Atmospheric Environment 210 (2019) 132–142

Contents lists available at ScienceDirect

Atmospheric Environment

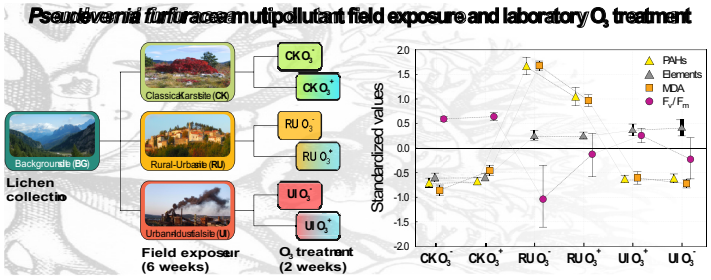
journal homepage: www.elsevier.com/locate/atmosenv

Beyond ozone-tolerance: Effects of ozone fumigation on trace element and PAH enriched thalli of the lichen biomonitor *Pseudevernia furfuracea*

Elva Cecconi[a], Lorenzo Fortuna[a], Elisa Pellegrini[b,c], Stefano Bertuzzi[d], Giacomo Lorenzini[b,c], Cristina Nali[b,c], Mauro Tretiach[a,*]

[a] Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, 34127, Trieste, Italy
[b] Department of Agriculture, Food and Environment, University of Pisa, Via Del Borghetto 80, 56124, Pisa, Italy
[c] CIRSEC, Centre for Climatic Change Impact, University of Pisa, Via Del Borghetto 80, 56124, Pisa, Italy
[d] Via Mazzini 2, 10066, Torre Pellice, Turin, Italy

GRAPHICAL ABSTRACT



Keywords:
Air pollution
O_3
Bioaccumulation
Malondialdehyde
Chlorophyll *a* fluorescence
Potassium leakage

ABSTRACT

In this study, the effects of ozone (O_3) on the physiology of the lichen *Pseudevernia furfuracea* var. *furfuracea* previously subjected to field stressing conditions were assessed. Samples collected in a pristine site were exposed for 6 weeks at 3 sites characterized by different pollution, *e.g.* elemental and PAH depositions (site RU, close to wood-burning house chimneys; site UI, close to cast-ironworks; site CK, in a semi-natural context). Afterwards, samples were transferred to controlled fumigation chambers, where they were either O_3-treated for 2 weeks (250 ppb O_3 for 5 h day^{-1}, O_3^+ samples) or not (0 ppb O_3, O_3^- samples). Three physiological markers (F_v/F_m, maximum quantum yield of primary photochemistry; MDA, malondialdehyde content; potassium leakage) as well as elemental and PAH concentrations were measured in matched sets of sample replicates at each experimental step. Data were explored by multivariate techniques and the effects of field exposure and fumigation were tested by generalized linear models (GLM). Detrimental effects on MDA and F_v/F_m were observed limited to samples exposed in RU and UI sites. Physiological parameters in O_3-treated samples showed heterogeneous variation patterns with respect to field-exposed ones. A recovery of F_v/F_m was observed in RU- and UI-exposed samples, whereas a significant increase of MDA was highlighted limited to CK O_3^+ and CK O_3^- samples, possibly related to a "chamber effect". Overall, the impairment caused by ozonation was limited, proving the strong O_3-tolerance of our test species. Interestingly, the content of the most abundant 4-ring PAHs in RU O_3^+ samples, which underwent the highest field enrichment of PAHs, was significantly lower than that of matched RU O_3^- samples. This suggested a possible role of ozone in degrading PAHs at thallus level, with interesting

Abbreviations: Polycyclic Aromatic Hydrocarbons, (PAHs); Ace, acenaphthene; Acy, acenaphthylene; Ant, anthracene; B[a]Ant, benzo[a]anthracene; B[ah]Ant, dibenzo[a,h]anthracene; B[a]Py, benzo[a]pyrene; B[b]Fl, benzo[b]fluoranthene; B[e]Py, benzo[e]pyrene; B[ghi]Per, benzo[g,h,i]perylene; B[j+k]Fl, benzo[j+k]fluoranthene; Chry, chrysene; F, fluorene; Fl, fluoranthene; I[cd]Py, indeno[1,2,3-cd]pyrene; N, naphthalene; P, phenanthrene; Py, pyrene

* Corresponding author.
E-mail address: tretiach@units.it (M. Tretiach).

https://doi.org/10.1016/j.atmosenv.2019.03.026
Received 21 February 2019; Received in revised form 21 March 2019; Accepted 23 March 2019
Available online 24 March 2019

interpretative repercussions in the context of transplant-based surveys aimed at evaluating PAH depositions when O_3 ground levels are high.

1. Introduction

Tropospheric ozone (O_3) is a strongly oxidizing secondary air pollutant and greenhouse gas (Logan, 1985; Wu et al., 2008), formed by a series of photochemical reactions between nitrogen oxides (NO_x) and volatile organic compounds (Hassan et al., 2013; Paoletti et al., 2017; Lefohn et al., 2018), and favoured by high temperatures. Ozone annual averages are increasing at both urban and rural sites, being an important component of global change (Paoletti et al., 2014).

In heavily polluted areas, ozone exposure effects are acknowledged at physiological, biochemical and molecular level (Goumenaki et al., 2010) on several organisms, including humans (Chen et al., 2017; Nuvolone et al., 2018) and plants (Nali et al., 2007; Paoletti, 2007; Saitanis, 2008; Sarkar et al., 2010). The O_3-sensitivity of plants is highly variable (Schraudner et al., 1998) and mostly related to their ability in detoxifying Reactive Oxygen Species (ROS; Frei et al., 2010). Several ROS detoxification mechanisms are known to occur in plants, and some of these are even acknowledged as variety-specific. A case in point in this regard is *Nicotiana tabacum*, with its O_3-supersensitive and O_3-tolerant cultivars (Bel-W3 and Bel-B, respectively), that allowed the development of a standardized protocol to biomonitor ambient air O_3 concentrations (EN 16789:2016), as well as to produce miniaturized plantlet kits for outdoor O_3 biomonitoring (Lorenzini, 1994; Nali et al., 2007; Lorenzini and Nali, 2018).

Besides plants, the effects of O_3 were also investigated in lichens. These symbiotic organisms, widely used as biomonitors of atmospheric pollution, are sensitive to a variety of gaseous pollutants, but their response to O_3 is still debated. Field studies carried out along oxidant gradients, coupled with the analysis of herbarium samples, highlighted residual amounts or no occurrence of once abundant species (mostly cyanolichens: *Collema nigrescens*, *Peltigera* spp., *Pseudocyphellaria* spp., but also chlorolichens of the genus *Usnea*) at sites that experienced a substantial increase of O_3 concentrations (Sigal and Nash III, 1983; Nash III, 2008). However, several recent studies carried out in open top chambers and fumigation chambers highlighted a noticeable O_3-tolerance of chlorolichens for a wide range of O_3 concentrations [(0-) 10–250(-50′000) ppb]. In these studies, the lichen response to O_3 was generally investigated (i) in fully or partially controlled environments (*e.g.*, Brown and Smirnoff, 1978; Nash et al., 1979; Ross and Nash III, 1983; Sigal and Johnston, 1986; Scheidegger and Schroeter, 1995; Tarhanen et al., 1997; Riddell et al., 2010; Bertuzzi et al., 2013, 2018; Pellegrini et al., 2014; Vannini et al., 2018), with the rationale of controlling/minimizing the effects related to accessory environmental causes of physiological stress, and (ii) directly in the field, "*en plein air*", with the aim of assessing and/or disentangling the physiological effects caused by O_3 when co-occurring with other gaseous pollutants (such as NO_x, gaseous HNO_3 and SO_2; Egger et al., 1993; Riddell et al., 2012; Tretiach et al., 2012). The evaluation of composite effects of pollutants on the physiology of biomonitors is receiving increasing interest in the last years, because there is an urgent need to study the behaviour of biomonitors in response not to single xenobiotics, but to their naturally occurring mixtures (*e.g.*, Sujetovienė and Galinytė, 2016). This knowledge is in fact necessary to correctly convert the information they can give into air quality assessment.

The fruticose macrolichen *Pseudevernia furfuracea* var. *furfuracea* (L.) Zopf. is a bioaccumulator of extensive use in active biomonitoring (*e.g.*, Sloof, 1995; Tretiach et al., 2011; Nascimbene et al., 2014; Kodnik et al., 2015), being locally abundant (Cecconi et al., 2018) and tolerant to several gaseous phytotoxic pollutants (Miszalski and Niewiadomska, 1993; Tretiach et al., 2007; Malaspina et al., 2018), on account of an efficient antioxidant machinery, that permits the survival of this species in environments characterized by high UV, high light, and low temperatures. In a study based on field fumigation chambers, this chlorolichen was defined as O_3-tolerant by Scheidegger and Schroeter (1995), because no detrimental effects were observed either at ultrastructural or at functional level (chlorophyll *a* fluorescence, Chl_aF, being used as a proxy of photosystem functionality). In their experiments, as done in the majority of the above cited works, the authors used healthy samples purposely collected in "pristine" environments.

Aim of this study was to evaluate the response to O_3 of *P. furfuracea* thalli, still collected in "pristine" environments as done by Scheidegger and Schroeter (1995), but preliminary subjected to a mixture of multi-origin pollutants and thus enriched in (*e.g.*) heavy metal-rich particulate matter, PAHs, etc. Our test hypothesis was that previously field-stressed thalli would exhibit physiological impairment due to subsequent ozonation treatment. The physiological status of samples was assessed after each experimental step using a multi-marker approach encompassing both symbiotic partners. Biomarkers included the chlorophyll *a* fluorescence (Chl_aF), the content of malondialdehyde (MDA), and the leakage of potassium ions (K^+ leakage), respectively considered as proxies of photosynthetic activity of algal population, peroxidation of membrane lipids, and membrane integrity.

2. Materials and methods

2.1. Lichen material, collection and sample pre-processing

Pseudevernia furfuracea is a meso-xerophilous lichen, growing on nutrient-poor, acid bark substrata (Nimis, 2016). The species is spread in cool temperate areas, where it may be locally very common (Rikkinen, 1997; Smith et al., 2009). The dorsi-ventral thallus is richly branched with upper surfaces often covered by finger-shaped outgrowths (isidia), which considerably increase the exchange surface per area and mass unit (Tretiach et al., 2005) and the particle entrapment (Bargagli and Mikhailova, 2002). The large thallus size and the easy identification in the field ensure fast sampling and preparation. *P. furfuracea* has also been targeted in several methodological studies, aimed at providing standardized methodologies (*e.g.*, Adamo et al., 2007, 2008; Incerti et al., 2017) and improving data quality in biomonitoring (Cecconi et al., 2018).

Thalli of *P. furfuracea* var. *furfuracea* were collected from isolated larch trees (*Larix decidua* L.) in a background area (henceforth BG; Cecconi et al., 2018) of the Carnic Alps (Lateis, NE Italy) at 1500 m a.s.l. Thalli, still attached to *c.*15–20 cm long twigs, were transported to the laboratory in paper bags and left to dry out in dim light at room temperature for 24 h. The lichen material was carefully cleaned from bark fragments, debris and other lichen and moss species. Moderately isidiate thalli of comparable size and branching, without sexual reproductive structures, were selected for the experimentation (Tretiach et al., 2007, 2011; Incerti et al., 2017). Different sets of 5 sample replicates each were obtained from the bulk lichen material with the aim of assessing physiological parameters, elemental and PAH content before the experimental treatments (henceforth 'pre-exposure' or BG samples): these samples were dehydrated in silica gel for 48 h, vacuum sealed and stored at −20 °C until the end of the experiment. The remaining lichen material was mounted on exposure devices. A single exposure device consisted in a 120 cm long wooden rod bearing thalli still attached on their twigs in a sufficient amount to build up sets of 3 sample replicates to assess physiological parameters, elemental and PAH content after field exposure and subsequent fumigation.

2.2. Experimental design: field exposure and O_3 fumigation

In order to evaluate the physiological response of our test species to O_3, a double-step experiment was planned. Firstly, *P. furfuracea* thalli were exposed for 6 weeks at three sites with different pollutant loads (mostly elemental and PAH depositions). The field exposure was followed by 2 week-stay in fumigation chambers, where samples were either O_3-treated or not. At each experimental step (*i.e.*, before and after the field exposure and after the controlled fumigation), the set of selected physiological markers, as well as elemental and PAH concentrations were measured in matched sets of sample replicates.

The field exposure was carried out in the Trieste province (NE Italy), between February 18th and April 4th, 2016 in order to avoid summer-like ambient O_3 levels (Nali et al., 2007). Lichen samples, mounted on the exposure devices, were transplanted at three sites, characterized by different land use: i) a semi-natural site in the Classical Karst (CK), far from major sources of anthropogenic pollution; ii) a rural-urban site (RU) in the Classical Karst, where the lichen samples were purposely placed close to wood-burning house chimneys with intense activity; iii) an urban-industrial site (UI) in the city of Trieste, close to a large operating cast-ironwork (ARPA FVG, 2018a; Supplementary Fig. S1). During field exposure, air temperature and relative humidity were continuously collected at the three sites by EL-USB-2 data loggers. Exposure devices were secured to artificial supports at 4 m above the ground. After 6 weeks, lichen samples were retrieved, sealed, and transported in cool bags to the laboratory. A first set of 3 samples per exposure site was immediately processed to measure the physiological parameters (sect 2.4), while a second set was processed and stored for the determination of elemental and PAH content (sect. 2.3) at the end of the experiment (field-exposed samples; Table 1). Further two matched paired groups of 3 samples per exposure site were sealed and transported to University of Pisa, where the fumigation was carried out.

Samples were placed for 2 weeks in a ventilated 0.90 × 0.90 × 0.65 cm Perspex chamber with the inlet air (two complete air changes min^{-1}) either subjected or not to 250 ± 4 ppb O_3 (herein, the notation O_3^+ and O_3^- refers to samples either O_3-treated or not; Table 1), provided for 5 h day^{-1} in form of a square wave generated by a Fisher 500 air-cooled apparatus (Zurich, CH) supplied with pure oxygen. The sample watering, necessary to secure a minimum metabolism for *c.* 3 h day^{-1}, was performed by spraying *c.* 0.01 mL cm^2 dH_2O in the morning, immediately before the input of O_3 (Bertuzzi et al., 2018). A photosynthetic photon flux density (PPFD) of 60 μmol photon m^{-2} s^{-1} was provided for 12 h day^{-1} by four quartz metal halide lamps with clear outer bulb (400 W, MASTER HPI-T Plus, Philips, NL) and by four high-pressure sodium lamps with clear tubular outer bulb (250 W, SON-T, Philips, NL).

After fumigation, samples were dried at room temperature in dim light for 12 h. Consequently, O_3^+ and O_3^- samples were processed to assess the physiological parameters, elemental and PAH content as described below.

Table 1
List of experimental factors and description of their levels.

Experimental factor	Levels and description	
Field exposure (*Exp.*)	CK	Samples exposed in the proximate-natural site in the Classical Karst.
	RU	Samples exposed in the rural-urban site, close to wood-burning house chimneys.
	UI	Samples exposed in the urban-industrial sites, close to an operating cast-ironworks.
Fumigation (*Fum.*)	CK O_3^+ RU O_3^+ UI O_3^+	Samples exposed in the field sites, then ozonated in fumigation chambers.
	CK O_3^- RU O_3^- UI O_3^-	Samples exposed in the field sites, non-ozonated but kept in fumigation chambers at the same environmental conditions of O_3^+ samples.

2.3. Analytical procedures

Pre-exposure (BG), field-exposed (CK, RU, UI) and fumigated (CK O_3^-, CK O_3^+, RU O_3^-, RU O_3^+, UI O_3^-, UI O_3^+) samples were singly processed for the determination of elemental and PAH content.

2.3.1. Elemental content

Samples earmarked for elemental analysis were dried out for 12 h, and terminal lobes of 2.5 cm were selected to assemble 1 g samples, which were pulverized with a planetary ball mill (Retsch PM100); the resulting powder was dried overnight at 40 °C and stored in microtubes (Bargagli and Nimis, 2002). Afterwards, the batch was submitted to the determination of the content of 25 elements (Al, As, Ba, Ca, Cd, Cr, Cu, Fe, K, Li, Mg, Mo, Mn, Na, Ni, P, Pb, S, Sb, Sc, Se, Sn, Ti, V, Zn). Replicate splits of 0.25 g were digested in a HNO_3—$HClO_4$—HF solution until fuming and then dried; the resulting residue was dissolved in 50% HCl solution and heated. The elemental content was determined by a Perkin Elmer Elan 6000 ICP mass spectrometer and all the values were expressed on a dry weight (DW) basis (μg g^{-1}). Accuracy was expressed in terms of mean recovery percentages, calculated as the ratio between the certified concentration values for the standard reference material BCR 482 (Quevauviller et al., 1996) and those measured in aliquots of the same standard blindly included in the batch (Supplementary Table S1).

2.3.2. PAHs

Samples earmarked for PAH analysis were dried out for 12 h, and terminal lobes of 2.5 cm were selected to assemble 1.5 g samples, which were finely chopped with ceramic scissors, sealed in glass jars and kept in the dark at 4 °C until analytical determination (Augusto et al., 2013). The content of 16 EPA priority PAHs (*i.e.*, Ace, Acy, Ant, F, P, Chry, Fl, Py, B[a]Ant, B[ah]Ant, B[a]Py, B[b]Fl, B[e]Py, B[j+k]Fl, I[cd]Py, B[ghi]Per) plus B[e]Py was measured. Prior to analysis, PAHs were extracted from lichen samples using 30 mL of a mixture of hexane/dichloromethane (1:1) in a Milestone Start E Microwave Extraction System, according to the US EPA method 3546 (EPA, 2018a). Subsequently, samples were purified by Supelclean™ LC-NH_2 SPE tube (bed wt. 500 mg), filtered, concentrated to 1 mL and further evaporated. The PAH content was determined by means of gas chromatography-mass spectrometer (GC-MS triple quadrupole, Bruker, model TQ300), in accordance to the US EPA method 8270 (EPA, 2018b). All the values were expressed on DW basis (ng g^{-1}). Accuracy of the analytical procedure was assessed in terms of measured-to-expected concentrations of deuterated PAH standards added to the experimental samples prior to the extraction.

2.4. Physiological measurements

The physiological status of lichen samples was assessed by measuring the chlorophyll *a* fluorescence (Chl_aF), the content of malondialdehyde (MDA), and the leakage of potassium ions (K^+ leakage).

2.4.1. Chlorophyll a fluorescence

Chl_aF was assessed in terms of the maximum quantum yield of primary photochemistry in dark adapted samples (F_v/F_m) and non-photochemical quenching (NPQ). Measurements were carried out on 6 lobes (60 ± 5 mg each) per sample, randomly selected for each experimental group. Before Chl_aF measurements, samples were hydrated in a glass jar at 100% relative humidity (RH) for 24 h (PPFD, 29 ± 2 μmol photons m^{-2} s^{-1}; 18 ± 1 °C; 12 h dark/12 h light) and consequently rinsed for 3 min in dH_2O. Afterwards, selected lobes were gently shaken to remove the excess of dH_2O and dark-adapted for 30 min. Chl_aF measurements were taken with a pulse-amplitude-modulated fluorometer PAM-2000 (Walz, Effeltrich), positioning the measuring fibre at 60° on the upper surface of the lobes. The modulated light was turned on to obtain the minimal Chl_aF level (F_0). A saturating

light pulse of *c*. 8000 μmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain the transient maximum Chl_aF level (F_m) and thus to calculate the variable Chl_aF level (F_v, *i.e.* $F_m - F_0$) and the maximum quantum efficiency of PSII photochemistry (F_v/F_m). An external actinic light provided by a light unit FL-460 (Walz, Effeltrich, D) with a halogen lamp was turned on to record the Kautsky effect at an intensity of 176 μmol photons m^{-2} s^{-1}. Such value is consistent with the species-specific $PPFD_{Ik}$ value, *i.e.* the photosynthetic photon flux at which the quantum yield of CO_2 assimilation is the highest (Piccotto and Tretiach, 2010). Once the emission peak was achieved (F'_m), saturating light pulses were applied at 60 s intervals during actinic illumination to determine NPQ (see *e.g.*, Baker, 2008; Bussotti et al., 2011). NPQ was calculated as $(F_m - F'_m)/F'_m$. Chl_aF measurements were repeated on the same lobes after 48 h recovery, at 100% RH and 29 μmol photons m^{-2} s^{-1}.

2.4.2. MDA assay

Lipid peroxidation was determined by the thiobarbituric acid reactive substances (TBARS) assay following the method proposed by Candotto Carniel et al. (2017) and based on Heath and Packer (1968). Three lobe samples (200 mg each) for each experimental group were pulverized with liquid nitrogen, lyophilized, homogenized in a mortar using 1.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12000*g* for 20 min at room temperature. An aliquot of 0.5 mL of the supernatant was collected and mixed with 1 mL of 20% TCA with 0.5% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 25 min, quickly cooled in an ice bath and centrifuged at 15000*g* for 10 min at room temperature. The supernatant was removed and used to determine MDA concentration. Absorbance readings were taken at 532 nm using a Jenway 7315 UV–vis spectrophotometer (Stone, UK) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results were expressed as nmol g^{-1} (DW).

2.4.3. K^+ leakage

In order to investigate membrane damage, K^+ leakage was measured on 3 lobes (60 ± 5 mg each) per sample, randomly selected for each experimental group. K^+ leakage was measured following the method proposed by Candotto Carniel et al. (2017). Terminal lobes were rinsed in 25 mL of dH_2O and continuously agitated at 100 rpm for 60 min. Afterwards, lobes were removed from suspensions, oven-dried at 80 °C for 24 h and weighed. The remaining dH_2O was filtered (0.45 μm) and stored at 4 °C until analysis. Acqueous K^+ concentration was determined by flame atomic adsorption spectroscopy (Perkin Elmer Analyst 400) with an uncertainaty less than 2%. The limit of detection (LOD) at the operative wavelength of 766.5 nm was 0.01 mg l^{-1}. Microtubes with dH_2O only were used as blanks. K^+ leakage was expressed as mg of leached K^+ per g (DW) of lichen lobes.

2.5. Data analysis

Data were organized in a matrix of 27 cases (3 replicates per 9 experimental groups) × 34 variables. Variables included 25 chemical elements (sect. 2.3.1), 5 PAH categories (2-, 3-, 4-, 5- and 6-rings), the sum of all PAHs (Σ PAHs), and 3 physiological parameters (F_v/F_m, MDA and K^+ leakage). Amongst the fluorescence parameters, NPQ was not included due to its high correlation with F_v/F_m values (Spearman's rho 0.91, $p < 0.01$). The matrix was preliminarily submitted to explorative multivariate analysis. In particular, a hierarchical Cluster Analysis (CA) was performed on the data matrix after a classic standardization of values (standardized values were obtained from the original values by subtraction of the mean of all samples, and dividing the result by the standard deviation), in order to ensure the full commensurability of variables (Podani, 2007). CA was performed for the set of 25 elements with Euclidean distance as distance measure and Ward's method as grouping algorithm. A Principal Component Analysis (PCA) based on correlations among the variables was performed on the data matrix reporting non-standardized values. In the PCA, the land use classes of the exposure sites (Table 1) were plotted as supplementary variables following the approach suggested by Legendre and Legendre (1998). In addition, significant differences among physiological parameters in experimental samples were tested using Kruskal-Wallis ANOVA and non-parametric Dunn's post hoc test (Dinno, 2017).

In order to disentangle the effects of the field exposure and O_3-treatment on the physiological state of lichen thalli, generalized linear models (GLMs) were fitted, limited to the data matrix of paired fumigated sample replicates. Main and interactive effects of field exposure (fixed effect with 3 levels, Table 1) and fumigation (fixed effect with 2 levels, Table 1) were tested, considering physiological variables, PAH and elemental content as dependent variables. In particular, standardized values for each target variable were used, separately considering each value as an individual observation.

All data analyses and graphics were performed with the software packages Statistica v. 10 (StatSoft Inc., Tulsa, OK, USA) and R (R Core Team, 2013). Statistical significance was tested at $\alpha = 0.05$ in all cases.

3. Results

3.1. Trace element and PAH enrichment in field-exposed thalli

Samples exposed for 6 weeks in the field sites CK, RU and UI (Supplementary Fig. S1) exhibited different PAH and elemental content.

Expectedly, samples exposed in the semi-natural site CK did not show significant variation with respect to background situation for the overall PAH and element content as well as for each of the three element clusters, as identified by the CA (Supplementary Fig. S2). On the contrary, samples exposed in site RU showed an increase in elemental content, especially for the elements of cluster I (Ca, Cd, Mg, Na, Sc and V; Fig. 1), but also a noteworthy increase of PAHs (except for PAH-2; see also post hoc results for PAH content in Table 2). Samples exposed in site UI also exhibited increased PAH and elemental content, however the PAH accumulation was less pronounced than in RU samples; in site UI, the highest elemental enrichment was observed for elements of cluster II (Al, Ba, Cr, Fe, Li, Mo, Pb, S, Se, Ti and Zn; Fig. 1). Overall, the elemental enrichment in both RU and UI samples was rather limited. Indeed, when expressing the element concentration data of Exposed samples (*E*) with respect to that of Unexposed samples (*U*) in terms of their ratio (*i.e.*, the so-called *EU* ratio, Cecconi et al., 2019), only few elements exceeded the value of 1.5 (*i.e.*, elemental content increased more than 50% in exposed samples). Namely, Na highly accumulated in all the exposure sites (2.72, 7.60 and 4.07 in CK, RU and UI samples, respectively), Ti and Sb (1.56 and 1.59) in site RU, and Al, Fe and Sb (1.67, 3.67 and 1.83) in site UI.

Concerning the relative contribution of different PAH categories to their overall accumulation, 3- and 4-ring PAHs showed the utmost significant accumulation in site RU, followed by site UI (only for 3-ring PAHs); 5- and 6-ring PAHs significantly increased in RU samples. Two-ring PAHs did not show any significant increase in sample groups (Table 2).

Different groups of samples segregated in the ordination space defined by the first two principal components (PCs), irrespective of the

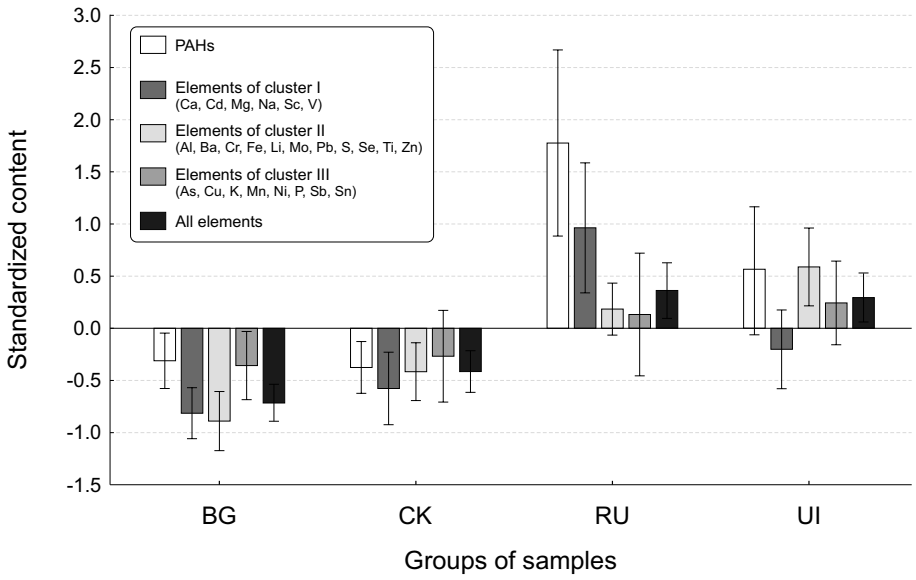


Fig. 1. Standardized PAH and elemental content (separately reported for the 3 element clusters derived from CA, and for all the 25 elements) in samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* collected in a background site (BG) and transplanted at the exposure sites (CK, Classical Karst; RU, rural-urban site; UI, urban-industrial site). Data are shown as mean and 95% confidence interval: positive and negative values of bars indicate the standard deviation offset from the mean value.

Table 2
Mean and standard deviation of the target variables (physiological parameters and PAH content) in different groups of samples (labelled as in Table 1) of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* (n = 5 for BG group, n = 3 for the rest of groups). Target variables include malondialdehyde content (MDA; nmol g^{-1}), potassium leakage (K^+ leakage; mg g^{-1}), maximum quantum efficiency of photosystem II (F_v/F_m), the sum of 18 polycyclic aromatic hydrocarbons as well as the sum of PAHs with 2-, 3-, 4-, 5- and 6-rings (respectively, Σ PAHs, PAH-2, PAH-3, PAH-4, PAH-5 and PAH-6; expressed in ng g^{-1}). Following one-way ANOVA, different letters indicate significantly different groups within each column (Dunn's post hoc test at $p < 0.05$).

Group	Physiological markers			PAH content					
	MDA	K^+ leakage	F_v/F_m	Σ PAHs	PAH-2	PAH-3	PAH-4	PAH-5	PAH-6
BG	15.9 ± 0.9 ^{a}	0.131 ± 0.023 ^{ab}	0.719 ± 0.009 ^{c}	200.2 ± 129.1 ^{a}	22.8 ± 30.0 ^{a}	79.3 ± 54.8 ^{a}	68.8 ± 57.3 ^{a}	22.0 ± 12.4 ^{ab}	7.3 ± 3.9 ^{a}
CK	12.5 ± 1.2 ^{a}	0.124 ± 0.014 ^{ab}	0.700 ± 0.021 ^{c}	245.5 ± 76.1 ^{a}	39.9 ± 13.3 ^{a}	96.8 ± 21.0 ^{a}	93.2 ± 39.8 ^{ab}	11.9 ± 4.3 ^{a}	3.8 ± 0.1 ^{a}
RU	56.4 ± 5.9 ^{d}	0.224 ± 0.013 ^{b}	0.505 ± 0.090 ^{a}	4023.2 ± 1164.4 ^{c}	30.1 ± 10.8 ^{a}	1374.5 ± 341.0 ^{c}	2510.5 ± 800.3 ^{d}	92.8 ± 24.5 ^{c}	15.3 ± 7.4 ^{b}
UI	24.5 ± 1.9 ^{bc}	0.145 ± 0.094 ^{ab}	0.593 ± 0.034 ^{ab}	813.4 ± 191 ^{b}	88.3 ± 22.5 ^{a}	309.2 ± 39.6 ^{b}	368.9 ± 128.0 ^{ab}	38.8 ± 24.9 ^{b}	8.2 ± 5.8 ^{ab}
CK O_3^-	21.6 ± 1.1 ^{b}	0.105 ± 0.016 ^{ab}	0.708 ± 0.007 ^{c}	185.4 ± 23.7 ^{a}	15.9 ± 7.2 ^{a}	82.9 ± 4.3 ^{a}	58.7 ± 4.2 ^{a}	21.6 ± 11.5 ^{ab}	7.1 ± 5.7 ^{a}
CK O_3^+	29.3 ± 0.6 ^{c}	0.093 ± 0.073 ^{a}	0.713 ± 0.013 ^{c}	206.0 ± 20.8 ^{a}	13.7 ± 7.5 ^{a}	75.1 ± 17.1 ^{a}	89.2 ± 12.9 ^{ab}	22.7 ± 5.9 ^{ab}	6.2 ± 4.2 ^{a}
RU O_3^-	71.2 ± 2.3 ^{e}	0.161 ± 0.059 ^{ab}	0.569 ± 0.178 ^{ab}	1492.7 ± 169.5 ^{b}	31.9 ± 1.8 ^{a}	567.1 ± 85.1 ^{b}	856.1 ± 61.7 ^{c}	39.0 ± 24.6 ^{ab}	6.9 ± 5.3 ^{a}
RU O_3^+	57.3 ± 3.9 ^{d}	0.199 ± 0.137 ^{ab}	0.646 ± 0.065 ^{bc}	1152.7 ± 175.6 ^{b}	35.1 ± 29.4 ^{a}	558.8 ± 41.5 ^{b}	530.2 ± 122.4 ^{b}	22.6 ± 11.4 ^{ab}	7.0 ± 5.5 ^{a}
UI O_3^-	26.4 ± 4.3 ^{bc}	0.205 ± 0.061 ^{ab}	0.680 ± 0.021 ^{bc}	234.5 ± 22.1 ^{a}	21.6 ± 6.6 ^{a}	86.9 ± 7.8 ^{a}	105.6 ± 15.3 ^{ab}	17.6 ± 7.8 ^{ab}	3.8 ± 0.1 ^{a}
UI O_3^+	24.0 ± 0.8 ^{b}	0.123 ± 0.079 ^{ab}	0.640 ± 0.062 ^{bc}	236.1 ± 17.3 ^{a}	21.3 ± 7.0 ^{a}	90.5 ± 13.2 ^{a}	110.9 ± 18.7 ^{ab}	10.5 ± 1.8 ^{a}	3.6 ± 0.2 ^{a}

fumigation treatment (Fig. 2A). Sample replicates exposed in site CK were placed at positive scores of PC 1, whereas RU and UI samples were placed at negative scores of PC 1, respectively characterized by positive and negative scores of PC 2 (Fig. 2A). Different PAH categories (3 - 6-rings) were placed in the IV quadrant of the ordination space, clearly associated to rural-urban land use, whereas 2-ring PAHs were positively associated to urban-industrial land use. Almost all of the 25 elements were placed in the 3rd and 4th quadrant: in particular, Na, Mg, V, and Sc (cluster I) were positively associated to rural-urban land use, whereas elements such as Fe, Pb and Zn (cluster II) were mostly associated to urban-industrial land use (Figs. 1 and 2B).

3.2. Effects of field exposure and O_3-fumigation on lichen physiology

The field sites were characterized by different meteoclimatic conditions during the 6 weeks of exposure. Expectedly, the proximate-natural site (CK) was characterized by the lowest daily temperature and the highest relative humidity, whereas the opposite situation was highlighted at the urban-industrial site (UI), close to the sea. Concerning the daily temperature, site RU was more similar to site CK than to site UI, whereas the relative humidity at RU was intermediate between than of sites CK and UI (Supplementary Fig. S3 A).

After field exposure, MDA content showed the uttermost variations with respect to pre-exposure (Table 2). Such physiological pattern was also highlighted by the PCA: the MDA content showed the highest negative correlation to PC 1 (−0.65) and positive to PC 2 (+0.58). Especially, MDA was clearly associated to increasing content of PAHs, in turn associated to the rural-urban land use of the exposure site (Fig. 2B). With respect to MDA, K^+ leakage showed a similar correlation pattern with the first two PCs, although with lower correlations in absolute figures with both axes (−0.32 with PC 1, and +0.29 with PC 2). F_v/F_m showed the opposite pattern, being positively correlated to PC 1 (+0.59) and negatively to PC 2 (−0.36), very close to the K content in the ordination space (Fig. 2B).

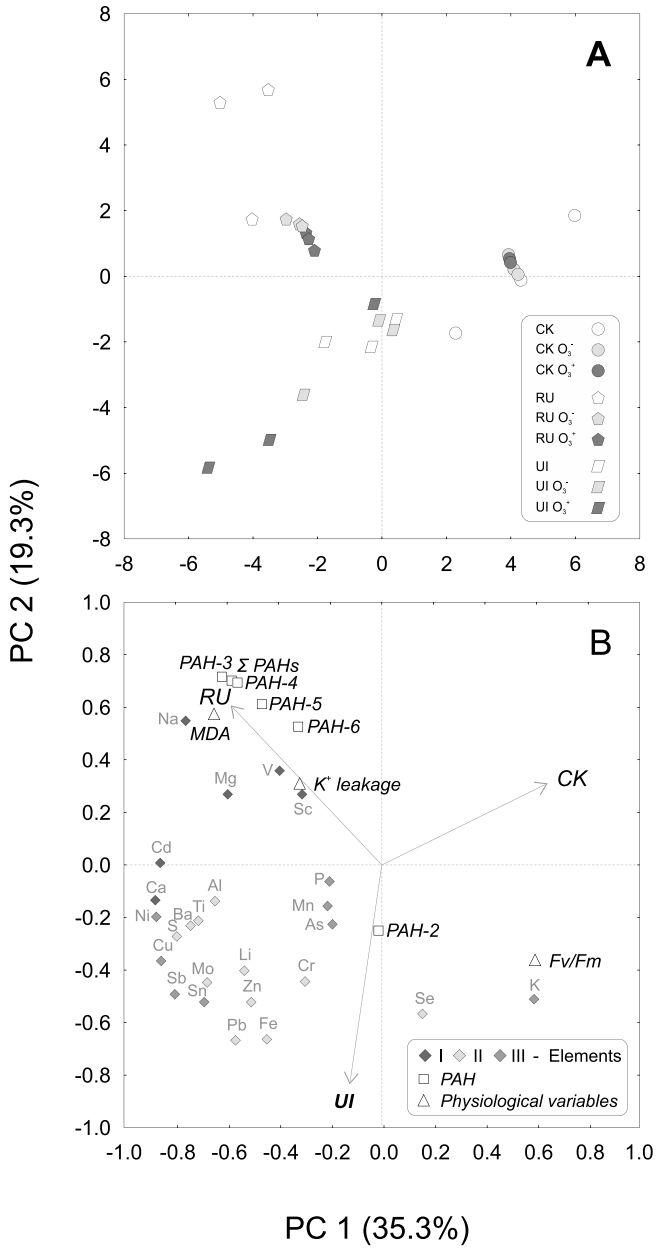


Fig. 2. (A) PCA plot showing factorial scores of replicates of samples groups of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*, labelled as in Table 1, and (B) loading vectors of PAHs, elements, physiological variables, and their relationships with exposure site type (CK, Classical Karst; RU, rural-urban; UI, urban-industrial) plotted as supplementary variables.

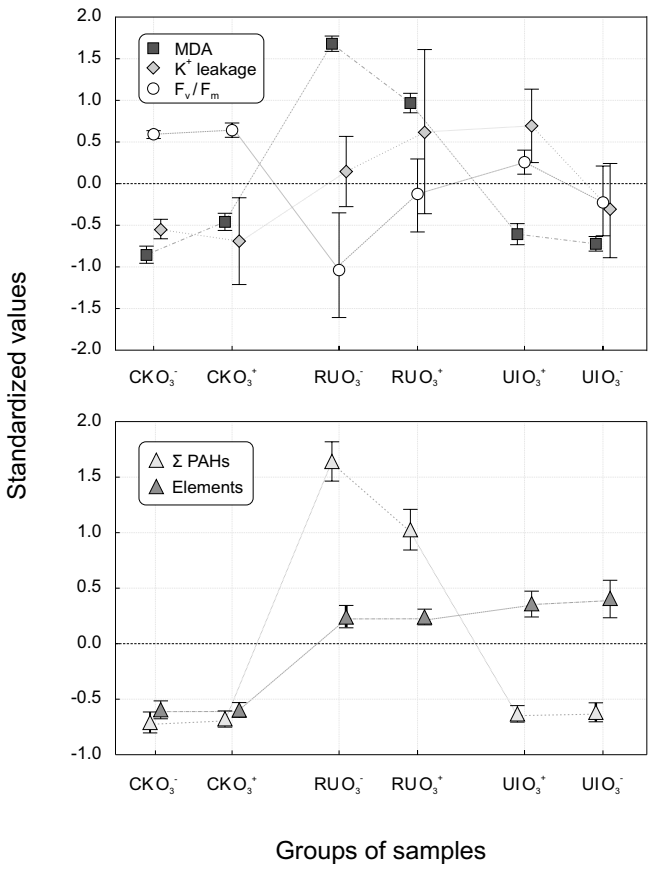


Fig. 3. Standardized values of physiological variables (top of the figure), overall PAH and elemental content (bottom of the figure). Different groups of samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea* are labelled as in Table 1. Data are shown as means and 95% confidence interval (positive and negative values of bars indicate the standard deviation offset from the mean value).

The CK samples showed unchanged values of MDA content, K^+ leakage and F_v/F_m with respect to BG samples (Table 2). By contrast, RU samples exhibited a significant increase of MDA content (+254.7%) and decrease of F_v/F_m (−29.8%), associated to an unchanged K^+ leakage. Finally, the UI samples exhibited a pattern similar to that of RU ones, although the physiological impairment was less pronounced (significant variation of MDA and F_v/F_m: +54.1% and −17.5%, respectively; Table 2).

The physiological parameters in fumigated samples showed a heterogeneous pattern of variation with respect to field-exposed samples. Their MDA levels significantly increased with respect to field-exposed samples for CK O_3^-, CK O_3^+ and RU O_3^- (+72.8% + 134.4% and +26.2%, respectively). By contrast, UI O_3^+ samples showed a non-significant decrease in MDA content. Also, F_v/F_m levels significantly increased for RU O_3^+ (+27.9%). Finally, the K^+ leakage remained unchanged, independently of O_3 treatment (Table 2).

When focusing on matched paired samples subjected to the fumigation treatment (O_3^+ and O_3^- samples), the magnitude and sign of the differences between physiological parameters were not conserved across different experimental groups. Limiting the description to the significant variations, CK O_3^+ samples showed higher levels of MDA than CK O_3^- ones (+35.6%), whereas the opposite was found for RU O_3^+ samples (−19.5%; Table 2, Fig. 3). Such pattern was in accordance with the outcome of GLMs. Indeed, GLM results showed that, amongst the tested markers, only MDA content was significantly affected by both the field exposure and the fumigation (Table 3). In addition, the content of potassium and phosphorus (*i.e.*, K and P in Fig. 3, major elements related to the physiological status of lichen thalli) was significantly affected not only by the field exposure, but also by the fumigation treatment and their interaction (Supplementary Table S2). When tested for significant differences using Wilcoxon's matched pair test, the content of K and P was significantly lower in UI O_3^+ samples than in matched paired UI O_3^- ones. The observed pattern for K content well agrees with that observed for K^+ leakage (see *supra*).

Table 3
Summary of Generalized Linear Model (GLM) testing for main and interaction effects of field exposure (*Exp.*) and fumigation (*Fum.*) on the physiological variables (malondialdehyde content, MDA; K^+ leakage; maximum quantum efficiency of photosystem II, F_v/F_m) and the content of 4-, 5- and 6-ring PAHs measured in samples of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*. F-statistics and *p*-values are reported for each factor and their interaction (*p*-values < 0.05 are reported in italic). The explained variance and statistical significance of the whole model are reported as adjusted r^2 and associated significance level below the variable name.

Physiological variables				PAHs							
Variable	Effect	F	p-value	Variable	Effect	F	p-value	Variable	Effect	F	p-value
MDA	Exp.	435.543	$< 10^{-4}$	**Σ PAHs**	Exp.	239.947	$< 10^{-4}$	**PAH-4**	Exp.	220.317	$< 10^{-4}$
$r^2 = 0.98$	Fum.	5.398	0.039	$r^2 = 0.97$	Fum.	4.936	0.046	$r^2 = 0.97$	Fum.	12.267	0.004
$p < 0.001$	Exp. × Fum.	25.312	$< 10^{-4}$	$p < 0.001$	Exp. × Fum.	6.037	0.015	$p < 0.001$	Exp. × Fum.	17.438	2.8×10^{-4}
K^+ leakage	Exp.	1.734	0.218	**PAH-2**	Exp.	3.016	0.087	**PAH-5**	Exp.	2.262	0.147
$r^2 = 0.03$	Fum.	0.240	0.633	$r^2 = 0.06$	Fum.	0.001	0.974	$r^2 = 0.11$	Fum.	1.357	0.267
$p > 0.05$	Exp. × Fum.	0.869	0.444	$p > 0.05$	Exp. × Fum.	0.063	0.939	$p > 0.05$	Exp. × Fum.	0.614	0.557
F_v/F_m	Exp.	2.353	0.137	**PAH-3**	Exp.	289.870	$< 10^{-4}$	**PAH-6**	Exp.	0.995	0.398
$r^2 = 0.07$	Fum.	0.126	0.728	$r^2 = 0.97$	Fum.	0.0491	0.828	$r^2 = -0.21$	Fum.	0.014	0.909
$p > 0.05$	Exp. × Fum.	0.764	0.487	$p < 0.001$	Exp. × Fum.	0.0428	0.958	$p > 0.05$	Exp. × Fum.	0.023	0.978

4. Discussion

4.1. A rather tolerant biomonitor

In this work, we evaluated the physiological response of *Pseudevernia furfuracea* var. *furfuracea* to a multi-pollutant field and laboratory exposure. The lichen physiological status was evaluated at each experimental step in terms of photosynthetic activity of the algal population (*i.e.*, F_v/F_m), peroxidation of membrane lipids (*i.e.*, MDA), and membrane integrity (*i.e.*, K^+ leakage).

Lichens collected in the BG area showed Chl_aF values fully consistent with those previously assessed for the same species. Indeed, F_v/F_m values lied within the range reported by several studies for unstressed *P. furfuracea* thalli (0.610–0.750: *e.g.*, Calatayud et al., 1997; Niewiadomska et al., 1998; Vidergar-Gorjup et al., 2001; Tretiach et al., 2007; Malaspina et al., 2018). Even for MDA our BG values were consistent with the few data available for *P. furfuracea* from background sites (*c.* 10 nmol g^{-1} DW: Corapi et al., 2014; Lucadamo et al., 2015), after correction of measurement units (*in litt.* to Lucadamo). Concerning K^+ leakage, reference values for *P. furfuracea* are missing; however, our values were lower than those reported for unstressed samples of the trebouxioid lichen *Flavoparmelia caperata* (0.170–0.300 mg g^{-1} DW; Candotto Carniel et al., 2017).

Overall, the physiological impairment of *P. furfuracea* after the field exposure was rather limited, although sometimes significant. Indeed, significant variations were highlighted for lipid peroxidation and chlorophyll fluorescence in samples exposed at the rural-urban site (RU), where PAH depositions were the highest (Fig. 1). Instead, the O_3-treatment produced a significant increase of MDA limited to samples previously exposed in the semi-natural site of the Classical Karst (*i.e.*, CK $O_3{}^+$ samples) (Table 2; Fig. 3). Our results showed that, amongst the tested physiological markers, MDA and F_v/F_m showed the clearest response to pollution and environmental changes (Fig. 2). K^+ leakage was previously used as a very effective biomarker of SO_2 (Tarhanen et al., 1996) and trace element pollution (Tarhanen et al., 1999; Cuny et al., 2002); however, in our case, K^+ leakage showed generally low mean values, and a non-significant pattern of variation (Table 2), as well as non-significant correlations with targeted elements (ranging from −0.26 to 0.40) and other markers (0.37 and −0.42 with MDA and F_v/F_m, respectively). On the one hand, this result suggests that SO_2 and metal pollution experienced by the exposed thalli was relatively low and, on the other hand, supports the idea that K^+ leakage may not uniquely reflect the loss of membrane integrity caused by lipid peroxidation, as previously hypothesized (Cuny et al., 2004). The F_v/F_m values after the field exposure were fully consistent with those reported by other authors for the same species (Tretiach et al., 2007; Malaspina et al., 2018). Indeed, the decrease of F_v/F_m was rather limited, confirming the good resistance of this lichen to the typical urban environmental conditions (such as high temperatures and irradiance, and low air humidity). Undoubtedly, this typical light-demanding lichen of exposed environments (Wirth, 1995) owns specific physiological and morpho-anatomical features which also allow it to cope with prolonged desiccation (Rikkinen, 1997).

When interpreting the physiological pattern of experimental samples, attention should be paid on the effects of possible confounders. As a matter of fact, the physiological response of lichens transplanted to urban environments is influenced by several factors, especially by the interplay of climatic conditions (see Supplementary Methods S1 for a climatic description of the exposure area) and air pollutants (Tretiach et al., 2012). For these reasons, monitoring meteoclimatic conditions, as well as the phytotoxic pollution loads during the exposure of lichen samples, is of primary importance (Piccotto et al., 2011). As far as meteoclimatic conditions are concerned, the sites in the Classical Karst (CK and RU) experienced higher rainfall than the UI site during the 6 weeks exposure (125 *vs* 96 mm; OSMER FVG, 2018), well reflecting climatic differences between these contexts (Supplementary Methods S1). The wind regime during the exposure period clarifies the generally low enrichment levels revealed in samples exposed at the UI site. The prevailing Bora wind, blowing from northeast towards the sea, definitely limits the lichen enrichment related to particulate matter depositions. Indeed, only Na and Fe were heavily accumulated (sect. 3.1), respectively reflecting the sea influence (all exposure sites) and the nearby presence of a large cast-ironwork (site UI) (Supplementary Fig. S1).

The more favourable conditions at CK and RU sites (see sect. 3.2 and Supplementary Fig. S3 A) suggest that the meteoclimatic gradient alone cannot explain the observed physiological pattern, since the highest detrimental effects were observed in lichens transplanted at site RU, meaning that pollutant gradients must be definitely considered as prominent. In this respect, we cannot exclude an effect of phytotoxic gaseous pollutants such as nitrogen oxides (NO_x) and SO_2, which are known to cause an impairment of the maximum quantum yield of primary lichen photochemistry (Rao and LeBlanc, 1966; Beckett et al., 2008; Piccotto et al., 2011). During the field exposure, the mean hourly concentrations of NO_2 and SO_2 measured at a monitoring station near the UI site were 32.0 ± 21.0 and 2.1 ± 3.4 μg m^{-3} (ARPA FVG, 2018b, 2018c; Supplementary Fig. S3 B), with maximum values far lower than the EU limits for human health (EU Directive, 2008/50/EC). It is not possible to fully disentangle the physiological effects due to different pollutants during the field exposure of lichen samples; however, NO_2 emissions in urban environments are mainly related to vehicular traffic (definitely prominent in UI site), whereas wood burning

activities, characterizing site RU, are acknowledged as sources of SO_2 (Cooper, 1980). Consequently, these sites can rightly be considered as affected by the highest winter levels of NO_2 and SO_2, respectively. Therefore, a combination of metal-rich particulate matter and NO_x plausibly caused the (rather limited) physiological impairment observed in samples exposed at site UI, whereas a combination of high PAH levels and SO_2 caused that of samples exposed at site RU, including the significant increase of MDA levels. However, in this respect, the strong match between the trends of PAHs and MDA content suggests a prominent role of PAHs with respect to SO_2 (Fig. 2; Fig. 3).

After the fumigation, the heterogeneous physiological pattern of samples (sect. 3.2) depicted an interesting scenario. Contrarily to our original hypothesis, the CK samples, that experienced the lowest pollution and the most favourable environmental conditions during the field exposure, suffered in relative terms the highest damage, reflected by a significant increase in MDA content affecting both CK O_3^+ and CK O_3^- samples. Considered the good health status of these samples immediately after the field exposure, it is highly feasible that these suffered a so-called "chamber effect" related to the regime of steady temperatures in the fumigation chambers (Bertuzzi et al., 2013), which were higher than those in the field (sect. 2.3, Supplementary Fig. S3 A). Nevertheless, this was not enough to damage the algal population, because the Chl_aF levels remained stable (Table 2).

Intriguingly, for UI samples (which experienced more stressing conditions than CK samples) a recovery of the algal population was observed, independently of O_3 treatment. Although variations were not significant, F_v/F_m increased by 15% and 8% in O_3^- and O_3^+ samples with respect to field-exposed ones. F_v/F_m values also exhibited slight differences between UI O_3^+ and UI O_3^- samples, the latter having value 6% higher than the former (Table 2). The fluorescence pattern of O_3-treated CK and UI samples well matches with the results of the other single work that addressed the physiological response of *P. furfuracea* to O_3 (Scheidegger and Schroeter, 1995). In accordance with our results, *P. furfuracea*, exposed for 80 days to 180 μg m^{-3} (day) and 80 μg m^{-3} (night) of O_3 (*c.* 90 and 40 ppb) in field fumigation chambers, always showed non-significant variation of F_v/F_m values (Scheidegger and Schroeter, 1995). This pattern is also consistent to that highlighted for other trebouxioid lichen species (Riddell et al., 2010; Pellegrini et al., 2014; Bertuzzi et al., 2018). However, an overall algal recovery occurred for fumigated UI samples. *P. furfuracea* is a desiccation tolerant species and a photobiont recovery was previously observed in fully controlled environment after a stressing field exposure (*e.g.*, Kranner et al., 2003). Thus, since we purposely avoided the exposure of wet thalli to relatively high light during their stay in the fumigation chambers (Bertuzzi et al., 2013; Pellegrini et al., 2014), the observed pattern contravenes our original hypothesis.

RU samples also exhibited an overall recovery of the algal population during their stay in the fumigation chambers, with F_v/F_m increased by 13% and 28% in O_3^- and O_3^+ samples with respect to field-exposed ones. In this case, the same considerations spelt out for UI samples apply, hence definitely proving the noteworthy O_3-tolerance of *P. furfuracea*. By contrast, the physiological pattern of F_v/F_m and MDA content in matched fumigated RU samples is opposite to that of UI samples. Indeed, RU O_3^- samples exhibited higher MDA content and lower F_v/F_m than RU O_3^+ ones (differences that were respectively significant or not; sect. 3.2). Most likely, such pattern has to be interpreted in relation to possible interaction phenomena between high levels of PAHs accumulated during the field exposure and the O_3 subsequently provided, which deserves further explanation.

4.2. Does summer-like ambient air O_3 affect PAH content in lichen biomonitors?

An intriguing aspect, with possible important implications for the interpretation of biomonitoring results, concerns the possible interactions between O_3 and Polycyclic Aromatic Hydrocarbons (PAHs). Indeed, the ability of O_3 in degrading either adsorbed or gas phase PAHs has repeatedly been demonstrated in different environmental matrices (Yao and Masten, 1992; Nam and Kukor, 2000) such as soils, sediments, water and sludges (*e.g.*, Kochany and Maguire, 1994; Bernal-Martinez et al., 2005; Haapea and Tuhkanen, 2006; Hong et al., 2008). Recently, Kodnik et al. (2015) hypothesized that such oxidative degradation reactions could also occur at thallus level in lichen transplants. In this respect we found an interesting pattern in relation to fumigated samples that experienced the highest field loads of PAHs.

Firstly, the content of PAHs in our lichen samples was generally lowered after their 2 week-stay in the fumigation chambers, especially in samples previously exposed at sites RU and UI, and such loss was spread amongst O_3^+ and O_3^- samples. Since all samples were carefully stored at the same conditions according to standardized procedures (Augusto et al., 2013) and contemporarily analysed, this loss (positively related to the level of PAH bioaccumulation occurred in the field) has most likely occurred during the fumigation. Interestingly, it was recently demonstrated that gas phase Fl and B[a]Py accumulate in the photosynthetic algal layer, but no major losses were observed during their stay in climatic chambers (Augusto et al., 2015). However, no results are available for other aromatic compounds and PAHs adsorbed to particulate matter. Since particulate is mainly deposited onto the lichen surface or trapped in the intercellular spaces of the medulla (Garty et al., 1979), it does make sense to assume that, depending on the compound-specific physicochemical characteristics, as well as on the environmental conditions and residence time in controlled environment, a loss of PAHs from lichen thalli could occur. Moreover, although PAHs have low solubility in water (Huang et al., 1993), it is also possible that these could be mechanically washed off due to daily rehydration procedures (sect. 2.2), as also expected by Augusto et al. (2015), but contextually not observed by the authors (Augusto et al., 2015).

Having said that, a clear and noticeable pattern emerged for RU O_3^+ and RU O_3^- samples previously exposed in a site where the wood burning in traditional fireplaces is the prevailing source of aerodisperse organic compounds (Mastral and Callén, 2000; Kodnik et al., 2015). Accordingly, these were characterized by far by the utmost content of Fl, Py, B[a]Ant and Chry (4-ring PAHs), the typical tracers of wood burning activities (Boström et al., 2002; Singh et al., 2013) (Fig. 4; Supplementary Fig. S4). Interestingly, after fumigation, F_v/F_m in RU O_3^+ samples was higher than in RU O_3^- ones, whereas the MDA and the 4-ring PAH content was significantly lower. In particular, the content of Fl, Py, Chry and B[a]Ant in RU O_3^+ samples was 23%, 46%, 86% and 89% lower than in the paired RU O_3^- samples (Wilcoxon matched paired test, $p < 0.05$; Supplementary Fig. S4). This suggests that ozonation caused the degradation of PAHs with subsequent physiological improvement.

The effectiveness of O_3 in degrading PAHs depends on the targeted matrix (Von Gunten, 2003; Masten and Davies, 1997; Goi and Trapido, 2004), the molecular weight and structure of the compounds, their physical state (Abdel-Shafy and Mansour, 2016) and oxidant doses (Siegrist et al., 2011). The structural and chemical complexity of the lichen matrix does not allow conclusive statements, and the experimental design was not conceived to test this specific hypothesis. Nonetheless, in light of such overt pattern affecting matched fumigated RU samples, it is highly feasible that O_3 treatment acted as a depletion agent for the most abundant residual PAHs retained at the algal layer. Indeed, lichens lack the waxy cuticle that characterizes the leaves of vascular plants, hence permitting the diffusion of O_3 towards the inner lichen layers (Pellegrini et al., 2014). It could be argued why a similar depletion pattern was not observed for the second most abundant class of the 3-ring PAHs. In this regard, it is known that the O_3 treatment of 4-ring PAHs generally produces 3-ring by-products (Cochran et al., 2016), that, according to their fate, may mask a lowering trend, hence misleading the interpretation of results due to possible chromatographic interferences with native PAHs (Janska et al., 2006; Fromberg

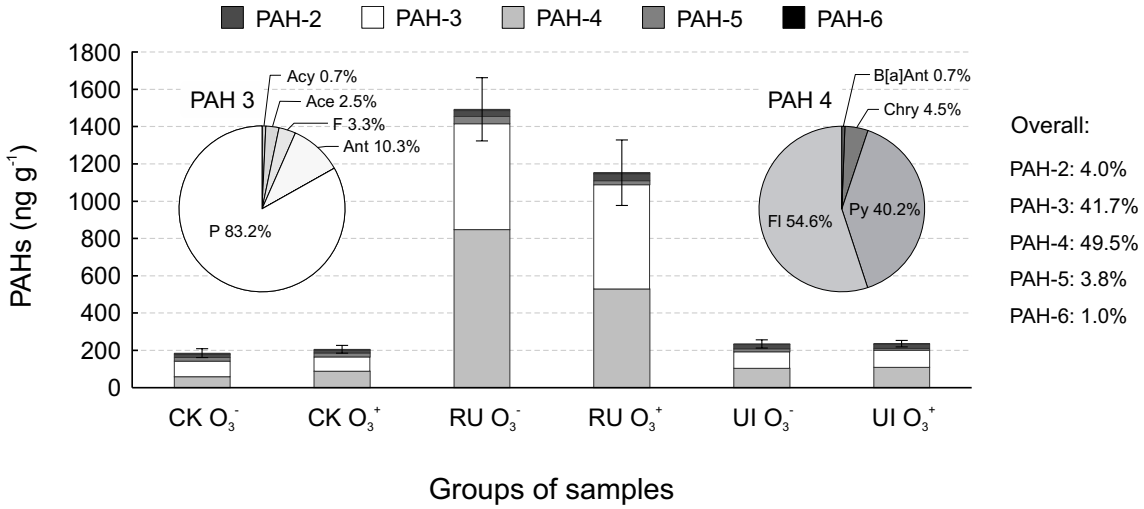


Fig. 4. PAH content in exposed thalli of the epiphytic lichen *Pseudevernia furfuracea* var. *furfuracea*, either ozonated or not (groups of samples are labelled as in Table 1). Data refer to mean values of 2-, 3-, 4-, 5- and 6-ring PAHs and overall standard deviations. The pie chart insets show the contribution of 3- and 4-ring PAHs, the most abundant polycyclic aromatic compounds revealed in exposed samples.

et al., 2007). Undoubtedly, further research is needed to clarify the fate of different phase PAHs in lichens, however, the monitoring of O_3 ground levels should become a routine precaution during summer campaigns with lichens as biomonitors, in order to avert potential underestimation of bioaccumulated PAHs.

5. Conclusions

This study provides another piece to the lichen O_3-tolerance puzzle faced in a number of recent studies (Riddell et al., 2010; Bertuzzi et al., 2013, 2018; Pellegrini et al., 2014). In particular, *Pseudevernia furfuracea* var. *furfuracea* stands as a rather tolerant lichen biomonitor, confirming its ability to cope with typical urban environmental conditions. A physiological impairment, mostly highlighted in terms of a significant increase in MDA levels, was caused by massive PAH loads (and possibly by SO_2) during the field exposure at wood-burning chimneys at the RU site. However, *P. furfuracea* did not experience major detrimental effects due to the O_3 treatment. In particular, samples that experienced more stressing field conditions, exhibited a recovery of the algal populations or, at most, no significant variations of other markers. This result contravenes our original hypothesis and highlights that the test species can be classified as fully tolerant to ozone.

Furthermore, limited to samples exposed to high PAH loads, the peculiar physiological and PAH pattern affecting fumigated samples suggested that a significant decrease of PAHs in ozonated samples was possibly ascribable to oxidative degradation occurring at the thallus system level, specifically consisting in the degradation of the most abundant 4-ring PAHs accumulated during the field exposure. Although further investigation is needed to clarify the issue, the possibility of an underestimation of PAH enrichment levels should seriously be considered when carrying out transplant-based surveys with contextually high O_3 ground levels.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Acknowledgements

Thanks are due to Nives Orlić and Daniel A. Puente Anzil (Trieste) for their help in sample processing and chlorophyll *a* fluorescence measurements. This study was financed under the PRIN project n. 20109E8F95 “TreeCity”, nat. resp. Prof. G. Lorenzini, and by D.T.N. (f.o.o.p) funds from Prof. M. Tretiach.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atmosenv.2019.03.026.

References

Abdel-Shafy, H.I., Mansour, M.S., 2016. A review on polycyclic aromatic hydrocarbons: source, environmental impact, effect on human health and remediation. Egypt. J. Petrol. 25, 107–123.

Adamo, P., Crisafulli, P., Giordano, S., Minganti, V., Modenesi, P., Monaci, F., Pittao, E., Tretiach, M., Bargagli, R., 2007. Lichen and moss bags as monitoring devices in urban areas. Part II: trace element content in living and dead biomonitors and comparison with synthetic materials. Environ. Pollut. 146, 392–399.

Adamo, P., Bargagli, R., Giordano, S., Modenesi, P., Monaci, F., Pittao, E., Spagnuolo, V., Tretiach, M., 2008. Natural and pre-treatments induced variability in the chemical composition and morphology of lichens and mosses selected for active monitoring of airborne elements. Environ. Pollut. 152, 11–19.

Augusto, S., Pereira, M.J., Máguas, C., Branquinho, C., 2013. A step towards the use of biomonitors as estimators of atmospheric PAHs for regulatory purposes. Chemosphere 92, 626–632.

Augusto, S., Sierra, J., Nadal, M., Schuhmacher, M., 2015. Tracking polycyclic aromatic hydrocarbons in lichens: it's all about the algae. Environ. Pollut. 207, 441–445.

Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu. Rev. Plant Biol. 59, 89–113.

Bargagli, R., Mikhailova, I., 2002. Accumulation of inorganic contaminants. In: Nimis, P.L., Scheidegger, C., Wolseley, W.A. (Eds.), Monitoring with Lichens - Monitoring Lichens. Kluwer Academic Publisher, Dordrecht, pp. 65–84.

Bargagli, R., Nimis, P.L., 2002. Guidelines for the use of epiphytic lichens as biomonitors of atmospheric deposition of trace elements. In: Nimis, P.L., Scheidegger, C., Wolseley, W.A. (Eds.), Monitoring with Lichens - Monitoring Lichens. Kluwer Academic Publisher, Dordrecht, pp. 295–299.

Beckett, R.P., Kranner, I., Minibayeva, F.V., 2008. Stress physiology and the symbiosis. In: Nash IIIT.H. (Ed.), Lichen Biology. Cambridge University Press, Cambridge, pp. 136–153.

Bernal-Martinez, A., Carrere, H., Patureau, D., Delgenes, J.P., 2005. Combining anaerobic digestion and ozonation to remove PAH from urban sludge. Process Biochem. 40, 3244–3250.

Bertuzzi, S., Davies, L., Power, S.A., Tretiach, M., 2013. Why lichens are bad biomonitors of ozone pollution? Ecol. Indicat. 34, 391–397.

Bertuzzi, S., Pellegrini, E., Candotto Carniel, F., Incerti, G., Lorenzini, G., Nali, C., Tretiach, M., 2018. Ozone and desiccation tolerance in chlorolichens are intimately

connected: a case study based on two species with different ecology. Environ. Sci. Pollut. Control Ser. 25, 8089–8103.
Boström, C.E., Gerde, P., Hanberg, A., Jernström, B., Johansson, C., Kyrklund, T., Rannug, A., Törnqvist, M., Victorin, K., Westerholm, R., 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environ. Health Perspect. 110, 451–488.
Brown, D.H., Smirnoff, N., 1978. Observations on the effect of ozone on *Cladonia rangiformis*. Lichenol. 10, 91–94.
Bussotti, F., Desotgiu, R., Cascio, C., Pollastrini, M., Gravano, E., Gerosa, G., Marzuoli, R., Nali, C., Lorenzini, G., Salvatori, E., Manes, F., Schaub, M., Strasser, R.J., 2011. Ozone stress in woody plants assessed with chlorophyll *a* fluorescence. A critical reassessment of existing data. Environ. Exp. Bot. 73, 19–30.
Calatayud, A., Deltoro, V.I., Barreno, E., del Valle-Tascon, S., 1997. Changes in *in vivo* chlorophyll fluorescence quenching in lichen thalli as a function of water content and suggestion of zeaxanthin-associated photoprotection. Physiol. Plantarum 101, 93–102.
Candotto Carniel, F., Pellegrini, E., Bove, F., Crosera, M., Adami, G., Nali, C., Tretiach, M., 2017. Acetone washing for the removal of lichen substances affects membrane permeability. Lichenol. 49, 387–395.
Cecconi, E., Fortuna, L., Benesperi, R., Bianchi, E., Brunialti, G., Contardo, T., Di Nuzzo, L., Frati, L., Monaci, F., Munzi, S., Nascimbene, J., Paoli, L., Ravera, S., Vannini, A., Giordani, P., Loppi, S., Tretiach, M., 2019. New interpretative scales for lichen bioaccumulation data: the Italian proposal. Atmosphere 10, 136–154.
Cecconi, E., Incerti, G., Capozzi, F., Adamo, P., Bargagli, R., Benesperi, R., Candotto Carniel, F., Favero-Longo, S.E., Giordano, S., Puntillo, D., Ravera, S., Spagnuolo, V., Tretiach, M., 2018. Background element content of the lichen *Pseudevernia furfuracea*: a supra-national state of art implemented by novel field data from Italy. Sci. Total Environ. 622, 282–292.
Chen, Y., Zang, L., Chen, J., Xu, D., Yao, D., Zhao, M., 2017. Characteristics of ambient ozone (O_3) pollution and health risks in Zhejiang Province. Environ. Sci. Pollut. Control Ser. 24, 27436–27444.
Cochran, R.E., Jeong, H., Haddadi, S., Derseh, R.F., Gowan, A., Beránek, J., Kubátová, A., 2016. Identification of products formed during the heterogeneous nitration and ozonation of polycyclic aromatic hydrocarbons. Atmos. Environ. 128, 92–103.
Cooper, J.A., 1980. Environmental impact of residential wood combustion emissions and its implications. J. Air Pollut. Control Assoc. 30, 855–861.
Corapi, A., Gallo, L., Nicolardi, V., Lucadamo, L., Loppi, S., 2014. Temporal trends of element concentrations and ecophysiological parameters in the lichen *Pseudevernia furfuracea* transplanted in and around an industrial area of S Italy. Environ. Monit. Assess. 186, 3149–3164.
Cuny, D., Pignata, M.L., Kranner, I., Beckett, R., 2002. Biomarkers of pollution-induced oxidative stress and membrane damage in lichens. In: Nimis, P.L., Scheidegger, C., Wolseley, W.A. (Eds.), Monitoring with Lichens - Monitoring Lichens. Kluwer Academic Publisher, Dordrecht, pp. 97–110.
Cuny, D., Van Haluwyn, C., Shirali, P., Zerimech, F., Jérôme, L., Haguenoer, J.M., 2004. Cellular impact of metal trace elements in terricolous lichen *Diploschistes muscorum* (Scop.) R. Sant. –Identification of oxidative stress biomarkers. Water Air Soil Pollut. 152, 55–69.
Dinno, A., 2017. Dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums. R package version 1.3.5.
Egger, R., Schlee, D., Türk, R., 1993. Changes of physiological and biochemical parameters in the lichen *Hypogymnia physodes* (L.) NYL. due to the action of air pollutants - a field study. Phyton 34, 229–242.
Frei, M., Tanaka, J.P., Chen, C.P., Wissuwa, M., 2010. Mechanisms of ozone tolerance in rice: characterization of two QTLs affecting leaf bronzing by gene expression profiling and biochemical analyses. J. Exp. Bot. 61, 1405–1417.
Fromberg, A., Højgård, A., Duedahl-Olesen, L., 2007. Analysis of polycyclic aromatic hydrocarbons in vegetable oils combining gel permeation chromatography with solid-phase extraction clean-up. Food Addit. Contam. 24, 758–767.
Garty, J., Galun, M., Kessel, M., 1979. Localization of heavy metals and other elements accumulated in the lichen thallus. New Phytol. 82, 159–168.
Goi, A., Trapido, M., 2004. Degradation of polycyclic aromatic hydrocarbons in soil: the Fenton reagent versus ozonation. Environ. Technol. 25, 155–164.
Goumenaki, E., Taybi, T., Borland, A., Barnes, J., 2010. Mechanism underlying the impacts of ozone on photosynthetic performance. Environ. Exp. Bot. 69, 259–266.
Haapea, P., Tuhkanen, T., 2006. Integrated treatment of PAH contaminated soil by soil washing, ozonation and biological treatment. J. Hazard Mater. 136, 244–250.
Hassan, I.A., Basahi, J.M., Ismail, I.M., Habeebullah, T.M., 2013. Spatial distribution and temporal variation in ambient ozone and its associated NO_x in the atmosphere of Jeddah City, Saudi Arabia. Aerosol Air Qual. Res. 13, 1712–1722.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.
Hong, P.A., Nakra, S., Kao, C.J., Hayes, D.F., 2008. Pressure-assisted ozonation of PCB and PAH contaminated sediments. Chemosphere 72, 1757–1764.
Huang, X.D., Dixon, D.G., Greenberg, B.M., 1993. Impacts of UV radiation and photomodification on the toxicity of PAHs to the higher plant *Lemna gibba* (duckweed). Environ. Toxicol. Chem.: Int. J. 12, 1067–1077.
Incerti, G., Cecconi, E., Capozzi, F., Adamo, P., Bargagli, R., Benesperi, R., Carniel, Candotto, Cristofolini, F., Giordano, S., Puntillo, D., Spagnuolo, V., Tretiach, M., 2017. Infraspecific variability in baseline element composition of the epiphytic lichen *Pseudevernia furfuracea* in remote areas: implications for biomonitoring of air pollution. Environ. Sci. Pollut. Control Ser. 24, 8004–8016.
Janska, M., Tomaniova, M., Hajšlová, J., Kocourek, V., 2006. Optimization of the procedure for the determination of polycyclic aromatic hydrocarbons and their derivatives in fish tissue: estimation of measurements uncertainty. Food Addit. Contam. 23, 309–325.
Kochany, J., Maguire, R.J., 1994. Abiotic transformations of polynuclear aromatic hydrocarbons and polynuclear aromatic nitrogen heterocycles in aquatic environments. Sci. Total Environ. 144, 17–33.
Kodnik, D., Candotto Carniel, F., Licen, S., Tolloi, A., Barbieri, P., Tretiach, M., 2015. Seasonal variations of PAHs content and distribution patterns in a mixed land use area: a case study in NE Italy with the transplanted lichen *Pseudevernia furfuracea*. Atmos. Environ. 113, 255–263.
Kranner, I., Zorn, M., Turk, B., Wornik, S., Beckett, R.P., Batič, F., 2003. Biochemical traits of lichens differing in relative desiccation tolerance. New Phytol. 160, 167–176.
Lefohn, A.S., Malley, C.S., Smith, L., Wells, B., Hazucha, M., Simon, H., Naik, V., Mills, G., Schultz, M.G., Paoletti, E., De Marco, A., Xu, X., Zhang, L., Wang, T., Neufeld, H.S., Musselman, R.C., Tarasick, D., Brauer, M., Feng, Z., Tang, H., Kobayashi, K., Sicard, P., Solberg, S., Gerosa, G., 2018. Tropospheric ozone assessment report: global ozone metrics for climate change, human health, and crop/ecosystem research. Elem. Sci. Anthropocene 6, 28.
Legendre, P., Legendre, L., 1998. Numerical Ecology. Elsevier, Amsterdam, pp. 853.
Logan, J.A., 1985. Tropospheric ozone: seasonal behaviour, trends, and anthropogenic influence. J. Geophys. Res.: Atmosphere 90 (D6), 10463–10482.
Lorenzini, G., 1994. A miniaturized kit for ozone biomonitoring. Appl. Biochem. Biotechnol. 48, 1–4.
Lorenzini, G., Nali, C., 2018. Ozone and plant life: the Italian state-of-the-art. Environ. Sci. Pollut. Control Ser. 25, 8069–8073.
Lucadamo, L., Corapi, A., Loppi, S., Paoli, L., Gallo, L., 2015. Spatial variation of ecophysiological parameters in the lichen *Pseudevernia furfuracea* transplanted in an area surrounding a cement plant (S Italy). Environ. Monit. Assess. 187, 500.
Malaspina, P., Modenesi, P., Giordani, P., 2018. Physiological response of two varieties of the lichen *Pseudevernia furfuracea* to atmospheric pollution. Ecol. Indicat. 86, 27–34.
Masten, S.J., Davies, S.H., 1997. Efficacy of in-situ for the remediation of PAH contaminated soils. J. Contam. Hydrol. 28, 327–335.
Mastral, A.M., Callen, M.S., 2000. A review on polycyclic aromatic hydrocarbon (PAH) emissions from energy generation. Environ. Sci. Technol. 34, 3051–3057.
Miszalski, Z., Niewiadomska, E., 1993. Comparison of sulphite oxidation mechanisms in three lichen species. New Phytol. 123, 345–349.
Nali, C., Balducci, E., Frati, L., Paoli, L., Loppi, S., Lorenzini, G., 2007. Integrated biomonitoring of air quality with plants and lichens: a case study on ambient ozone from central Italy. Chemosphere 67, 2169–2176.
Nam, K., Kukor, J.J., 2000. Combined ozonation and biodegradation for remediation of mixtures of polycyclic aromatic hydrocarbons in soil. Biodegradation 11, 1–9.
Nascimbene, J., Tretiach, M., Corana, F., Schiavo, F.L., Kodnik, D., Dainese, M., Mannucci, B., 2014. Patterns of traffic polycyclic aromatic hydrocarbon pollution in mountain areas can be revealed by lichen biomonitoring: a case study in the Dolomites (Eastern Italian Alps). Sci. Total Environ. 475, 90–96.
Nash III, T.H., 2008. Lichen sensitivity to air pollution. In: Nash IIIT.H. (Ed.), Lichen Biology. Cambridge University Press, Cambridge, pp. 301–316.
Nash III, T.H., Sigal, L.L., 1979. Gross photosynthetic response of lichens to short-term ozone fumigations. Bryologist 82, 280–285.
Niewiadomska, E., Jarowiecka, D., Czarnota, P., 1998. Effect of different levels of air pollution on photosynthetic activity of some lichens. Acta Soc. Bot. Pol. 67, 259–262.
Niewiadomska, E., Jarowiecka, D., Czarnota, P., 1998. Effect of different levels of air pollution on photosynthetic activity of some lichens. Acta Soc. Bot. Pol. 67, 259–262.
Nimis, P.L., 2016. The Lichens of Italy. A Second Annotated Catalogue. EUT, Trieste, pp. 739.
Nuvolone, D., Petri, D., Voller, F., 2018. The effects of ozone on human health. Environ. Sci. Pollut. Control Ser. 25, 8074–8088.
Paoletti, E., 2007. Ozone impacts on forests. CAB Rev.: Perspect. Agricul. Veter. Sci. Nutr. Nat. Resour. 2, 13.
Paoletti, E., De Marco, A., Beddows, D.C., Harrison, R.M., Manning, W.J., 2014. Ozone levels in European and USA cities are increasing more than at rural sites, while peak values are decreasing. Environ. Pollut. 192, 295–299.
Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., Badea, O., 2017. A new-generation 3D ozone FACE (free air controlled exposure). Sci. Total Environ. 575, 1407–1414.
Pellegrini, E., Bertuzzi, S., Candotto Carniel, F., Lorenzini, G., Nali, C., Tretiach, M., 2014. Ozone tolerance in lichens: a possible explanation from biochemical to physiological level using *Flavoparmelia caperata* as test organism. J. Plant Physiol. 171, 1514–1523.
Piccotto, M., Tretiach, M., 2010. Photosynthesis in chlorolichens: the influence of the habitat light regime. J. Plant Res. 123, 763–775.
Piccotto, M., Bidussi, M., Tretiach, M., 2011. Effects of the urban environmental conditions on the chlorophyll a fluorescence emission in transplants of three ecologically distinct lichens. Environ. Exp. Bot. 73, 102–107.
Podani, J., 2007. Analisi ed Esplorazione Multivariata dei Dati in Ecologia e Biologia. Liguori Editore, Napoli, pp. 515.
Quevauviller, P., Herzig, R., Muntau, H., 1996. Certified reference material of lichen (CRM 482) for the quality control of trace element biomonitoring. Sci. Total Environ. 187, 143–152.
R Core Team, 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Rao, D.N., LeBlanc, F., 1966. Effects of sulfur dioxide on the lichen alga, with special reference to chlorophyll. Bryologist 69, 69–75.
Riddell, J., Nash III, T.H., Padgett, P., 2010. Responses of the lichen *Ramalina menziesii* Tayl. to ozone fumigations. Bibl. Lichenol. 105, 113–123.
Riddell, J., Padgett, P.E., Nash III, T.H., 2012. Physiological responses of lichens to factorial fumigations with nitric acid and ozone. Environ. Pollut. 170, 202–210.
Rikkinen, J., 1997. Habitat shifts and morphological variation of *Pseudevernia furfuracea* along a topographical gradient. Symb. Bot. Ups. 32, 223–245.
Ross, L.J., Nash III, T.H., 1983. Effect of ozone on gross photosynthesis of lichens.

Environ. Exp. Bot. 23, 71–77.
Saitanis, C., 2008. Tropospheric ozone: a menace for crops and natural vegetation in Greece. Ital. J. Agron. 3, 71–77.
Sarkar, A., Rakwal, R., Bhushan Agrawal, S., Shibato, J., Ogawa, Y., Yoshida, Y., Agrawal, G.K., Agrawal, M., 2010. Investigating the impact of elevated levels of ozone on tropical wheat using integrated phenotypical, physiological, biochemical, and proteomics approaches. J. Proteome Res. 9, 4565–4584.
Scheidegger, C., Schroeter, B., 1995. Effects of ozone fumigation on epiphytic macrolichens: ultrastructure, CO_2 gas exchange and chlorophyll fluorescence. Environ. Pollut. 88, 345–354.
Schraudner, M., Moeder, W., Wiese, C., Camp, W.V., Inzé, D., Langebartels, C., Sandermann Jr., H., 1998. Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. Plant J. 16, 235–245.
Siegrist, R.L., Crimi, M., Brown, R.A., 2011. In situ chemical oxidation: technology description and status. In: Siegrist, R.L., Crimi, M., Simpkin, T.J. (Eds.), In Situ Chemical Oxidation for Groundwater Remediation. Springer, New York, pp. 1–32.
Sigal, L.L., Johnston Jr., J.W., 1986. Effects of acidic rain and ozone on nitrogen fixation and photosynthesis in the lichen *Lobaria pulmonaria* (L.) Hoffm. Environ. Exp. Bot. 26, 59–64.
Sigal, L.L., Nash III, T.H., 1983. Lichen communities on conifers in Southern California: an ecological survey relative to oxidant air pollution. Ecology 64, 1343–1354.
Singh, D.P., Gadi, R., Mandal, T.K., Saud, T., Saxena, M., Sharma, S.K., 2013. Emissions estimates of PAH from biomass fuels used in rural sector of Indo-Gangetic Plains of India. Atmos. Environ. 68, 120–126.
Sloof, J.E., 1995. Lichens as quantitative biomonitors for atmospheric trace-element deposition, using transplants. Atmos. Environ. 29, 11–20.
Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., Gilbert, O.L., James, P.W., Wolseley, P.A., 2009. The Lichens of Great Britain and Ireland. British Lichen Society, London, pp. 1046.
Sujetovienė, G., Galinytė, V., 2016. Effects of the urban environmental conditions on the physiology of lichen and moss. Atmos. Pollut. Res. 7, 611–618.
Tarhanen, S., Holopainen, T., Poikolainen, J., Oksanen, J., 1996. Effect of industrial emissions on membrane permeability of epiphytic lichens in northern Finland and the Kola Peninsula industrial areas. Water Air Soil Pollut. 88, 189–201.
Tarhanen, S., Holopainen, T., Oksanen, J., 1997. Ultrastructural changes and electrolyte leakage from ozone fumigated epiphytic lichens. Ann. Bot. 80, 611–621.
Tarhanen, S., Metsärinne, S., Holopainen, T., Oksanen, J., 1999. Membrane permeability response of lichen *Bryoria fuscescens* to wet deposited heavy metals and acid rain. Environ. Pollut. 104, 121–129.
Tretiach, M., Crisafulli, P., Pittao, E., Rinino, S., Roccotiello, E., Modenesi, P., 2005. Isidia ontogeny and its effect on the CO_2 gas exchanges of the epiphytic lichen *Pseudevernia furfuracea* (L.) Zopf. Lichenol. 37, 445–462.
Tretiach, M., Piccotto, M., Baruffo, L., 2007. Effects of ambient NO_x on chlorophyll *a* fluorescence in transplanted *Flavoparmelia caperata* (Lichen). Environ. Sci. Technol. 41, 2978–2984.
Tretiach, M., Candotto Carniel, F., Loppi, S., Carniel, A., Bortolussi, A., Mazzilis, D., Del Bianco, C., 2011. Lichen transplants as a suitable tool to identify mercury pollution from waste incinerators: a case study from NE Italy. Environ. Monit. Assess. 175, 589–600.
Tretiach, M., Pavanetto, S., Pittao, E., Di Toppi, L.S., Piccotto, M., 2012. Water availability modifies tolerance to photo-oxidative pollutants in transplants of the lichen *Flavoparmelia caperata*. Oecologia 168, 589–599.
Vannini, A., Paoli, L., Ceccarelli, S., Sorbo, S., Basile, A., Carginale, V., Nali, C., Lorenzini, G., Pica, M., Loppi, S., 2018. Physiological and ultrastructural effects of acute ozone fumigation in the lichen *Xanthoria parietina*: the role of parietin and hydration state. Environ. Sci. Pollut. Control Ser. 25, 8104–8112.
Vidergar-Gorjup, N., Sircelj, H., Pfanz, H., Batic, F., 2001. Some physiological effects of biocide treatment on the lichen *Pseudevernia furfuracea* (L.) Zopf. Symbiosis 31, 123–140.
Von Gunten, U., 2003. Ozonation of drinking water: Part I. Oxidation kinetics and product formation. Water Res. 37, 1443–1467.
Wirth, V., 1995. *Die Flechten*. Baden-Wurttembergs, Teil 1, 2 Ulmer, 1-1006. Stuttgart. pp. 1244.
Wu, S., Mickley, L.J., Jacob, D.J., Rind, D., Streets, D.G., 2008. Effects of 2000–2050 changes in climate and emissions on global tropospheric ozone and the policy relevant background ozone in the United States. J. Geophys. Res.: Atmosphere 113 (D18).
Yao, J.J., Masten, S.J., 1992. Use of ozone for the oxidation of VOCs and PAHs in the presence of naturally occurring organic matter. In: Proceedings Of the Industrial Waste Conference (USA), 46th Purdue Industrial Waste Conference. Purdue University, West Lafayette, Indiana, pp. 642–651.

Web references

ARPA FVG, 2018a. http://www.arpa.fvg.it/cms/focus_ambientali/Ferriera_Servola, Accessed date: 12 December 2018.
ARPA FVG, 2018b. http://www.arpaweb.fvg.it/qagis/stfolder.asp?folder=TimeSeries_NO2, Accessed date: 12 December 2018.
ARPA FVG, 2018c. http://www.arpaweb.fvg.it/qagis/stfolder.asp?folder=TimeSeries_SO2, Accessed date: 12 December 2018.
EPA, 2018a. https://www.epa.gov/sites/production/files/2015-12/documents/3546.pdf, Accessed date: 11 December 2018.
EPA, 2018b. https://www.epa.gov/sites/production/files/2015-07/documents/epa-8270d.pdf, Accessed date: 11 December 2018.
OSMER FVG, 2018. http://www.osmer.fvg.it/archivio.php?ln=&p=dati, Accessed date: 12 December 2018.