effects of SO_2 , NO_2 , and O_3 (Heagle and Johnston 1979; Lefohn and Orinrod 1984) and SO_2 and HF (McCune 1984) on vascular plants. It has been proposed that McCune's response-surface model may be applicable not only for predicting interactions among those pollutants but also for SO_2 and NO_3^- or O_3 and heavy metals, and even that all such interactions are chemically interrelated (Runeckles 1984). Using heterogeneous data which relate to the interactive effects of binary combinations of SO_2 , H_2SO_4 , HNO_3 , NO_3^- , NO_2 , O_3 , Ni, Cr, and Pb on diverse physiological processes, ranging from nitrogenase activity to visible foliar

injury in a wide range of autotrophs, from cyanobacteria to angiosperms and including lichens containing either cyanobacteria or green algae, it is conspicuous that nonadditive effects are highly correlated ($r = -0.72$, $p < 0.00001$) with the individual impact of the most damaging pollutant in the mixture (Fig. 3).

Although the mechanisms are still not known, such a generalized response depicts an empirical pattern which suggests the participation of unspecific common causes, such as the convergence of these interactions in the production of free radicals. These highly reactive molecular species arise as a secondary consequence of exposure to oxidative stresses (Alscher and Amthor 1987; Wellburn 1990), and their involvement in toxicity mechanisms of SO_2 has also been reported in lichens (Modenesi 1993; Richardson and Nieboer 1983).

The results of the present study could have several implications in the interpretation of data derived from field studies. First the fact that lichen-impoverished zones have been reported to exceed the area where levels of SO_2 are above the toxicity threshold (Nash and Sigal 1981) might be associated with the greater than additive effect expected when the most damaging component is below or at its threshold of toxicity in a mixture of pollutants. Synergistic interactions could also explain the more damaging effect of NO_2 observed on lichens in field studies than that following controlled fumigations (Blum et al. 1989). A complex pattern of interaction, such as that displayed by *R. farinacea* in response to SO_2 and NO_3^- in the present study, could explain the decline of *Ramalina menziesii* in areas where SO_2 levels are low and NO_3^- dry deposition is important but does not reach a potential NO_3^- toxicity threshold (Boonpragob and Nash 1991). This pattern of interaction would support the involvement of codeposition of nitrogenous pollutants and SO_2 in triggering lichen responses at annual mean concentrations of SO_2 below the theoretical toxicity threshold of the pollutant (Bates et al. 1996). Finally, the response-surface model proposed in the present study would elucidate, to some extent, the causes of impeded lichen recolonization (Bates et al. 1990; McCune 1993) in a changing air pollution scenario where atmospheric SO_2 concentrations are declining in parallel with increasing levels of nitrogen oxides (Bates et al. 1990; Slovik et al. 1996).

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