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Nickel Exposure Enhances the Susceptibility of Lichens *Usnea amblyoclada* and *Ramalina celastri* to Urban Atmospheric Pollutants

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Abstract. In the present study, the effect of nickel incorporation on the chemical response of *Ramalina celastri* (Spreng.) Krog & Swinsc. and *Usnea amblyoclada* (Müll. Arg.) Zahlbr. exposed to urban atmospheric pollutants was assessed. Lichen thalli were treated with Ni solutions and then transplanted to two areas of the city of Córdoba with different levels of pollution. After exposure, the concentration of chlorophyll a, chlorophyll b, phaeophytin a, hydroperoxy conjugated dienes, malondialdehyde, sulphur content, electric conductivity of the immersion water, and nickel content were measured. Also, a Pollution Index was calculated for each lichen species. The exposure to Ni altered the physiological response to atmospheric pollutants in both lichen species, making them more sensitive to any damage caused by them. When the species were compared, it was observed that *R. celastri* accumulated more sulphur than *U. amblyoclada*.

Air pollution is a serious problem in many densely populated and industrialized areas of the world. Terrestrial environments polluted with mine waste from smelters or urban environments commonly have high concentrations of heavy metals in association with other elements such as gaseous SO_2 and acidity (Seaward and Richardson 1990; Purvis and Halls 1996). In particular, heavy metals have been the object of many studies because they are persistent and belong to the most widely dispersed industrial pollution (Wriaga and Palyna 1998).

Several studies have emphasized that lichens are effective air quality biomonitors in urban–industrial environments (Carreras and Pignata 2002; González et al. 2003; González and Pignata 1997, 2000). Thus, the use of transplanted lichens allows study of the response of living organisms exposed to different atmospheric conditions including pollutants such as heavy metals, SO_2, fluorides, ozone, nitrogen oxides, and peroxyacetyl nitrate (Zambrano and Nash 2000; Garty et al. 2003; González et al. 2003). Lichens are among the most widely used biomonitors in the terrestrial environment because they have ideal characteristics, as, for example, the presence of special organs for nutrient uptake instead of roots, they are perennial, the absence of cuticle on their thalli, and their worldwide distribution (Nimis and Purvis 2002). Although all lichens have the capacity to fix heavy metals on their surface, the levels of accumulation vary according to species, morphology, and structural characteristics as well as environmental conditions (Garty 2001).

Nickel is emitted from several anthropogenic sources such as combustion, high temperature metallurgical operations, and nickel primary production operations (European Commission 2000). In urban environments, Ni is derived not only from industrial activity but also from vehicular sources (Garty 1993). Metallic nickel has many economically important uses; it is used in alloys, including stainless steel, nickel–cadmium batteries, and nickel plating (IARC 1976, 1990; NIOSH 1977). Occupational exposure of humans to mixtures of insoluble and soluble nickel compounds is correlated with an increased incidence of lung, sinus, and pharyngeal tumors (Landolph et al. 1996; Clemens and Landolph 2003). In mouse fibroblasts, nickel compounds bind to DNA as well as to DNA-binding proteins (Kasprzak et al. 1986). Hence, nickel ions bound to DNA polymerases which then bind to DNA probably cause an alteration in the DNA structure at the replication fork, facilitating gene amplification. Thus, Ni compound–induced transformed cell lines carry a carcinogen-induced primary molecular lesion, one of eight primary events that occur to give rise to a tumorous cell (Clemens et al. 2005).

Garty (2000) points out that nickel emitted by stationary or mobile sources is accumulated in lichen thalli producing different physiological damages such as damage to cell membranes (Eskblad et al. 1998; Hyvärinen et al. 2000) and alterations in urease synthesis (Pérez Urria et al. 1986). Also, a variety of biological parameters have been used to assess pollution damage to transplanted lichens, including the content and degradation of chlorophyll (González et al. 1996; Garty et al. 2000, 2001), the increase of membrane leakage (Garty et al. 2003), the peroxidation of membrane lipids (González and Pignata 1994; Egger et al. 1994) and the accumulation of various air pollutants as heavy metals and sulphur (Showman and Long 1992; González et al. 2003).

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Although there is an extensive history concerning the accumulation of heavy metals in lichens (Garty 1993), there is not enough information on the effect of these elements on some chemical responses of thalli transplanted to urban areas, as for *Ramalina celastri* and *Usnea amblyoclada*, species frequently employed as biomonitors of atmospheric pollution in urban and industrial environments. Therefore, the aims of the present study are i) to evaluate the influence of nickel incorporation on the chemical response of *Usnea amblyoclada* and *Ramalina celastri* thalli transplanted to an urban atmosphere, and ii) to compare the response patterns to urban pollutants of both species.

Materials and Methods

Study Area

Córdoba City is located in the center of the Argentine Republic at 31°24′S, 64°11′W, 440 m above sea level. The climate is subhumid, with an average annual rainfall of 790 mm, concentrated mainly in summer, with a mean annual temperature of 17.4°C and prevailing winds from the NE, S, and SE. No precipitation was registered during the transplantation period, and the mean monthly temperature was 14°C with prevailing winds from the NE (20%), S (13%), and calm (30%).

Two different zones were selected for transplanting i) a nonpolluted zone (control zone), with hardly any traffic, no surrounding industries, and abundant natural and exotic vegetation; and ii) a polluted zone (urban zone) located downtown, a densely populated area with heavy traffic, high buildings limiting circulation of wind, and scarce vegetation.

Biological Material and Collection Procedures

Thalli of the fructicose lichens *Usnea amblyoclada* (Müll. Arg.) Zahlbr. and *Ramalina celastri* (Spreng.) Krog & Swinsc. were collected from Los Gigantes, 70 km west of Córdoba city, an area considered to be ''clean,'' with a very low level of pollution and far from emission sources. Thalli of *U. amblyoclada*, found on rocky substrates, were collected in Los Gigantes, west of the city of Córdoba, whereas *R. celastri* found on bark substrates was collected in La Calera, to the northwest of Córdoba city. The basal parts of the lichen thalli were detached with the adhering pieces of substrate and stored in the laboratory at room temperature in the dark for 48 h until treatment.

Treatment of Lichens and Sample Preparation

Lichen thalli were immersed and incubated for 30 min at room temperature in one of the following salt solutions: 10 mM, 5 mM, and 0.5 mM $NiSO_4.6H_2O$. Previous studies showed that the incorporation of heavy metals can be affected by the acidity of the environment (Kauppi et al. 1998) so the acidity of every solution was adjusted to pH 3.5 with H_2SO_4 to guarantee incorporation and simulate an extreme condition. As there is no data on the levels of Ni in the city of Córdoba, the concentrations of Ni corresponded to the environmental concentrations in other urban or industrial areas (McKinney 1993).

After treatment with $NiSO_4.6H_2O$, the thalli were rinsed with ultrapure water and wiped gently to remove excess moisture. Lichen-bags (20 cm × 20 cm) were prepared with 6.0 g fresh material loosely packed in a fine nylon net (1 mm × 1.5 mm) so that each bag included several thalli. Bags with thalli immersed only in ultrapure water and bags with thalli without any previous treatment were prepared as treatment controls.

To obtain baseline levels, part of the freshly picked material was submitted to the same chemical analysis carried out in the transplanted material.

Lichen Transplantation

In August 2001, lichen-bags were transplanted to the polluted and the nonpolluted areas in Córdoba city. The exposure season was chosen considering that in Córdoba city the most severe pollution problem occurs in winter, a season with thermic inversions that cause pollutant accumulation in a layer close to the surface (Olcese and Toselli 1998).

At each sampling zone, three lichen bags per treatment were tied with a nylon rope on different posts 3 m above the ground, a method employed in previous studies (González and Pignata 1994; González et al. 2003; Carreras et al. 2005; Carreras and Pignata 2002). Four weeks later, at the end of the exposure period, part of the lichen material was separated to measure its electric conductivity and nickel content. The rest of the material was homogenized in a mortar with a pestle and freeze-dried in polythene bags. From each lichen bag, three subsamples of the homogenized material were taken to obtain a mean arithmetic value ± standard deviation for each chemical determination.

Chemical Determinations

All determinations were expressed on a dry-weight basis.

Chlorophylls. One hundred milligrams of lichen material was homogenized in 10 ml of ETOH at 96% v/v with an Ultra Turrax homogenizer, T18. 1KA Works, Inc. USA. Subsequently, the supernatant was separated. Afterwards, HCl 0.06 M was added to the clear chlorophyll extract (1 ml HCl and 5 ml chlorophyll extract) in order to produce phaeophytin formation. Absorption of chlorophylls and phaeophytins, and phaeophytins alone (after addition of HCl) was measured with a spectrophotometer Beckman DU 7000, USA. Concentrations of chlorophylls and phaeophytins were calculated on a dry weight basis (Wintermans and De Mots 1965). The ratios chlorophyll b/chlorophyll a (Chl b/Chl a) and phaeophytin a/chlorophyll a (Phaeoph a/Chl a) were also calculated. (González et al. 1996; Carreras et al. 1998).

Sulphur content. Five milliliters of a Mg $(NO_3)_2$-saturated solution was added to 0.5 g of freeze-dried lichen and dried in an electric heater. Subsequently, the sample was heated in an oven for 30 min at 500°C. The ashes were then suspended in 10 ml of 6 M HCl, filtered, and the resulting solution boiled for 3 min. The solution was brought to 50 ml with distilled water. The amount of SO_4^{2-} in the solution was determined by turbidimetric method with barium chloride (González and Pignata 1994). Results were expressed in mg g^{-1} dry wt.

Peroxidation product. Fifty milligrams of freeze-dried lichen was homogenized in 2.5 ml of distilled H_2O. An equal volume of 0.5% TBA (2-thiobarbituric acid) in 20% trichloroacetic acid solution was added and samples were incubated at 95°C for 30 min. The reaction was stopped by placing the experimental tubes in an ice bath. Samples were then centrifuged at 10,000*g* for 30 min. The supernatant was

removed and read at 532 nm, and the value for nonspecific absorption at 600 nm was read and subtracted from this. The amount of MDA present was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} (Kwon et al. 1965). Results were expressed as μmol g^{-1} dry wt.

Hydroperoxy conjugated dienes (HPCD) were extracted by homogenization of the lichen material (50 mg) in 96% v/v ethanol at a ratio 1:50 FW/v with an Ultra Turrax homogenizer. The absorption was measured at 234 nm in the supernatant and its concentration was calculated by means of the extinction coefficient of 2.65×10^4 M^{-1} cm^{-1} (Boveris et al. 1980). Results were expressed in μmol g^{-1} dry wt.

Electrical conductivity. The electrical conductivity (EC) parameter is a relative measure of membrane integrity (Garty et al. 2002). Conductivity measurements were performed in lichen thalli that had been previously incubated in a humidity chamber (80%) for 2 h. After this, whole thalli were divided into samples of 2 g and immersed in 50 ml ultrapure water for 2 h at room temperature. The EC of the water was measured by an EC-meter with a glass electrode (Oakton WD-35610) and expressed as $\mu S.m^{-1}$ mLg^{-1}.

Nickel

An accurately weighed portion of each sample (0.5 g dry weight) was reduced to ashes in an oven at 500°C for 60 min. The ashes were then digested using a 5:1 mixture of HCl (18%) and HNO_3 (65%) and heated until boiling. The solid residue was separated by centrifugation and the sample was diluted to a final volume of 50 ml with ultrapure water. Nickel content was analyzed with a Buck 210-VGP atomic absorption spectrophotometer, using the air/acetylene flame. Nickel concentration was calculated on a dry-weight basis. In order to check the accuracy of the analytical method, certified reference material (IAEA/V-10 Hay Powder) was measured every 10 samples.

Pollution Index

The pollution index (PI) for *R. celastri* was determined using the equation cited by González et al. (1996). For *U. amblyoclada* the PI was determined according to Carreras et al. (1998). Both indexes have previously been statistically checked and used in biomonitoring studies with the same species (González and Pignata 1999; Carreras et al. 1998; Carreras and Pignata 2001; González et al. 2003).

$$PI_{R.celastri} = [(Phaeoph - a/Chl - a) + (S_T/S_F)][(MDA_T/MDA_F) + (HPCD_T/HPCD_F)]$$

$$PI_{U.amblyoclada} = [(Chl - b/Chl - a) + (S_T/S_F)](HPCD_T/HPCD_F)$$

The subindex T belongs to the determinations about transplanted samples and the subindex F, to the ones about freshly picked material (baseline).

Statistical Analysis

Results are expressed as the mean value ± standard deviation of three independent determinations for each of the three sampling sites in each experimental zone. Physiological parameters and metal concentrations of each species were submitted to an analysis of variance (one-way analysis of variance [ANOVA] using as factor variation: the transplantation zone; the different Ni treatments). Whenever the ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means was undertaken using the Least Significant Difference test. The ANOVA assumptions were previously verified graphically (residual vs. fitted values, box plots, and steam leaf plots).

A Principal Component Analysis (PCA) was performed using species and area as classification criteria in order to assess the relationship between the area of transplantation and the treatment (with/without the highest Ni concentration) and the chemical response of each species studied.

Results and Discussion

Before analyzing the effect of Ni treatment by ANOVA, we assessed the effect of transplantation (comparing the mean values of unexposed and untreated baseline thalli with those of untreated thalli transplanted to the nonpolluted zone); immersion (comparing samples immersed in ultrapure water with nonimmersed samples, from each sampling area); and the effect of the SO_4^{2-} anion on Ni incorporation (comparing samples immersed in different H_2SO_4 solutions with samples immersed in ultrapure water, from each transplantation area). However, no significant differences were observed in any of the cases (data not shown), indicating the absence of these effects.

Effect of Ni on the Physiological Response of R. celastri

Table 1 shows the mean values of the parameters measured in *R. celastri* and the ANOVA results. In order to assess chlorophyll degradation (phaeophytin a/chlorophyll a ratio), the content of photosynthetic pigments in the lichen species was determined. This index has previously been used as a damage indicator for *R. celastri* (González et al. 1996; González and Pignata 1999). Although the highest values of Phaeo-a/Chl-a ratio were observed in samples transplanted to the polluted area, these differences were not significant among treatments nor between transplantation zones, suggesting that this ratio is not a good indicator of damage for the exposure period studied.

Regarding peroxidation products HPCD and MDA, significant differences were observed only for HPCD values. Treatment with different Ni solutions had a significant effect on the level of HPCD in samples retrieved from both the polluted and nonpolluted zone. When the sampling zones were compared, HPDC content was greatest in the polluted zone in samples treated with the highest nickel concentration. HPCD appeared to be a better estimator of damage, because no significant changes were detected in the MDA content. This increase related to urban pollutants has previously been observed for these species (Carreras and Pignata 2001; González et al. 2003). Moreover, the changes observed in HPCD content indicate that the effects of pollutants on the lichen membranes are even worse in the presence of Ni, because the highest values of this compound were found in thalli retrieved from the polluted zone and treated with Ni solution. In agreement with these observations, Hyvärinen et al. (2000) found

Table 1. Mean values of the concentration ± standard deviation and results of the analysis of variance (ANOVA) of the chemical parameters of *Ramailina celastri* transplanted to a polluted and nonpolluted area and submitted to immersion in different Ni solutions or purified water (controls)

	Mean ± SD				
	Control	Ni 0.5 mM	Ni 5 mM	Ni 10 mM	ANOVA[a]
Phaeo-a/Chl-a					
Non polluted	1.270 ± 0.183	1.328 ± 0.075	1.164 ± 0.107	1.152 ± 0.111	ns
Polluted	1.477 ± 0.193	1.559 ± 0.151	1.293 ± 0.056	1.350 ± 0.169	ns
ANOVA[b]	ns	ns	ns	ns	
HPCD μmol g^{-1} DW					
Non polluted	54.111 ± 0.027 b	69.224 ± 0.051 a	56.174 ± 0.087 b	67.465 ± 0.011 a	*
Polluted	58.600 ± 0.028 c	62.933 ± 0.019 bc	63.474 ± 0.038 b	76.150 ± 0.003 a	*
ANOVA[b]	ns	ns	ns	***	
MDA μmol g^{-1} DW					
Non polluted	0.109 ± 0.007	0.101 ± 0.018	0.112 ± 0.024	0.113 ± 0.006	ns
Polluted	0.104 ± 0.027	0.111 ± 0.002	0.109 ± 0.005	0.107 ± 0.014	ns
ANOVA[b]	ns	ns	ns	ns	
Sulphur mg g^{-1} DW					
Non polluted	1.279 ± 0.057 d	1.359 ± 0.039 c	1.500 ± 0.057 b	1.698 ± 0.075 a	*
Polluted	1.466 ± 0.096 b	1.572 ± 0.071 b	1.619 ± 0.083 b	2.264 ± 0.105 a	*
ANOVA[b]	***	**	*	***	
Nickel mg g^{-1} DW					
Non polluted	3.871 ± 0.328 c	12.354 ± 0.413 b	12.148 ± 0.263 b	23.307 ± 0.484 a	*
Polluted	6.261 ± 0.271 d	11.893 ± 0.344 c	31.048 ± 0.389 b	46.018 ± 0.325 a	*
ANOVA[b]	***	ns	***	***	
EC μS.m^{-1}					
Non polluted	2.500 ± 0.194 b	2.338 ± 0.065 b	2.852 ± 0.115 a	2.887 ± 0.172 a	*
Polluted	2.914 ± 0.251 c	5.796 ± 0.244 b	6.497 ± 0.137 b	7.678 ± 0.153 a	*
ANOVA[b]	*	***	***	***	
PI					
Non polluted	3.989 ± 0.075 c	8.097 ± 0.527 b	7.512 ± 0.505 b	9.200 ± 0.558 a	*
Polluted	5.165 ± 0.238 d	9.203 ± 0.867 b	8.174 ± 0.390 c	11.057 ± 0.679 a	*
ANOVA[b]	**	*	*	*	

Note: Values on each horizontal line followed by the same letter do not differ significantly ($p = 0.05$). *ns* not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

[a]ANOVA between treatments.

[b]ANOVA between areas.

indications of prejudicial effects on membrane integrity in thalli treated with high Ni concentrations.

The concentration of sulphur was significantly different among treatments and in the nonpolluted zone it increased as the Ni content of the solutions was greater. Sulphur content was significantly higher in all the samples transplanted to the polluted zone. The fact that sulphur accumulation was independent of Ni treatment suggests that this species accumulates sulphur more efficiently. Sulphur accumulation of lichens under urban atmospheric conditions in Argentina has previously been mentioned for *R. celastri* (Levin and Pignata 1995; González et al. 1996, 2003; González and Pignata 1999).

The concentration of Ni measured in the lichen thalli was significantly different among treatments. In the samples exposed to the polluted zone, it increased significantly as did its concentration in the solutions. In thalli from the nonpolluted area, the Ni content was only significantly higher in samples treated with the highest concentration of Ni, with no significant differences between the two other Ni treatments. The lowest content of Ni was observed in the control samples. Higher levels of Ni were generally found in the samples transplanted to the polluted zone.

Both Ni treatment and the area of transplantation significantly affected the EC of the water in which thalli were immersed. In thalli retrieved from the polluted area, the highest value corresponded to samples treated with the highest concentration of Ni. A similar pattern was observed in thalli transplanted to the nonpolluted area. The variations indicate that damage originated by air pollutants is even worse in the presence of high levels of Ni cations. These results suggest that previous incorporation of metallic cations increases the damage of pollutants to cell membranes, and as a result also increases ion leakage (Garty et al. 2003).

PI values varied significantly with both the transplantation area and Ni treatment. The highest values were observed in thalli treated with the highest concentration of Ni in samples transplanted to polluted and nonpolluted areas, although the values of the polluted zone were significantly higher. This variation indicates that damage observed in lichen thalli is mainly originated by pollutants and enhanced by the presence of Ni. This is even more evident in thalli transplanted from the polluted zone, probably due to an additive effect of previously incorporated Ni and urban pollutants. Higher values of PI associated to urban pollutants have been observed before by González et al. (2003) using *R. celastri* as biomonitors.

Table 2. Mean values of concentration ± standard deviation and results of the analysis of variance (ANOVA) of the chemical parameters of *Usnea amblyoclada* transplanted to a polluted and nonpolluted area and submitted to immersion in different Ni solutions or purified water (controls)

	Mean ± SD				
	Control	Ni 0.5 mM	Ni 5 mM	Ni 10 mM	ANOVA[b]
Chl-b/Chl-a					
Non polluted	1.294 ± 0.039	1.329 ± 0.042	1.311 ± 0.035	1.365 ± 0.047	Ns
Poluted	3.544 ± 0.341 a	2.636 ± 0.119 b	3.443 ± 0.169 a	3.476 ± 0.297 a	*
ANOVA[b]	***	***	***	**	
HPCD μmol g^{-1} DW					
Non polluted	35.109 ± 0.354 b	22.190 ± 0.193 c	50.415 ± 0.301 a	50.816 ± 0.098 a	*
Polluted	37.232 ± 0.400 c	48.985 ± 0.712 b	58.586 ± 0.755 a	52.669 ± 0.165 ab	*
ANOVA[b]	ns	*	*	ns	
MDA μmol g^{-1} DW					
Non polluted	0.132 ± 0.005	0.114 ± 0.018	0.132 ± 0.019	0.131 ± 0.018	ns
Polluted	0.115 ± 0.027	0.108 ± 0.011	0.113 ± 0.010	0.112 ± 0.011	ns
ANOVA[b]	ns	ns	ns	ns	
Sulphur mg g^{-1} DW					
Non polluted	1.197 ± 0.034 b	1.237 ± 0.089 b	1.316 ± 0.038 b	1.456 ± 0.149 a	*
Polluted	1.284 ± 0.097 b	1.327 ± 0.019 b	1.420 ± 0.023 b	2.283 ± 0.224 a	*
ANOVA[b]	ns	ns	***	**	
Nickel mg g^{-1} DW					
Non polluted	3.955 ± 0.090 c	11.265± 0.697 b	22.737 ± 0.871 a	21.697± 0.927 a	*
Polluted	4.792 ± 0.055 d	15.991± 0.880 c	28.346± 0.815 b	60.165 ± 0.443 a	*
ANOVA[b]	***	***	**	***	
EC $\mu S.m^{-1}$					
Non polluted	1.879 ± 0.096	2.194 ± 0.084	2.226 ± 0.162	2.114± 0.179	ns
Polluted	2.244± 0.042 c	2.725 ± 0.101 a	2.502 ± 0.085 b	2.860 ± 0.210 a	*
ANOVA[b]	***	**	***	**	
PI					
Non polluted	1.318 ± 0.153 c	0.853 ± 0.077 d	1.984 ± 0.081 b	2.139 ± 0.158 a	*
Polluted	2.843 ± 0.241 b	2.901 ± 0.259 b	4.489 ± 0.488 a	4.694 ± 0.205 a	*
ANOVA[b]	***	***	***	***	

Note: Values on each horizontal line followed by the same letter do no differ significantly ($p = 0.05$); nsnot significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

[a] ANOVA between treatments.

[b] ANOVA between areas.

Effect of Ni on the Physiological Response of U. amblyoclada

Table 2 shows the mean values of the parameters measured for *U. amblyoclada* and the results of the ANOVA. The values of Chl-b/Chl-a ratio were significantly different among treatments, but only in thalli from the polluted zone. These changes are probably caused by the influence of air pollutants on the integrity of chlorophyll a, which is more sensitive to oxidations than chlorophyll b (Gries 1996). Moreover, the Chl-b/Chl-a ratio in *U. amblyoclada* thalli increased in all the samples transplanted to the polluted zone, indicating a decrease of Chl-a concentration due to degradation. This variation has been previously been observed in the same species (Carreras et al. 1998, 2005; Carreras and Pignata 2002). On the other hand, when the different transplantation areas were compared, all the treatments and controls were significantly different and the values corresponding to the polluted area were the highest.

Regarding HPCD content, significant differences were observed among treatments within both areas. The highest value corresponded to thalli pretreated with medium and high Ni concentrations and transplanted to the polluted zone, which once again suggests an additive effect between the presence of pollutants and Ni cations. The lowest values corresponded to thalli treated with the least concentrated Ni solution and transplanted to the nonpolluted zone and to control samples retrieved from the polluted zone. No differences were observed between areas in the control samples and in the most concentrated Ni solution. Similarly to what was observed in *R. celastri*, no differences in MDA content were evidenced in any of the samples.

Nickel treatment had significant effects on the content of sulphur in thalli exposed to the polluted and the nonpolluted zone. In both cases, the highest values corresponded to thalli treated with the highest concentration of Ni, possibly because of the presence of the SO_4^{-2} anion. Differences between sampling zones only were significant in samples treated with medium and high Ni concentrations, indicating an enhancement of accumulation in thalli transplanted to the polluted area.

The content of Ni in lichen thalli retrieved from the polluted zone significantly increased proportionally to its concentration in the solutions. In the nonpolluted zone, a similar variation was observed, but no differences were found between the two most concentrated solutions. Differences between the sampling areas were significant for all treatments, with higher values measured in thalli retrieved from the polluted zone.

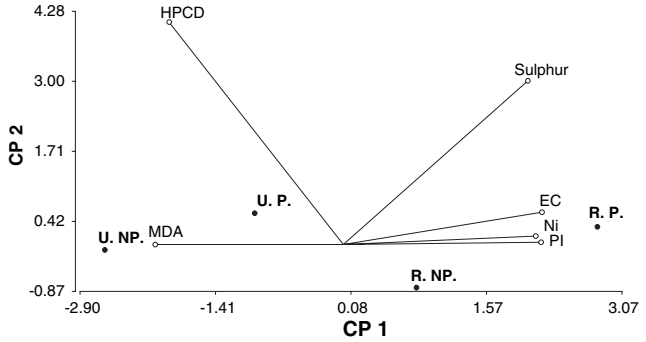

Fig. 1. Biplot based on the two principal components of the Principal Component Analysis for control samples, using area of transplantation and species as classification criteria. *U Usnea amblyoclada*, *R Ramalina celastri*, *NP* nonpolluted zone; P, polluted zone; *HPCD* hydroperoxy conjugated dienes, *MDA* malondialdehyde, *EC* electric conductivity, *Ni* nickel, *PI* pollution index

EC values were significantly different among treatments, but only in samples transplanted to the polluted zone. The highest values corresponded to thalli pretreated with Ni concentrations. In all cases, the highest values were found in the polluted zone. Previous studies in lichens have shown that toxic concentrations of metallic cations can increase the intracellular leakage of ions (Garty et al. 2001).

Both the area of transplantation and the immersion treatment showed significant effects on PI values. In lichens from the polluted zone, the highest values corresponded to thalli treated with the two most concentrated Ni solutions. In lichens retrieved from the nonpolluted area, the PI values were significantly different for all cases. Furthermore, PI values from the polluted area were significantly higher in all the treatments. Together with the aforementioned EC values, these results suggest that, as observed in *R. celastri*, both Ni and urban pollutants could be responsible for alterations in cell wall integrity. Similarly, Hyvärinen et al. (2000) found that only thalli treated with a concentrated Ni^{2+} solution showed prejudicial effects on some indicators of membrane permeability. Higher values of PI associated with urban pollutants were observed before by Carreras and Pignata (2002).

Effect of Ni on the Response Patterns to Environmental Conditions of R. celastri *and* U. amblyoclada

A PCA analysis was performed in order to compare the response patterns to environmental conditions in the presence or absence of Ni cations (extreme conditions: treatment with the highest concentration of Ni and controls, respectively). This analysis was only performed in order to summarize the ANOVA results and have a clear view of the patterns. Although PI equations are specific for each species, they were included in the PCA analysis because they were both extensively verified and clearly represent the global specific damage.

The analysis was undertaken using samples treated with the highest concentration of Ni and control samples, with area and species as classification criteria. Eigenvalues corresponding to the first two components of control samples are presented in Table 3 and the results are represented in biplots (Figure 1). This analysis show that the studied species differ in the parameters that best explain their response to the conditions of the area of transplantation. Control thalli of *R. celastri* transplanted to the polluted zone were mainly positively related with the content of Ni, PI, and EC values and, to a less degree, with sulphur content. A similar pattern was observed in samples transplanted to the nonpolluted zone, but the association was weaker. The parameters that best reflected the response of *U. amblyoclada* from the polluted area were MDA and HPCD, whereas the pattern of response in samples from the nonpolluted zone was mainly determined by the MDA content only. These results suggest that the ability to incorporate Ni from the atmosphere is more efficient for *R. celastri* because this parameter was mainly associated with this species.

Table 3. Eigenvectors obtained in principal component analysis of the chemical variables measured in control samples of *Ramalina celastri* and *Usnea amblyoclada*

	Component	
Chemical variables	1	2
Hydroperoxy conjugated dienes	−0.37	0.80
Malondialdehyde	−0.41	1.2×10^{-4}
Sulphur	0.40	0.59
Nickel	0.41	0.03
Electric conductivity	0.43	0.12
Pollution Index	0.43	0.01
Eigenvalues	5.43	0.36

Table 4. Eigenvectors obtained by principal component analysis of the chemical variables measured for samples treated with 10 mM solution nickel of *Ramalina celastri* and *Usnea amblyoclada*

	Component	
Chemical variables	1	2
Hydroperoxy conjugated dienes	0.34	0.58
Malondialdehyde	0.48	−0.08
Sulphur	−0.41	0.46
Nickel	−0.37	0.53
Electric conductivity	−0.40	−0.15
Pollution Index	−0.43	−0.37
Eigenvalues	4.07	1.53

Eigenvalues corresponding to the two first components of samples treated with the highest concentration of Ni are presented in Table 4 and they demonstrate that the specific pattern of response was altered when thalli from these two species were previously exposed to the Ni solution. The biplot (Fig. 2) shows that the response of *R. celastri* transplanted to the polluted zone was mainly determined by EC and PI and, to a lesser extent, by sulphur, whereas samples transplanted to the nonpolluted zone were weakly associated with these parameters. *U. amblyoclada* thalli from the nonpolluted zone were associated with MDA content, whereas the thalli from the polluted zone were related to the content of Ni and sulphur. These results indicate that the highest experimental concentration of Ni increases damage caused by atmospheric pollutants and hides the specific responses elicited by each lichen species. Furthermore, under these conditions, *R. celastri* is

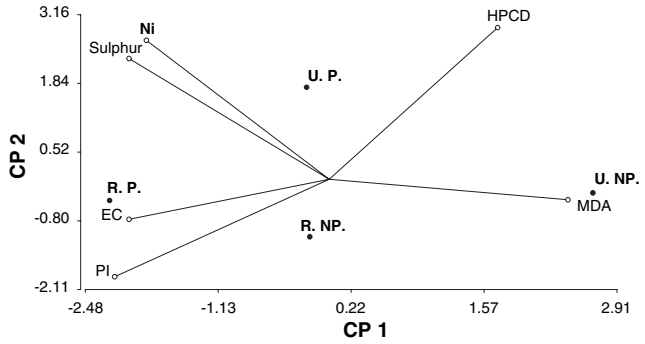

Fig. 2. Biplot based on the two principal components of the Principal Component Analysis for samples treated with 10 mM solution nickel, using area of transplantation and species as classification criteria. *U Usnea amblyoclada*, *R Ramalina celastri*, *NP* nonpolluted zone, *P* polluted zone, *HPDC* hydroperoxy conjugated dienes, *MDA* malondialdehyde, *EC* electric conductivity, *Ni* nickel, *PI* pollution index

seemingly a more sensitive indicator of damage caused by urban pollutants, as evidenced by the close association between PI and thalli transplanted to the polluted area, regardless of the Ni treatment. Further evidence results from the ANOVA of samples from the polluted area, which indicate the capacity of PI to differentiate control samples from Ni-treated thalli.

Conclusions

The results of the present study indicate that the pattern of response to air pollutants can be altered in both *R. celastri* and *U. amblyoclada*, enhancing their susceptibility to pollutants by short periods of exposure to high levels of Ni. The chemical response to urban pollutants was increased in both species with the previous incorporation of Ni. Moreover, the comparison of the specific pattern of response indicates that *R. celastri* is a more efficient sulphur accumulator.

The results of this preliminary study about Ni effect on the chemical response of studied species will guide future studies by screening species and variables for their relationship with air pollutants in biomonitoring programs.

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