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Physiological response and sulfur accumulation in the biomonitor *Ramalina celastri* in relation to the concentrations of SO_2 and NO_2 in urban environments

A.C. Mateos *, C.M. González

Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET and Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina

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ABSTRACT

This study extends the current knowledge regarding the use of different tools for monitoring atmospheric pollution. The chemical response of the lichen *Ramalina celastri* was evaluated through physiological parameters and sulfur accumulation in relation to the SO_2 and NO_2 concentrations present in the air at the monitoring sites with different emission sources, in order to assess the atmospheric pollution in urban environments. In this way, it was possible to establish different levels of air quality using simultaneous measurements of gaseous pollutants in the air and of parameters for the exposed biomonitor, as well as to determine the relationship among them and their association with the different emission sources present. Thalli of *Ramalina celastri* were transplanted at sites with different pollutant emission sources within the city of Córdoba, and simultaneously the concentrations of SO_2 and NO_2 were measured at the same sampling sites. Pigment content, hydroperoxy-conjugated dienes, malondialdehyde, and sulfur accumulation were quantified in the lichen and the pollution index was calculated. The results showed that high concentrations of atmospheric SO_2 in urban environments can be detected with certainty by measuring sulfur accumulation on the biomonitor. Although this relationship has often been assumed, it has not been confirmed by using simultaneous measurements. This biomonitor allowed different air quality levels to be identified in relation to the concentrations of NO_2 and SO_2 in Córdoba city. The biomarkers that best reflected the damage caused by pollutants on the lichen were pollution index, hydroperoxy-conjugated dienes, and malondialdehyde, which revealed a strong association with the most polluted areas in the city (industrial and heavy traffic sites).

1. Introduction

Many developing countries, including Argentina, have experienced a progressive degradation in air quality as a consequence of population growth, industrial development, agricultural practices, motor vehicles and fuels with a poor environmental performance, old transportation systems, and deficient environmental regulations [1,2]. These urban environments might be viewed as dense sources of vast anthropogenic emissions of pollutants that alter the atmospheric composition, chemistry, and life cycles [3]. The gaseous criteria pollutants NO_2 and SO_2 are markers of traffic emission which contribute to poor atmospheric quality and produce, among other effects, wet and dry acidic deposition [4]. Anthropogenic emissions of SO_2 are produced by fossil fuel combustion, biomass burning, and the smelting of sulfur containing ores [5], with the high levels of urban atmospheric NO_2 being due to vehicle emissions, as well as industrial processes and power plants, among other sources [6,7]. NO_2 and other nitrogen oxides are precursors of harmful secondary air pollutants such as ozone and particulate matter, and they also have an important role in the acid rain phenomenon [8,9].

In Córdoba city, although there are multiple sources that emit a wide range of air pollutants, no systematic measurements of these pollutants have been carried out and currently there are no systems for monitoring air quality. As a few isolated and sporadic measurements have indicated that it is indeed a city with high levels of air pollution [1,10,11], the use of alternative monitoring methods such as the use of biomonitors [12–15] appears to be a suitable alternative for the estimation of air quality related to the high concentration of some urban pollutants [16]. Lichens are well-known for being reliable monitors of the effects of air pollution, due to their sensitivity to environmental changes [17–22]. Moreover, lichen associations are particularly attractive for use as bioindicators because they have slow growth, lack a cuticle, and are perennial organisms that indiscriminately uptake elements from the atmosphere and are almost completely dependent on wet and dry atmospheric deposition for their nutritional requirements [23–25]. Furthermore, as they possess a distinctive capability to accumulate elements in excess of their nutritional demand [26], it can be assumed that the concentration of the elements in the lichen reflect the concentration of elements in the atmosphere [27,28]. The lichen *Ramalina celastri* is a

* Corresponding author. Tel.: +54 351 5353800 int. 29771.
E-mail address: caromateos@gmail.com (A.C. Mateos).

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well-recognized biomonitor of air quality utilized in the measurements of changes in the physiological response and/or the accumulation of pollutants [20,25,29–33].

In most European countries, despite that a significant decrease in SO_2 from industrial sources has been observed, the levels of NO_2 still generally exceed the limit values [34–37]. In Córdoba, the current levels of SO_2 and NO_2 are unknown, since the last measurements were performed in the '90s and at only a few sites [1]. This lack of instrumental measurements and the absence of environmental policies to control air quality make it imperative to use any other tool that can detect dangerous levels of these gasses. Many previous studies have established that the levels of SO_2 in the air are reflected by the accumulation of sulfur in lichens [38–42] and specifically in *R. celastri* [17,18,29,30,43]. However, this has never been proven by performing instrumental measurements while this lichen was exposed. In addition, it is known that these pollutants cause damage to the lichens [44–46] as has been shown in studies under controlled conditions in the laboratory [46, 47], but the effects of different levels of SO_2 and NO_2 in real atmospheres with a complex composition and multi-pollutants on lichenic physiology have not yet been studied [24].

The main objectives of the study were to (1) evaluate the physiological response of *Ramalina celastri* when exposed to urban environments by considering different air pollutant emission sources, and (2) evaluate the physiological parameters and sulfur accumulation in *Ramalina celastri* in relation to the air concentrations of SO_2 and NO_2 in order to determine the biomonitor's ability to reflect the atmospheric pollution associated with these gaseous pollutants in urban environments.

2. Materials and methods

2.1. Study area and sampling sites

Cordoba is located in the central area of Argentina at 31°24′S; 64°11′ W, 400 m above sea level and has an irregular topography with a population of 1.4 million [48]. The city lies in a depression, with an increasing positive slope from the center toward the surrounding areas. This concave formation reduces the air circulation and causes intense thermal inversions in both autumn and winter [49]. The climate is sub-humid, with an average annual rainfall of 790 mm that is concentrated mainly in summer. The mean annual temperature is 17.4 °C, with prevailing winds coming from the NE, S, and SE. Detailed information regarding the meteorological variables of the study area during the sampling period can be found in Table S1 in the Supplementary Material. Traffic is the most important factor contributing to urban air pollution with high seasonal average values of NOx, CO, and PM_{10} [1] and the downtown area has heavy and very slow vehicular traffic. There are also industrial sites and agricultural areas in the surroundings of the city.

Eight monitoring sites were selected according to the principal activity carried out at each site (land use). Then, the sites were grouped according to the environmental conditions that they represented. These corresponded to four heavy traffic sites (T), of which two were in large avenues where traffic was intense (TA), and another two were located in the downtown area (TD); two industrial sites (I) with metallurgical (MI) and metal-mechanic industries (MMI). Finally, we selected a typically residential site with average traffic (R) and a site away from any source of pollution designated control (C), which had low traffic and was far from any industrial source (Fig. 1).

2.2. Biological material and lichen transplantation

The lichen *Ramalina celastri* (Spreng.) Krog. and Swinsc. were collected from an unpolluted site, located 49 km to the south of Córdoba city. A brief description of the lichen species can be found in the Supplementary Material. Lichen samples were removed from the bark substrate, and lichen bags were prepared by weighing 6.0 g of fresh weight and packing this loosely in a nylon net (1 × 1.5 mm), according to González and Pignata [50]. Part of the fresh material was separated and analyzed the same way as the transplanted samples in order to determine the physiological state prior to transplantation of the lichen (baseline). The lichen bags were then transplanted to the different monitoring sites (n = 3) and placed at a height of 3 m above the ground in June 2014 (beginning of winter time). The exposure season was chosen considering that in Córdoba city, the most severe pollution occurs in winter due to thermal inversion causing pollutant accumulation in a layer close to the surface [51]. Three months later, at the end of the exposure period, the lichen material from each bag was separated into three sub-samples (each sample consisting of several thalli) and a mean arithmetic value ± standard deviation was derived for each chemical determination. The thalli were shredded, using a blender with a blade, to sizes of about 0.5 and 2 mm to achieve homogeneity and then freeze-dried. In Figs. S1 and S2 (Supplementary material), pictures of *R. celastri* in the recollection zone and after the exposure period are presented.

2.3. Nitrogen dioxide and sulfur dioxide monitoring

During this period of lichen transplantation (June 21/September 21, 2014), instrumental electrochemical sensors (Cairpol® micro-sensors) were placed at a height of 3 m from ground at each site to determine the real-time concentrations of SO_2 and NO_2. These sensors were placed inside a stainless weather protection case (CairTub®) designed for exterior use even in bad weather conditions. This instrumental monitoring system has a battery with autonomy of 21 days. The SO_2 sensor has a detection limit of 50 ppb; uncertainty <25%, and a range of 0–250 ppb, and the NO_2 sensor has a detection limit of 20 ppb; uncertainty <30%, and a range of 0–1000 ppb. The SO_2 and NO_2 concentrations were recorded every 15 minutes, with all the recorded data being downloaded via micro-USB onto a PC using specific software (CairSoft®). The filter was replaced once every 2 weeks and quality control checks were performed every 3 days which included inspection of the shelter and instruments as well as a zero concentration check and precision and span checks.

2.4. Chemical determinations in R. celastri

2.4.1. Pigments

One hundred milligrams of lichen material were homogenized in 10 mL of ethanol (ETOH) at 96% V/V with an Ultra Turrax homogenizer, T18. 1KA Works, Inc., USA. The supernatant was separated by centrifugation and then 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. The absorption of chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids (Carot), phaeophytin a (Ph a), phaeophytin b (Ph b), and phaeophytins alone (after addition of HCl) was measured with a spectrophotometer Beckman DU 7000, USA. Concentrations of chlorophylls, carotenoids, and phaeophytins were calculated on a dry weight (DW) basis with results being expressed as mg g^{-1} DW [52]. The phaeophytin a/chlorophyll a (Ph a/Chl a) ratio was also calculated [17,40].

2.4.2. Peroxidation product estimation

One hundred milligrams of freeze-dried lichen were 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. This reaction was stopped by placing the experimental tubes in an ice bath. Samples were then centrifuged at 10,000 g for 30 min. after which, the supernatant was removed and read at 532 nm, with the value for non-specific absorption at 600 nm being measured and subtracted from this. The amount of malondialdehyde (MDA) present was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} [53] with results being expressed in μmol g^{-1} DW. Hydroperoxy-conjugated dienes (HPCD)

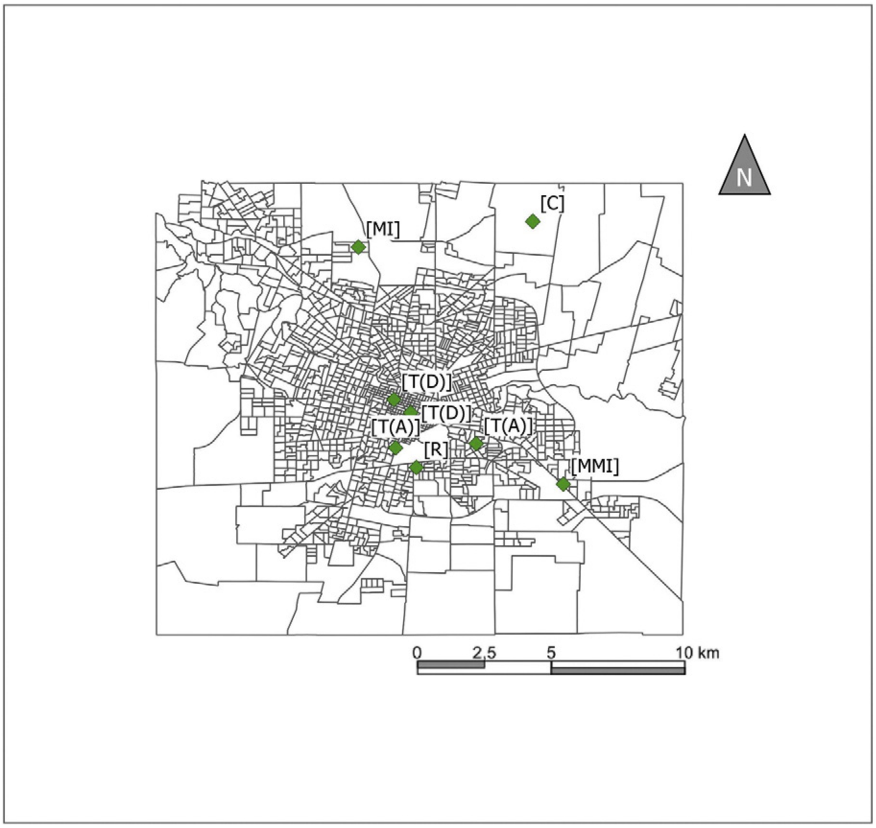

Fig. 1. Location of the study area and identification of the sampling sites (TD, TA, MMI, MI, R, C) in the city of Córdoba, Argentina.

were extracted by homogenization of the lichen material (50 mg) in 96% v/v ETOH at a ratio of 1:50 FW/v with an Ultra Turrax homogenizer. The absorption was measured in the supernatant at 234 nm, and its concentration was calculated by means of $\varepsilon = 2.65 \times 10^4\ M^{-1}\ cm^{-1}$ [54] with results being expressed as μmol g^{-1} DW.

2.4.3. Sulfur content

Five milliliters of $Mg\ (NO_3)_2$ saturated solution were added to 0.5 g of freeze-dried lichen and dried in an electric heater. Then, the sample was heated in an oven for 30 min at 500 °C and the ashes were suspended in 10 mL of 6 M HCl, filtered, and the resulting solution boiled for 3 min. The solution was brought up to 50 mL with distilled water and the amount of SO_4^{2-} in the solution was determined by the turbidimetric method using barium chloride [50] with results being expressed in mg of total sulfur (S) g^{-1} DW.

2.5. Pollution index

The pollution index (PI) for *R. celastri* was determined using the following equation cited by González et al. [17]:

$$\mathbf{PI} = [(\mathrm{Pha}/\mathrm{Chla}) + (\mathrm{S_T}/\mathrm{S_F})][(\mathrm{MDA_T}/\mathrm{MDA_F}) + (\mathrm{HPCD_T}/\mathrm{HPCD_F})]$$

where the subindex T denotes determinations of transplanted samples, and the subindex F refers to the freshly picked material (baseline). This index has previously been statistically checked and used in biomonitoring studies with the same species [17,18,25,30–33,53,55].

2.6. Data analysis

Results were expressed as the mean value ± standard deviation of three independent determinations for each of the eight sampling sites, the physiological parameters and the NO_2 and SO_2 concentrations were used in an analysis of variance (one-way analysis of variance, ANOVA) using as the factor variation: Condition (based on land use) with five levels (control, residential, traffic (downtown), traffic (avenues), metallurgical industry, and metal-mechanic industry with traffic). Whenever the ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means was undertaken using the least significant difference (LSD) test. The ANOVA assumptions were previously verified graphically (residual vs. fitted values, box plots, and steam leaf plots). In addition, Pearson's coefficients of correlation were used to evaluate the degree of correlation among variables. Finally, a multivariate analysis: principal component analysis (PCA) was performed using condition as the classification criteria in order to assess the association of contaminants with the emission sources present at the sampling sites as well as the simultaneous relationships among lichen chemical variables and the gaseous pollutants measured in air.

3. Results and discussion

3.1. SO_2 and NO_2 monitoring

Table 1 shows the mean 24-hour values of SO_2 and NO_2 measured by the electrochemical micro-sensors for the different environmental conditions in the city of Córdoba. Due to the previous lack of measurements of pollutants and air quality standards in the city of Córdoba, we used as a reference the National Ambient Air Quality Standards (NAAQS) of the Environmental Protection Agency of the United States [56]. According to NAAQS, the primary standard for a 1-hour averaging time is 75 ppb and 100 ppb for SO_2 and NO_2, respectively. At all heavy traffic sites, the limit values for NO_2 were exceeded several times (TD: 206.25 ppb maximum value; TA: 150.25 ppb maximum value), which was similar to the findings of Lanzafame et al. [57] who reported above-limit values of NO_2 in areas with heavy traffic in Catania (Italy). Related to this, Olcese and Tosseli [1] found values measured in Córdoba during 1996 which

Table 1
Mean values ± standard deviation, minimal and maximal values of SO_2 (ppb) and NO_2 (ppb) measured by the automatic monitoring sensors at sites with different environmental conditions in the city of Córdoba.

	SO_2 (1-hour primary standard: 75 ppb)				NO_2 (1-hour primary standard: 100 ppb)			
Condition	Mean	S.D.	Min	Max	Mean	S.D.	Min	Max
TA	19.83	2.23	17.00	23.00	133.96	11.85	121.25	150.25
TD	32.50	10.21	22.00	45.00	172.79	25.87	138.00	206.25
MI	112.33	15.31	103.00	130.00	60.58	14.56	49.25	77.00
MMI	25.33	1.53	24.00	27.00	76.25	4.09	72.75	80.75
R	16.33	1.16	15.00	17.00	77.66	5.90	71.50	83.25
C	16.33	2.52	14.00	19.00	74.67	3.76	70.75	78.25

were higher than those in other cities. In the case of SO_2, the NAAQS primary standard was only exceeded in the condition corresponding to metallurgical industries (MI: 130 ppb maximum value). Our results were also in good agreement with a study of air pollution carried out near a lignite-burning power plant in Greece [27]. As the standards used are designed to provide public health protection, especially for the health of sensitive populations such as asthmatics, children, and the elderly [56], then these high-concentration values imply a risk for the health of the population.

3.2. Biomonitoring and SO_2 and NO_2 concentrations

Before analyzing the effect of transplanting to urban-industrial conditions, we assessed the effect of transplantation in the lichen by comparing the mean values of unexposed baseline thalli with lichens re-transplanted to the recollection zone. However, no significant differences were observed (data not shown), indicating an absence of any effect.

The mean values (±S.D.) of the SO_2 and NO_2 concentrations measured in air, chlorophyll a (Chl a), phaeophytin a (Ph a), carotenoids (Carot), hydroperoxy-conjugated dienes (HPCD), malondialdehyde (MDA), Ph a / Chl a ratio, sulfur (S), and pollution index (PI) measured in *R. celastri* transplanted as well as the ANOVA results corresponding to the different environmental conditions are shown in Table 2.

Higher concentration values for SO_2 were observed in both industrial and heavy traffic in the downtown (TD) area, while lower values were found in heavy traffic in avenues (TA), residential and control conditions, as expected due to the principal emission sources of this pollutant being industrial processes and vehicle exhausts [58,59]. The highest concentrations of NO_2 were found at traffic sites downtown (TD) followed by traffic in avenues (TA), with both showing significant differences with the other conditions: residential (R), industrial (MI and MMI), and with the least concentrations occurring in the control condition (C). These variations were expected because NO_2 emissions arise primarily from high temperature combustion of fossil fuels which originate from power plants and traffic using either gasoline or diesel fuel [9,60].

The samples of the biomonitor transplanted to heavy traffic in avenues (TA) revealed the highest pigment content but also the greatest value of pigment degradation as shown by the Ph a/ Chl a ratio. For these indicators, the control (C) condition had the lowest pigment concentrations and the lowest degradation ratio. The chlorophyll degradation (Ph a/Chl a) was higher at all urban sites compared with the control site, regardless of the pigment concentration (Table 2). This phenomenon was also observed in other studies with another lichen species (*Usnea amblyoclada*) where the total pigment content was higher in the lichen thalli exposed in areas with heavy traffic levels, indicating that the pigment content increases with the level of air pollutants [40, 61]. These authors attributed this to a fertilizing influence of pollutants, especially that of NO_x. Moreover, von Arb et al. [62] found an association between NO_2 from traffic and an increase in chorophyll content in lichens which was also the case in the present study, since the heavy traffic conditions resulted in the highest values of pigment content in thalli and the greatest concentrations of atmospheric NO_2.

The ratio Ph a / Chl a has previously been used as a damage indicator for *R. celastri* [17,18,32,33]. However, as the differences for chlorophyll degradation in the present study were not significant among traffic (TD and TA) and industrial (MI and MMI) conditions, then this ratio would not be a good indicator for discriminating the environmental conditions associated with the emission sources considered. This suggests that the presence of atmospheric oxidants in all conditions, except the control, provoked similar oxidative damage to the photosynthetic pigments.

MDA and HPCD are biomarkers related to lipid peroxidation of cell membranes in lichens [63], and consequently, to the cell membrane integrity. Thus, increases in their values indicate the oxidative damage level caused by pollutants [16,29,40,50]. The comparison among conditions showed significant differences for these parameters (Table 2) with a similar variation pattern (traffic > metallurgical industry > residential > control) for HPDC and MDA being observed. However, the highest values of HPDC were observed in the condition of heavy traffic in avenues (TA), whereas the greatest concentration of MDA occurred in the condition of heavy traffic in the downtown area (TD). González et al. [18] postulated that one of the environmental conditions that best explains variations in HPCD is traffic and the distance to the emission source, while MDA concentrations depend more on environmental variables that affect the pollutant spreading. Thus, it was expected that MDA would show higher concentrations in lichens transplanted to heavy traffic sites in the city center (TD), where the wind circulation is scarce (due to high buildings and the area topography) and therefore

Table 2
Mean values ± standard deviation and analyses of variance (ANOVA) of physio-chemical parameters measured in *R. celastri* and the SO_2 and NO_2 concentrations in the air for the different conditions: TA: high traffic in avenues, TD: high traffic in downtown area, MI: metallurgical industry, MMI: metal-mechanic industry, R: residential area, and C: Control, during the exposure period (21 June 2014 to 21 September 2014) in Córdoba city.

	TA		TD		MI		MMI		R		C		ANOVA
SO_2 (ppb)	19.83 ± 2.23	C	32.50 ± 10.21	B	112.33 ± 15.31	A	27.00 ± 0.00	BC	16.33 ± 1.15	C	16.33 ± 2.52	C	***
NO_2 (ppb)	133.96 ± 11.85	A	172.79 ± 25.87	B	60.58 ± 14.56	C	76.25 ± 4.09	C	77.66 ± 5.90	C	74.67 ± 3.76	C	***
Chl a (mg/g DW)	1.38 ± 0.13	A	0.79 ± 0.07	CD	0.92 ± 0.05	C	0.90 ± 0.09	C	1.08 ± 0.09	B	0.69 ± 0.07	D	***
Ph a (mg/g DW)	1.47 ± 0.13	A	0.85 ± 0.07	C	1.28 ± 0.25	AB	0.94 ± 0.10	C	1.16 ± 0.10	B	0.66 ± 0.03	D	***
Carot (mg/g DW)	0.39 ± 0.05	A	0.20 ± 0.02	C	0.36 ± 0.07	A	0.23 ± 0.02	BC	0.29 ± 0.03	B	0.19 ± 0.02	C	***
HPCD (μmol/g DW)	11.70 ± 1.29	A	8.01 ± 0.62	C	12.30 ± 0.27	A	8.34 ± 0.25	BC	9.52 ± 1.04	B	7.36 ± 0.51	C	***
MDA (μmol/g DW)	0.07 ± 0.01	BC	0.08 ± 0.01	A	0.08 ± 0.00	A	0.08 ± 0.00	ABC	0.08 ± 0.00	AB	0.07 ± 0.00	C	*,**
Ph a/ Chl a	1.06 ± 0.02	A	1.07 ± 0.01	A	1.07 ± 0.02	A	1.04 ± 0.00	A	1.07 ± 0.01	A	0.95 ± 0.15	B	*
Sulfur (mg/g DW)	0.99 ± 0.04	B	0.88 ± 0.03	C	1.13 ± 0.02	A	1.17 ± 0.03	A	0.69 ± 0.05	D	0.61 ± 0.01	E	***
PI	8.43 ± 0.42	B	6.83 ± 0.45	D	9.76 ± 0.18	A	7.61 ± 0.04	C	6.59 ± 0.58	D	4.82 ± 0.41	E	***

ns: not significant. nd: no data. Values in each horizontal line followed by the same letter do not differ significantly.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level.

Table 3
Correlation matrix (Pearson correlation coefficient) for physio-chemical parameters measured in *R. celastri* thalli and concentrations of SO_2 and NO_2 measured by automatic monitoring of the air in Córdoba city.

	Chl a	Ph a	Carot	HPCD	MDA	Ph a/ Chl a	Sulfur	PI	SO_2	NO_2
Chl a	1									
Ph a	0.89***	1								
Carot	0.86***	0.97***	1							
HPCD	0.75***	0.87***	0.92***	1						
MDA	ns	ns	ns	ns	1					
Ph a/ Chl a	ns	ns	ns	ns	0.41*	1				
Sulfur	ns	0.42*	ns	0.46*	ns	ns	1			
PI	0.51*	0.73***	0.71***	0.83***	ns	0.51*	0.82***	1		
SO_2	ns	ns	ns	0.41*	ns	ns	0.49*	0.60**	1	
NO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns	1

ns: not significant.
* Significant at 0.05 probability level.
** Significant at 0.01 probability level.
*** Significant at 0.001 probability level.

hinders the dispersion of pollutants. Other studies on this species have reported variation in HPCD associated with distance to vehicular traffic or power plants [29–31]. Despite these differences, both parameters were found at higher concentrations in dense traffic conditions that coincided with greater NO_2 concentrations, indicating that high levels of NO_2 may induce the formation of peroxidation products in the lichen cell membrane [64]. These results suggest that MDA content might reflect a synergistic effect of NO_2 and SO_2, according to their level in the air, since thalli revealed the highest values at conditions with the greatest SO_2 concentration. Therefore, MDA would appear to be the best indicator of the potent oxidant presence among pollutants.

Regarding sulfur accumulation, significant differences were observed among conditions with the values being significantly higher in the industrial areas, followed by heavy traffic sites, residential and control condition (Table 2). This was also previously observed in *R. celastri* by Gonzalez et al. [17,18,30] in biomonitoring studies in urban areas. The association between sulfur bioaccumulation and atmospheric pollutant emission sources is known for different biomonitor species and reflects different sensibilities in the presence of industrial, urban, or both emissions. Moraes et al. [65] found elevated foliar sulfur content due to emissions from chemical and petrochemical industries in a biomonitoring study using tropical tree leaves, whereas Wannaz and Pignata [66] reported the greatest values of sulfur accumulation in the leaves of the biomonitor *Tillandsia capillaris* in industrial areas and at heavy traffic sites, as observed by Bermudez et al. [67] in *T. recurvate*. In the present study, the sulfur accumulation in samples transplanted to different conditions reflected the SO_2 concentrations in the air, which is consistent with the correlations found between them (Table 3).

The pollution index (PI) is considered to be a good indicator of physiological damage to the biomonitor employed [18,25,33] and was calculated to establish which conditions produced the greatest damage to the exposed thalli. This revealed the highest value in metallurgical industries (MI) and the lowest values at the control site (C), thus the poorest condition of air quality in the study area corresponded to the industrial zone located within the city. With respect to gas concentration in the air, the highest values of sulfur accumulation and PI were observed in metallurgical industries (MI). These sites also had the highest concentrations of SO_2 in the air, in agreement with other authors [42,68,69] who assumed that sulfur accumulation in lichen is positively correlated with SO_2 concentration in the air. It should be noted, however, that this relationship has not been confirmed before the current study in relation to real-time measurements of urban air SO_2 concentration. Here, the variations of PI and sulfur accumulation with respect to SO_2 atmospheric concentrations may indicate that the damage caused by the presence of sulfur in the air is significant, which when taken together with the increase in MDA and HPCD content suggests an important oxidative effect.

With the aim of studying the association between the air concentration of gaseous pollutants and the damage observed in lichens, the Pearson correlation coefficients were calculated between the SO_2 and NO_2 concentrations in the air and the physiological parameters measured on the lichen thalli (Table 3). The NO_2 atmospheric concentrations did not correlate with any of the parameters measured in the lichen or with the SO_2 concentrations measured in the air. Similar results were also found for NO_2 by Frati et al. [70] for the lichen *Evernia prunastri* exposed near a highway in Italy and by Carreras and Pignata [61] using *U. amblyoclada*. On the other hand, SO_2 concentrations correlated positively with the accumulation of sulfur in the lichen, HPCD content, and PI, indicating that the high levels of atmospheric SO_2 were related not only with sulfur accumulation in the lichen but also with the damage to the lichen detected through the biomarkers of physiological damage HPCD and PI. The significant positive correlation of sulfur accumulation and the concentration of SO_2 in the air corroborates the assumption mentioned above that bioaccumulation of sulfur may reflect an SO_2 atmospheric concentration. Therefore, this validates the use of *R. celastri* as a biomonitor of the atmospheric levels of sulfur (SO_2 plus SO_3, SO_4^{2-}, HSO_4^-, SO_3^{2-}, HSO_3^-, etc.).

In order to identify the parameters that best explain the variability of the data and the response patterns of the lichen with respect to environmental conditions and the concentrations of the gaseous pollutants, a multivariate analysis was performed (PCA) using the environmental conditions as the classification criteria. This analysis was undertaken using the parameters measured in the lichen (HPCD, MDA, Ph a/Chl, PI, and S) and the atmospheric concentrations of SO_2 and NO_2, measured at the same transplantation sites of the biomonitor. Eigenvalues corresponding to the first two components are shown in Table 4 with the results being presented in a bi-plot (Fig. 2). The eigenvalues associated with the axis and the lichen-environment conditions are indicators of to what extent the species response explains the environmental variables. The first two axes retained 76.10% of the total variance with the first axis being mostly determined by PI and HPCD, followed by

Table 4
Eigenvectors obtained by principal component analysis (PCA) of the physio-chemical variables measured in *Ramalina celastri* using the condition as the classification criteria.

	Component	
Variables	1	2
HPCD	0.42	−0.11
MDA	0.32	0.43
Sulfur	0.39	−0.19
PI	0.49	−0.10
SO_2	0.40	−0.27
NO_2	−0.04	0.72
Ph a/ Chl a	0.41	0.42

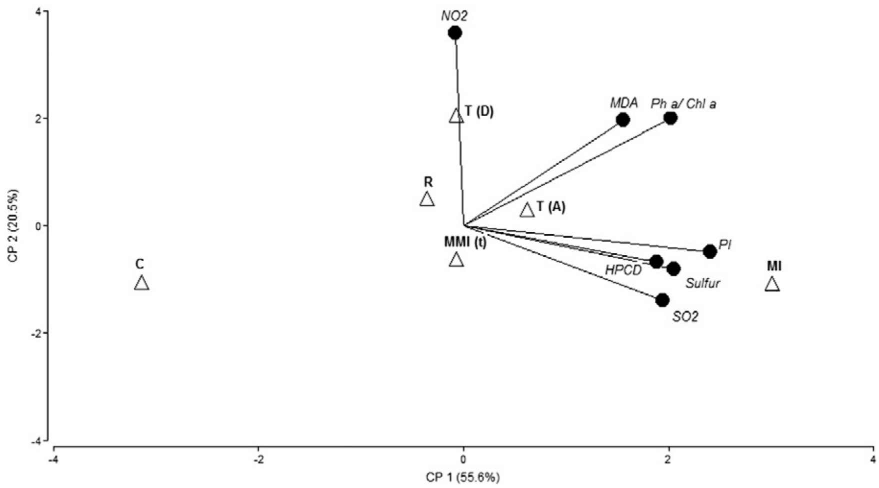

Fig. 2. Bi-plot based on the two principal components of the principal component analysis (PCA) for physio-chemical parameters measured in *R. celastri* and for the SO_2 and NO_2 concentrations in the air, using the condition as the classification criteria. MMI: metal-mechanic industry; MI: metallurgical industry; TD: heavy traffic in downtown; TA: heavy traffic in avenues; R: residential; C: control; HPCD: hydroperoxy-conjugated dienes; MDA: malondialdehyde; PI: pollution index; Ph a / Chl a: phaeophytin a / chlorophyll a ratio.

sulfur accumulation and SO_2 concentrations to similar extents, which confirms that PI can be used as a biomarker of air quality and that sulfur accumulation as a biomarker of the SO_2 concentration in the air. The second axis is strongly defined by NO_2 concentration, MDA, and to a lesser extent, Ph a/ Chl a ratio, reflecting that the impact of NO_2 on the lichen is lower than that of SO_2. It is possible that NO_2 and SO_2 combined had a synergist effect on the lichen, or that NO_2 enhanced the effect caused by SO_2. The bi-plot obtained from the first two components (Fig. 2) revealed a strong positive association among PI, sulfur accumulation, HPCD, and SO_2, all parameters primarily associated with the metallurgical industries (MI) and to a lesser degree with traffic in avenues (TA) and metal-mechanic industries (MMI). This association was also observed in the Pearson's correlation analysis which showed a strong relationship between sulfur accumulation in the lichen and the concentration of SO_2 in the air. Thus, it may be suggested that the measurement of sulfur accumulation in *R. celastri* can be used to distinguish areas within the city with different levels of atmospheric SO_2. Moreover, high levels of SO_2 were related to lipid peroxidation products, especially HPCD in the biomonitor. This strong relationship confirms that not only the sulfur accumulation in the lichen thalli reflects the atmospheric SO_2 concentrations but also that the high levels of SO_2 induced the formation of peroxidation products, especially HPCD. In addition, the strong correlation between the PI and SO_2 concentrations suggests that this index can be used as an indicator of the air quality in the city. The ANOVA of PI and SO_2 indicated similar variation patterns within the different conditions under study (Table 2), with the PCA analysis reflecting the close relationship between the concentration of MDA and Ph a/ Chl a with traffic conditions and NO_2 indicating that these parameters could be used as biomarkers of traffic emissions. The condition of heavy traffic in downtown area (TD) showed an association with NO_2 concentration with a similar pattern being observed with residential with average traffic (R), but with a weaker association. Finally, the metal-mechanic industry (MMI) revealed a weak association with SO2 concentration, but no clear association with any of the other variables measured.

4. Conclusions

The results of this study show that atmospheric SO_2 concentration in urban environments was very well reflected by measuring the sulfur accumulation in the exposed thalli of *R. celastri*, thereby being a useful biomarker of SO_2 concentration when no instrumental monitoring is available. Moreover, the lichen revealed differing levels of air quality in relation to the concentrations of NO_2 and SO_2 at different sites in the city of Córdoba which was strongly associated with land use. The association observed of SO_2 concentration with sulfur accumulation, HPCD, and PI indicates that *R. celastri* provides important information not only about the SO_2 concentration in the air, but also concerning the oxidative effect on the biomonitor as shown by HPCD and the global physiological damage reflected by PI. The application of the Pollution Index, evaluated in this study for the first time in relation to the concentrations of SO_2 and NO_2 in the air, was able to discriminate air quality according to the different emission sources present in the conditions considered, thereby confirming this index to be an excellent biomarker. The pattern response revealed the importance of this species for biomonitoring programs, since it can be used successfully to assess air quality and consequently detect the most harmful conditions by measuring only a few chemical parameters in the lichen. Thus, it is possible not only to obtain information about air quality but also to associate each parameter with the gaseous concentrations and emission sources. Biomonitoring at the same sites and in the same periods in which the instrumental monitoring of SO_2 and NO_2 was performed allowed the validation of the use of *R. celastri* as a biomonitor of the atmospheric levels of SO_2 and its use to establish different levels of air quality in urban environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.microc.2015.11.025.

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