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ASSESSMENT OF DIESEL EXHAUST POLLUTANTS EFFECTS IN *Tillandsia capillaris* AND *Ramalina celastri* BY LABORATORY TRIALS

Evaluación de los efectos de contaminantes emitidos por motores diésel en *Tillandsia capillaris* y *Ramalina celastri* mediante ensayos de laboratorio

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Key words: vehicular air pollutants, physiochemical damage, PM bound metals, biomonitors, controlled experimental conditions.

ABSTRACT

Traffic-related air pollution is one of the most relevant environmental problems in urban areas. Several cryptogams (i.e., lichens and mosses) and vascular species have been employed to monitor urban air pollution since they allow the assessment of air quality in a large number of sampling sites simultaneously at low cost. In large urban cities, vehicle emissions are frequently the major source of air pollution along with residential energy (for cooking and heating), industry, power generation, and waste incineration. Biomonitors in these urban environments are exposed to a mixture of pollutants making it difficult to identify which pollutant causes the greatest damage to organisms. However, studies that analyze the effect of pollutants emitted by vehicle exhaust are scarce and in the particular case of the most used biomonitor species in Argentina, no analysis of how they are affected by vehicle emissions has been carried out so far. So, the aim of this work was to analyze changes in physiochemical parameters (pigment content, pro-oxidant products, and sulfur accumulation) in *Ramalina celastri*, and heavy metal accumulation in *Tillandsia capillaris*, exposed to diesel exhausts under laboratory conditions. A strong damage in the photosynthetic apparatus of *R. celastri* was observed as well as metal concentration in *T. capillaris* after 20 min of exposure and 48 h of permanence in the exposure chambers. The results indicate that not only the particles and metals cause damage to these two well-known biomonitors, but the interaction of these pollutants with other components of the atmosphere that form different secondary pollutants, together with a longer exposure time, could cause the highest level of damage in them.

Palabras clave: contaminantes vehiculares del aire, daño físico y químico, metales unidos a partículas, biomonitores, condiciones experimentales controladas.

RESUMEN

La contaminación del aire relacionada con el tráfico es uno de los problemas ambientales más relevantes en las zonas urbanas. Se han empleado varias criptógamas (líquenes y musgos) y especies vasculares para monitorear la contaminación del aire urbano, ya que permiten evaluar la calidad de éste en una gran cantidad de sitios de muestreo simultáneamente y a bajo costo. En las grandes ciudades urbanas, las emisiones vehiculares son generalmente la principal fuente de contaminación del aire junto con la energía residencial (para cocinar y calentar), la industria, las centrales de energía y la incineración de residuos. Los biomonitores en estos entornos urbanos están expuestos a una mezcla de contaminantes, lo que dificulta identificar qué contaminante causa el mayor daño a los organismos. Sin embargo, los estudios que analizan el efecto de los contaminantes emitidos por los gases de escape de los vehículos son escasos y, en el caso particular de las especies de biomonitores más utilizadas en Argentina, hasta el momento no se ha realizado ningún análisis del efecto de dichas emisiones. Por lo tanto, el objetivo de este trabajo fue analizar en dos biomonitores ampliamente utilizados, los cambios en los parámetros físicos y químicos (contenido de pigmentos, productos de peroxidación y acumulación de azufre) en *Ramalina celastri* y la acumulación de metales pesados en *Tillandsia capillaris*, expuestos a gases de escape de diésel en condiciones controladas de laboratorio. Se observó un marcado daño en el aparato fotosintético de *R. celastri*, así como concentración de metales en *T. capillaris* después de 20 min de exposición y 48 h de permanencia en las cámaras de exposición. Los resultados indican que no sólo las partículas y los metales causan daño a los biomonitores, sino que la interacción de estos contaminantes con otros componentes de la atmósfera que forman diferentes contaminantes secundarios, junto con un mayor tiempo de exposición, podría causar el mayor nivel de daño en los biomonitores.

INTRODUCTION

In urban environments, motor vehicles are one of the main emission sources that contribute to air pollution at local, regional, and global scale along with residential energy (for cooking and heating), industry, power generation, and waste incineration (Jain et al. 2016, Álvarez-Vázquez et al. 2017, Goyal et al. 2021). Over the last years, many urban areas from developing countries, such as Córdoba city (Argentina), showed a strong population growth that derived in an intense traffic flow. This greater number of vehicles generated an increase in the levels of urban air pollutants, which is heightened during the frequent traffic congestions (Puliafito et al. 2011, Morales et al. 2012, Mateos et al. 2018a).

One of the main pollutants emitted by vehicles are suspended particles with aerodynamic diameters less than 10 and 2.5 µm (PM_{10} and $PM_{2.5}$) (Maricq et al. 1999, Puliafito et al. 2011), although a major contribution of particles can be attributed to emission from diesel-powered vehicles (Maher et al. 2008, Giordano et al. 2010). PM are produced by the engine due to incomplete fuel combustion, lubricant volatilization, and wear and tear on auto parts. Also, brake lining, tire wear, and road dust contribute to vehicle-related PM emissions (Vouitsis et al. 2009). It has been proven that in urban areas the major source of ultrafine particles are low-mass aerosols from diesel combustion exhausts (Verma et al. 2014), and over 80 % of PM_{10} present in large cities originates from freight and passenger transport that are mainly diesel power vehicles.

PM emissions can be classified as exhaust and non-exhaust (Lawrence et al. 2016). The first category considers particles produced due to incomplete fuel combustion and lubricant volatilization (Vouitsis et al. 2009), while non-exhaust emissions are particles generated by re-suspension of road dust but also by corrosion of vehicle components or during mechanical processes, such as braking, using the clutch, or tire wear (Lawrence et al. 2013, Ravindra et al. 2015). According to a national transport report (Puliafito and Cartesana 2010), in the 1960s 56 % of freight and passenger transportation in Argentina used diesel as fuel and the other 44 % gasoline, while in 2008, 74 % of the transportation was diesel-powered and only 26 % used gasoline. Thus, diesel consumption has been increasing over the years, being always the most consumed fuel in the country. Although a partial control of PM emissions is performed with after-treatment devices, such as catalytic converters and particle filters, several studies have shown an increase in particle numbers due to enhanced nucleation downstream (Giordano et al. 2010). The disadvantage of these devices is that they release into the air large amounts of platinum group elements (Pt, Pd, Rh) due to thermal and chemical mechanisms that occur while the vehicle is running (Moldovan et al. 2002). Moreover, the replacement of the catalytic converter is not a usual practice in the country due to the lack of regulations (Sbarato and Rubio 2017). Organic matter and elemental carbon account for most of the exhaust particles, being the polycyclic aromatic hydrocarbons (oxy-, and nitro-PAHs) the dominant organic compounds identified (Valavanidis et al. 2006, Phuleria et al. 2007, Najmeddin and Keshavarzi 2018) as the most widespread mutagenic and carcinogenic particulate environmental pollutants. The United States Environmental Protection Agency (US-EPA) has listed 16 PAHs as priority pollutants due to their photomutagenicity (Yan et al. 2004). For example, when the exposure concentration to B[a]P (benzopyrene), one of the 16 PAHs, exceed 1 ng/m^3, the DNA would be damaged (Han et al. 2021).

Also, vehicular emissions contribute to atmospheric levels of several toxic metals (Monaci et al. 2000, Singh et al. 2002, Lough et al. 2005). An example of this is the emission of Zn, Cu and Fe (which are used as a coating due to their heat conduction properties) during mechanical abrasion of vehicular brakes (Zechmeister et al. 2006, Raparthi and Phuleria 2021). Zn is also released in the combustion of motor oil and tire wear (Huang et al. 1994, Akbar et al. 2006, Manno et al. 2006). Ni is also related to vehicular air pollution due to the corrosion of bearings, shafts and crankshafts, and it also is emitted from vehicular exhaust (Kumar et al. 2021). Even though the use of leaded gasoline has been banned since the 1990s, vehicular traffic continues to be a source of emission of this metal (Mishra et al. 2004, Kabata-Pendias and Mukherjee 2007) due to the removal of residues accumulated for years in the ground and to resuspension in what is called vehicular dust (Pandey et al. 2014). Another metal associated with vehicular emissions is Mn, since it is used as an additive to increase the octane levels of gasoline and in the manufacture of brake tapes (Keskin et al. 2007, Swietlik et al. 2015). There is also a group of heavy metals related to non-tailpipe emissions, like Co (Wang et al. 2021), which are present in auto parts. Another group of toxic species emitted are gaseous compounds, such as carbon monoxide (CO), nitrogen oxides (NOx), volatile organic compounds (VOCs), and sulfur dioxide (SO_2), mainly from diesel exhaust (Dogruparmak et al. 2014), as well as some greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (Hu et al. 2009, Nel and Cooper 2009, Zhang et al. 2016).

In Argentina, as in many other developing countries, despite the harmful effects of the aforementioned pollutants emitted by diesel exhaust, air pollutants are scarcely monitored and only isolated data from researchers is available. The lack of instrumental monitoring has led to the growing use of biomonitors since they allow the assessment of air quality in a large number of sampling sites simultaneously, with relatively very low cost (Gombert et al. 2006, Augusto et al. 2010, Wannaz et al. 2013, Abril et al. 2014a, Mateos et al. 2018b). In addition, biomonitors like mosses and lichens can evidence the presence of substances that are difficult to monitor in real time with instruments, such as PM-bound metals (Monaci et al. 2000). Despite its great utility, it is very difficult to find species that can be used globally, so it is interesting to test biomonitors that are widely distributed in the study region. In particular, *Tillandsia capillaris* has been frequently employed in many air quality studies in Argentina (Pignata et al. 2002, Wannaz and Pignata 2006, Wannaz et al. 2006, Bermúdez et al. 2009, Rodríguez et al. 2011, Abril et al. 2014b, Mateos et al. 2018b), and *Ramalina celastri* has demonstrated to be an excellent biomonitor in urban environments (Garty et al. 1993, 2007, González and Pignata 1994, 1999, Levin and Pignata 1995, González et al. 1998, 2003, Carreras and Pignata 2002, Augusto et al. 2007, Pignata et al. 2007, Carreras et al. 2009, Álvarez et al. 2012, Mateos and González 2016).

Previous studies have demonstrated that vehicular emissions are the main source of pollutants in Córdoba (Stein and Toselli 1996, Olcese and Toselli 2002, Mateos et al. 2018a). On the other hand, other studies with *T. capillaris* (Mateos et al. 2018b) and *R. celastri* in the city (Mateos and González 2016) have demonstrated severe damage to this biomonitors after exposition to the urban atmosphere. However, they were exposed to the mixture of urban pollutants, therefore it is not possible to attribute the observed damage to a specific pollutant. In the present study we focused on the effect of diesel exhaust emissions on physiochemical parameters and heavy metal accumulation in two frequently used biomonitors. After a bibliographic review, we decided to analyze the variations in physiochemical parameters in *R. celastri* and the accumulation of heavy metals in *T. capillaris*, since in several studies lichen is indicated as an efficient physiological biomonitor, and the vascular epiphyte as a good biomonitor for heavy metal accumulation. Our hypothesis it is that the pollutants emitted by diesel exhaust induce adverse changes in the values of the physiochemical parameters in *R. celastri* and cause an increase in the levels of heavy metals accumulated in *T. capillaris*.

MATERIALS AND METHODS

Biomonitors

***Tillandsia capillaris* Ruiz and Pav.**

Bromeliaceae is a monocotyledon family with a wide distribution in South America that includes the *Tillandsioideae* subfamily, with numerous slow growth epiphytic species that live in trees or inert substrates. They are completely independent from soil to get nutrients, being the adhesion to substrate the only function of their roots (Papini et al. 2010). Due to the epiphytic nature of the *Tillandsia* genus, they are suitable for atmospheric quality monitoring studies (de Souza et al. 2007).

Several samples of *T. capillaris* (**Fig. 1**) were collected from tree branches using plastic gloves to prevent any risk of sample contamination in a non-polluted, natural reserve area (La Quebrada) located 38 km NW from Córdoba city (Abril et al. 2014a). Part of the collected material was separated to be analyzed as baseline or control group (unexposed) and the rest was used as test organisms in the different trials with diesel exhausts.

Fig. 1. *Tillandsia capillaris* Ruiz and Pav.

***Ramalina celastri* (Spreng.) Krog and Swinsc**

Ramalina celastri (**Fig. 2**) is an abundant lichen in Argentina growing mainly on tree branches and shrubs, although it is common to find posts covered by *Ramalina thalli* (Nash 2008, Estrabou et al. 2011). It is a very sensitive lichen species, one of the first to disappear when air pollution increases (González and Pignata 1994).

Fig. 2. *Ramalina celastri* (Spreng.) Krog and Swinsc.

R. celastri was collected in Despeñaderos, within a native relict forest located 45 km SW from Córdoba city. The collection of lichens was carried out using plastic gloves in order to avoid any contamination (González and Pignata 1994). Part of the collected material was separated as baseline or control group (unexposed) and the rest was used as test organisms in the different trials with diesel exhausts.

Exposure chambers and experimental setup

To investigate the effects of diesel exhaust emissions, we exposed the biomonitors in closed glass chambers connected to a diesel truck engine (**Fig. 3a**). Each chamber consisted of a closed glass jar (5 L) with an air intake for the input of diesel emissions and an air output for the exhaust of gases, which generates a constant current flow inside the chambers (**Fig. 3b**). Thus, 10 net bags (per trial) containing samples either of *R. celastri* (8-10 g/bag) or *T. capillaris* (200 g/bag) were put inside the chambers and exposed to diesel emissions during different time periods. A water-cooling system (**Fig. 3c**) was employed to reduce the temperature of the emissions and avoid a high temperature stress on the biomonitors. The exhaust temperature after cooling ranged between 22-26 ºC. The diesel engine was an MWM-International

Fig. 3. Exposure chambers and experimental equipment. (a) Diesel engine; (b) glass chambers with biomonitors bags; (c) cooling system.

Maxion S4, with the following characteristics: displacement: 3990 cm^3; compression ratio: 18.5:1; power: 88 hp in 2800 rpm; cuplemax: kg × m in 1600 rpm: 27.5; consumption every 100 cm^3: 96 s; specific weight of diesel fuel: 0.85 g/cm^3; volumetric performance: 0.7; number of rpm: 1200 rpm.

First, a trial test was performed to adjust the exposure times until differences were observed with the baseline or control group (unexposed). Then, three trials were conducted twice varying the exposure time and the permanence of biomonitors inside the chamber after exposition: (i) 10 min exposure (ii) 20 min exposure, and (iii) 20 min exposure + 48 h inside the exposure chambers, closed hermetically. All the physiological and chemical determinations in the biomonitors after the trials, were performed also in samples of the control groups that were not exposed to any of the trials, in order to obtain baseline conditions.

Physiological variables
Ramalina celastri
Pigments content

One hundred milligrams of lichen material were homogenized in 10 mL of ethanol at 96 % V/V with an Ultra Turrax homogenizer, T18 (1KA Works, USA). By centrifugation, the supernatant was separated and 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 DU 7000 spectrophotometer (Beckman, USA). Concentrations of chlorophylls, carotenoids and phaeophytins were calculated considering the dry weight (DW). The results were expressed as mg g^{-1} DW (Wintermans and de Mots 1965). The Ph a/Chl a ratio was also calculated (Carreras et al. 1998, González et al. 1998).

Peroxidation products

Lichen samples were freeze-dried and 100 mg were homogenized in 2.5 mL of distilled H_2O. An equal volume of 0.5 % 2-thiobarbituric acid in a 20 % trichloroacetic acid solution was added, and samples were incubated at 95 ºC for 30 min. The reaction was cut by placing the tubes in an ice bath and then samples were centrifuged for 30 min at 410 rad/s. After that, the supernatant was removed and the absorption at 532 nm was registered; the value for non-specific absorption at 600 nm was subtracted. Malondialdehyde (MDA) concentration was calculated from the extinction coefficient of 155 mM/cm (Kosugi et al. 1989) with results being expressed in μmol/g DW. Hydroperoxy conjugated dienes (HPCD) were extracted by homogenization of 50 mg of the lichen in 96 % v/v ethanol at 1:50 FW/v ratio with an Ultra Turrax homogenizer. The supernatant was separated and the absorption was measured at 234 nm; the concentration was calculated with the formula $\mathcal{E} = 2.65 \times 10^4$ M/cm (Boveris et al. 1980) and the results expressed as μmol/g DW.

Bioaccumulation variables
Sulfur content in R. celastri

A $Mg(NO_3)_2$ saturated solution was used where 5 mL were added to 0.5 g of freeze-dried lichen and dried in a stove. Then, the sample was heated in an oven for 30 min at 500 ºC and ashes were suspended in 10 mL of a 6 M HCl solution. Then, they were filtered and the resulting solution boiled for 3 min. The solution was brought up to 50 mL with distilled H_2O and the amount of SO_4^{2-} in the solution was determined by the turbidimetric method using barium chloride (González and Pignata 1994). Results were expressed in mg of total sulfur [(S)/g DW].

Heavy metal content in T. capillaris

Approximately 2.5 g of *T. capillaris* dry weight leaf were put at 450 ºC for 4 h in a muffle furnace,

TABLE I. MEAN TEMPERATURE (ºC), ATMOSPHERIC PRESSURE (hPa), FUEL CONSUMPTION (cm^3) AND VOLUME OF GASES EMITTED (m^3) DURING EACH TRIAL.

Trial	Temperature (ºC)	Atmospheric pressure (hPa)	Fuel consumption (cm^3)	Gases volume (m^3)
10 min	19.1	955.0	625	16.75
20 min	20.0	954.8	1250	33.50
20 min + 48 h	19.8	951.1	1237	33.20

then digested with 5 mL concentrated HNO_3 (65 % Merck, Germany) and kept 24 h in the dark (Wannaz and Pignata 2006). Samples were filtered twice using a 2 µm filter paper (Munktell, Germany) and brought to a final volume of 25 mL with ultrapure water (MilliQ). The accumulation of Pb, Cu, Co, Fe, Mn, Zn and Ni were determined by atomic absorption spectrophotometry (AAS, Perkin-Elmer AA3110). The same procedure was done in baseline samples, certified reference material (CTA-OTL-1, Institute of Nuclear Chemistry and Technology) and laboratory blanks to acknowledge the baseline heavy metal content of the species and to evaluate the digestion procedure, respectively. Only residual standard deviation values less than 10 % were accepted with samples outside this range being re-analyzed. Results were expressed in µg/g DW.

Pollution index

The pollution index (PI) for *R. celastri* was determined using the equation 1 (González et al. 1996):

$$PI = [(Ph\ a / Chl\ a) + (S_E / S_F)]\ [(MDA_E / MDA_F) + (HPCD_E / HPCD_F)] \quad (1)$$

Where subindex E indicates the exposed samples and subindex F refers to the freshly picked material (baseline). This index has been widely checked and used in biomonitoring studies with the same species (González and Pignata 1994, González et al. 1996, 1998, 2003, Olcese and Toselli 2002, Rodríguez et al. 2007, Mateos and González 2016).

Data analysis

Statistical analyses were carried out with mean values corresponding to three subsamples from each bag. Assumptions for normality were tested by the modified Shapiro-Wilks normality test and the homogeneity of variance by the Levene's test. The mean and standard deviation for each physiochemical parameter determined in *R. celastri* and heavy metals measured in *T. capillaris* in each trial, were calculated. An analysis of the variance (ANOVA) was performed to compare the results of the four different trials (unexposed samples, exposition during 10 min, exposition during 20 min and exposition during 20 min + 48 h). When the ANOVA null hypothesis was rejected ($p < 0.05$) post-hoc comparisons were performed using the LSD (least significant difference) Fisher posteriori test. In order to analyze relationships between the parameters determined in the biomonitors and the different trials, a multivariate analysis (principal component analysis [PCA]) was carried out. All the analyses were performed employing InfoStat (Di Rienzo et al. 2011).

RESULTS AND DISCUSSION

Trials parameters

Table I shows the temperature, atmospheric pressure, fuel consumption and gas volumes during the different expositions. Emitted gases concentrations were estimated according to engine characteristics, combustion period and amount of fuel consumed (**Table II**). These results are in good agreement with the data showed in Mbuligwe and Kassenga (1997) for a 3.5 t truck/bus with a diesel engine similar to the one employed in this study.

TABLE II. CONCENTRATION RANGES OF DIESEL EXHAUST GAS COMPONENTS IN IDLING CONDITIONS.

Components	NOx (ppm)	NO_2 (ppm)	CO (ppm)
This study	120-150	2-10	520-640
Mbuligwe and Kassenga 1997	< 1.5-300	< 0.5 > 60	440-600

Physiochemical parameters

We first run a 10 min trial exposure, but no significant differences were observed with unexposed samples in any of the parameters measured in *R. celastri* (**Table III**). When we exposed the biomonitors

TABLE III. MEAN VALUES ± STANDARD DEVIATION OF PHYSIOCHEMICAL PARAMETERS MEASURED IN *Ramalina celastri* AND ANALYSES OF VARIANCE (ANOVA) BETWEEN TRIALS.

	Unexposed		10 min		20 min		20 min + 48 h		ANOVA
Chl a	1.129 ± 0.067	A	1.126 ± 0.180	A	1.211 ± 0.142	A	0.834 ± 0.101	B	***
Chl b	0.350 ± 0.017	A	0.382 ± 0.071	A	0.379 ± 0.044	A	0.276 ± 0.033	B	**
Ph a	1.405 ± 0.090		1.200 ± 0.183		1.198 ± 0.249		1.563 ± 0.003		ns
Ph b	0.360 ± 0.019		0.367 ± 0.058		0.374 ± 0.036		0.330 ± 0.026		ns
Carot	0.304 ± 0.012	A	0.303 ± 0.046	A	0.315 ± 0.039	A	0.206 ± 0.027	B	***
Ph a/Chl a	1.062 ± 0.004	B	1.067 ± 0.014	B	1.065 ± 0.013	B	1.205 ± 0.060	A	***
Chl b/Chl a	0.339 ± 0.008	AB	0.338 ± 0.012	AB	0.325 ± 0.016	B	0.402 ± 0.067	A	*
HPCD	5.347 ± 0.060	B	11.831 ± 0.769	AB	12.495 ± 1.817	A	15.318 ± 0.255	A	***
MDA	0.109 ± 0.015	C	0.112 ± 0.017	C	0.136 ± 0.012	B	0.156 ± 0.013	A	**
Sulfur	0.808 ± 0.034	C	0.895 ± 0.097	BC	1.003 ± 0.111	B	1.281 ± 0.030	A	**
PI	3.787 ± 0.052	B	3.854 ± 0.047	B	3.727 ± 0.156	B	4.209 ± 0.307	A	*

Chl a: chlorophyll a; Chl b: chlorophyll b; Ph a: phaeophytin a; Ph b: phaeophytin b; Carot: carotenoids; HPCD: hydroperoxy conjugated dienes; MDA: malondialdehyde; PI: pollution index; ns: not significant.
Values in each horizontal line followed by the same letter do not differ significantly.
*Significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level.

to a 20 min period, no significant differences were observed in pigment contents. However, an increase in MDA levels, PI and sulfur content was observed, indicating the presence of compounds that produced some physiological damage in the biomonitor. When lichens were exposed for 20 min and left 48 h in the closed chamber, all physiological parameters were significantly altered. A notable decrease in Chl a, Chl b and carotenoids concentration was observed, which is consistent with the findings of Langmann et al. (2014), who observed in another lichen species that diesel exhaust affects the photosynthetic apparatus, decreasing pigments content and altering its ability to perform photosynthesis. Chl a is the most important photosynthetic pigment, while Chl b and carotenoids function as photoprotective pigments (Han et al. 2017). Besides, carotenoids are capable of eliminating some free radicals from the chloroplast, therefore a decrease in these pigments increases the vulnerability of the chloroplast to the oxidative damage of free radicals, causing a severe damage to membranes and associated molecules. Moreover, an alteration of antioxidant systems in plants exposed to atmospheric pollutants have been already observed (González and Pignata 1994, Levin and Pignata 1995, Carreras et al. 1998, Munzi et al. 2012). The decrease in carotenoids concentration could explained the increase in biomarkers related to lipid peroxidation of cell membranes, MDA and HPCD, in the 20 min + 48 h trial.

One of the main gases emitted by diesel exhaust is SO_2 (Liu et al. 2016), in addition to sulfates formed by oxidation of sulfur in fuels and lubricant (Tan et al. 2017); therefore, the highest sulfur values measured in the 20 min + 48 h trial could be attributed to diesel emissions. Like all the parameters analyzed above, the PI also showed the highest values in the 20 min + 48 h trial. This prolonged permanence of biomonitors in the exposed chamber could be compared with the exposition in heavy polluted urban environments.

In order to identify the parameters that best explain the variability of the data, a multivariate analysis (PCA) was performed using the physiochemical determinations as variables and the different trials as classification criteria (excluding the 10 min that showed no effect). Eigenvalues corresponding to the first two components are shown in **table IV**. The eigenvalues associated with the axis and the different trials are indicators of the extent to which the lichen response explains laboratory trial variables. The first two axes (PC1 and PC2) explained most of the total variance, where the first axis (PC1) was mostly determined by individual pigments, the ratios and the sulfur accumulation, and the second (PC2) was driven by the PI and HPCD (**Table IV**), reflecting that these two biomarkers are less useful to identify diesel exhaust effects on lichens. The biplot (**Fig. 4**) shows that all damage indicators (Chl b/Chl a, Ph a/Chl a, sulfur, MDA, PI and in a lesser extent HPCD) were associated with the 20 + 48 h trial, in agreement with the results informed in **table III**. It is interesting to note that the PI was associated to a lesser extent to the 20 min exposure, confirming this indicator is sensitive enough to reflect physiological damage caused by vehicular emissions, as seen in previous studies with the same species (González et al. 1996,

TABLE IV. EIGENVECTORS CORRESPONDING TO THE FIRST TWO PRINCIPAL COMPONENTS OBTAINED IN THE PRINCIPAL COMPONENT ANALYSIS FOR THE PHYSIOCHEMICAL PARAMETERS DETERMINED IN *Ramalina celastri* EXPOSED TO DIESEL EXHAUST.

Parameters	Component	
	1	2
Chl a	0.31	0.10
Chl b	0.31	–0.03
Ph a	0.31	–0.08
Ph b	0.29	0.30
Carot	0.31	0.09
HPCD	–0.12	0.69
MDA	–0.31	0.15
Ph a/Chl a	–0.30	–0.25
Chl b/Chl a	–0.28	–0.32
Sulfur	–0.31	–0.09
PI	–0.26	0.42

Chl a: chlorophyll a; Chl b: chlorophyll b; Ph a: phaeophytin a; Ph b: phaeophytin b; Carot: carotenoids; HPCD: hydroperoxy conjugated dienes; MDA: malondialdehyde; PI: pollution index.

1998, 2003, Rodríguez et al. 2007, Mateos and González 2016). These results indicate that 20 min of high exposure to diesel exhaust are not enough to produce a considerably damage in the lichen.

Heavy metals accumulation

The mean concentrations (μg/g DW) of Pb, Cu, Ni, Co, Fe, Mn and Zn accumulated in samples of *T. capillaris* as well as the ANOVA results between trials (unexposed; 10 min; 20 min; 20 min + 48 h) are shown in **table V**. A significant increase in the concentration of all elements was observed in the 20 min + 48 h trial. These results could be explained by the fact that besides primary particles that are directly emitted from a source, PM can also be formed in the air due to some physical and chemical processes (secondary particles) where condensation or homogenous nucleation of gas species onto existing particles are among the physical processes that can result in secondary particle formation (Esmaeilirad and Hosseini 2018). Moreover, particles of diesel exhaust have large and irregular surfaces that facilitate the adsorption of different organic and inorganic materials from the environment or from the engine exhaust itself (Wernke 2014). Also, the values found in this study are in good agreement with those shown in Mateos et al. (2018b) for the same specie exposed for six months in the downtown area of Córdoba city.

The levels of Ni and Fe were also high in the 20 min trial, suggesting that even short exposure periods can induce changes in elemental concentrations. These results are in good agreement with studies that indicate that besides gases, particle-bound heavy metals are also abundant in diesel emissions (Wernke 2014, Wang et al. 2018). Even though the Co emission from diesel fuel is not frequently reported (Fuga et al. 2008), in the present study we observed an increase even in samples exposed during 10 min to diesel emissions. This difference could be attributed to the fact that the in the present study we did not use a catalytic converter neither a

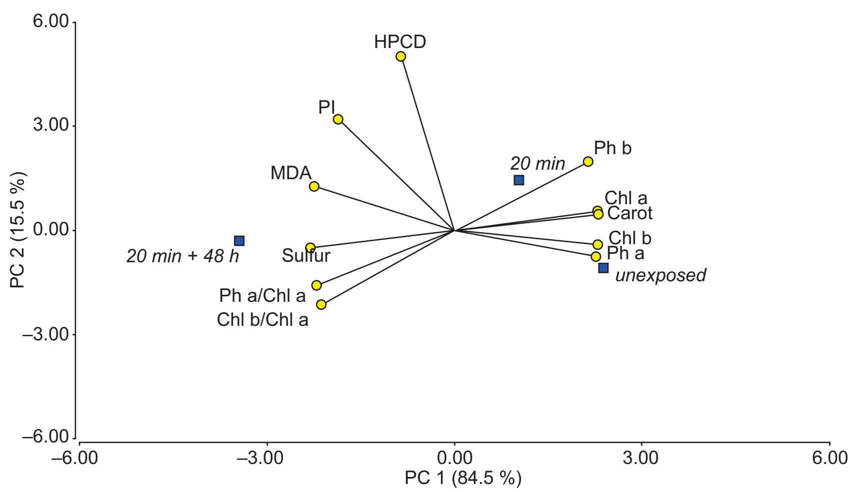

Fig. 4. Biplot based on the two principal components (PC1 and PC2) of the principal component analysis for physiochemical parameters determined for *Ramalina celastri* in the trials' categories (unexposed; 20 min; 20 min + 48 h).

TABLE V. MEAN VALUES (µg/g DW) ± STANDARD DEVIATION OF HEAVY METAL ACCUMULATED IN *Tillandsia capillaris* AND ANALYSES OF VARIANCE (ANOVA) BETWEEN TRIALS.

	Unexposed				10 min				20 min				20 min + 48 h				ANOVA
Pb	10.73	±	0.81	C	12.15	±	0.90	BC	14.20	±	1.49	B	18.31	±	0.66	A	***
Cu	5.37	±	0.86	B	5.09	±	0.66	B	5.19	±	0.99	B	7.35	±	1.42	A	***
Ni	2.11	±	0.01	B	3.02	±	0.70	B	5.60	±	1.32	AB	7.31	±	3.30	A	*
Co	1.16	±	0.12	B	2.15	±	0.93	AB	2.36	±	1.18	A	2.90	±	0.66	A	*
Fe	974.68	±	102.10	B	1598.23	±	365.12	B	3773.33	±	378.25	A	3962.54	±	321.65	A	**
Mn	49.44	±	2.82	B	94.48	±	15.850	AB	96.97	±	47.26	AB	137.60	±	18.30	A	*
Zn	12.28	±	1.07	C	18.27	±	1.460	BC	23.39	±	1.79	B	29.82	±	2.07	A	***

Values in each horizontal line followed by the same letter do not differ significantly.
*Significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level.

particle filter, as is the situation of public transport in Córdoba city.

It is noteworthy that the concentrations of Pb, Mn and Zn were significantly higher in exposed samples, even during short time periods in agreement with the fact that these metals have been found in high levels in particles collected from areas with intense traffic (Mateos et al. 2018b). Metals in diesel emissions can derive from different sources such as fuel alone or from the use of additives; however, most of them derive from lubricating oil and by-products of engine wear that enter the combustion chamber (Giordano et al. 2010). Elements that can be attributed to traffic have been found at remarkably high levels in the exhaust of diesel vehicles and to a lesser extent in gasoline powered vehicles (Monaci et al. 2000). Particularly, the presence of Pb, Cu, Ni, Fe, Mn and Zn in diesel emissions has been confirmed before (Monaci et al. 2000, Geller et al. 2006, Fuga et al. 2008, Hu et al. 2009); therefore, the effect of some of these metals together with some powerful oxidant gases, such as SO_2, could be the responsible for the noticeable damage observed in the biomonitor *R. celastri*, as reflected earlier by the PI. To globally observe the relationship of heavy metals accumulated in the biomonitor with the different trails, a PCA was performed. **Table VI** shows the eigenvectors values, where PC1 is mostly explained by the concentrations of Pb, Ni, Co, Mn, and Zn, all of which have the greatest association with diesel emissions (Giordano et al. 2010). PC2 was associated with Cu and Fe concentrations in the biomonitor, which is consistent with the findings of Bermúdez et al. (2012), who suggested that Fe and Cu concentrations are indicators of anthropogenic emissions related to soil resuspension and metallurgical and metal-mechanical activities, respectively. **Figure 5** presents the biplot obtained with the PCA analysis showing the strong association of heavy metals with the 20 + 48 h trial, confirming what was observed in the ANOVA test.

TABLE VI. EIGENVECTORS CORRESPONDING TO THE FIRST TWO COMPONENTS OBTAINED IN THE PRINCIPAL COMPONENT ANALYSIS FOR THE HEAVY METAL ACCUMULATION DETERMINED IN *Tillandsia capillaris* EXPOSED TO DIESEL EXHAUST.

Heavy metal	Component	
	1	2
Pb	0.41	–0.16
Cu	0.31	–0.58
Ni	0.42	0.05
Co	0.42	0.07
Fe	0.29	0.63
Mn	0.38	0.37
Zn	0.39	–0.33

CONCLUSIONS

The laboratory trials performed in the present study demonstrate that pollutants coming from diesel engines might cause physiological damage in biomonitors, even with exposures as short as 20 min. In addition, an increase in the damaging effect and larger metal accumulation was observed when the exposure time of biomonitors was augmented. Thus, larger concentrations of secondary pollutants and more damaging effects on biomonitors are expected with time inside the chamber, since secondary pollutants are much more reactive and damaging than primary ones.

On the other hand, a clear effect of pollutants from diesel engines was observed on the integrity

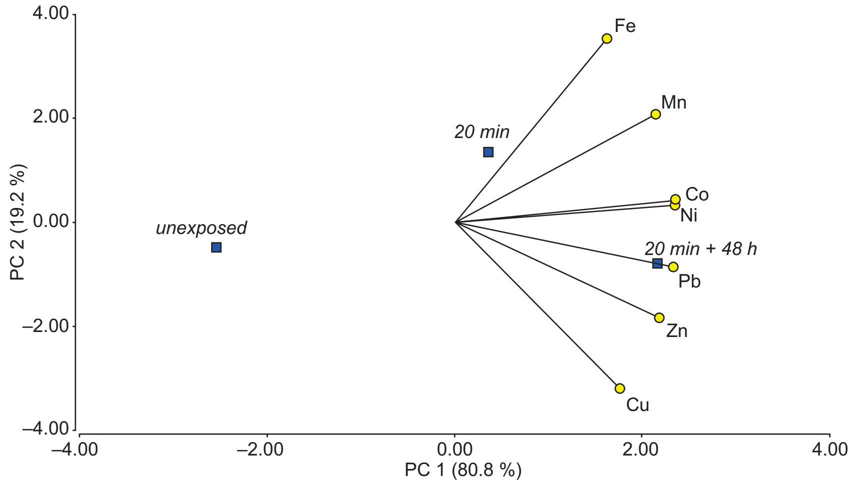

Fig. 5. Biplot based on the two principal components (PC1 and PC2) of the principal component analysis for heavy metals accumulation determined for *Tillandsia capillaris* in the trials' categories (unexposed; 20 min; 20 min + 48 h).

of the photosynthetic apparatus. This is consistent with previous studies where it was observed that diesel emissions were significantly related to the chlorophyll degradation index. In addition, diesel exhaust pollutants cause a marked decrease in carotenoid content, which increases the susceptibility of biomonitors to oxidative damage caused by free radicals.

Another noticeable result were the high levels of Pb accumulated by *T. capillaris* after a 20 min exposition to vehicle emissions. Despite the fact that this metal is no longer used in fuels since the 1990s, it is possible that vehicles are still sources of Pb from lead plates that line the fuel tanks, lead in vulcanized gasoline tubes or in the lining of pistons, valve seats and spark plugs. The results of the present study evidence the need to regulate Pb emissions coming from vehicles, considering that this metal has known toxicological and carcinogenic effects. On the other hand, the results obtained here are a first approximation to establish that pollutants emitted by diesel exhaust have a notable effect on biomonitors. This confirms the assumption that traffic is one of the main and most worrying sources of air pollutants in urban areas. More studies evaluating other air pollutants present in urban areas are necessary to complement the preliminary results of this work.

Overall, the results presented in here are particularly relevant for cities with a large proportion of old vehicles, whose atmospheres could be similar in composition to the one simulated in the chambers employed in this study.

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