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Lichen and moss bags as monitoring devices in urban areas. Part I: Influence of exposure on sample vitality

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The lichen Pseudevernia furfuracea was more resilient than the moss Hypnum cupressiforme in two exposure experiments on trace metal uptake.

Abstract

Samples of the lichen *Pseudevernia furfuracea* (L.) Zopf and the moss *Hypnum cupressiforme* Hedw. were exposed for 6 weeks in nylon bags in two air pollution monitoring stations in Trieste and Naples (Italy) with different climates and pollution loads to evaluate influence of environmental conditions on sample vitality. This was assessed before and after exposure by transmission electron microscopy observations, K cellular location, and measurements of C, N, S and photosynthetic pigments content, CO_2 gas exchange, and chlorophyll fluorescence. Almost all data sets indicate that exposures caused some damage to the species, considerably heavier in the moss, especially in Naples. The two cryptogams differed significantly in accumulation and retention of C, N, and S, the lichen clearly reflecting NO_2 availability. The difference in vitality loss was related to the different ecophysiology of the species, because concentrations of phytotoxic pollutants were low during exposure. Critical notes on the analytical techniques are also given.

Keywords: Biomonitoring; Air pollution; Trace element; Transplant; *Pseudevernia furfuracea*; *Hypnum cupressiforme*

1. Introduction

Italy is one of the European countries with the highest emission of trace elements into the atmosphere, but the knowledge of their deposition intensity on the territory is very scarce and fragmentary (Bargagli, 1998) since concentrations (except for lead) are not recorded by most of the automated monitoring stations. In the last decade, however, thanks to the use of lichens and mosses as bioaccumulators, it has been possible to map deposition patterns not only near single pollution sources, but also over relatively large areas at municipal or even regional scale (Giordano et al., 2004; Nimis et al., 1999) using species which are relatively tolerant to phytotoxic pollutants and occur frequently in the survey areas. Standard protocols for sampling, analysis and data treatment have been proposed (Bargagli and Nimis, 2002), and are now widely adopted. Although this approach cannot be followed in areas where the recommended species are absent, the problem may be overcome by the use of transplants: lichens and mosses collected elsewhere are exposed in small nylon bags in stations chosen by the operators, and analysed after an appropriate period of time (Adamo et al., 2003; Castello et al., 1999; Vingiani

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et al., 2004). Some recent studies highlighted the link between metal concentrations in transplanted lichens and those measured directly in atmospheric deposition (Bari et al., 2001), and deposition maps based on moss transplants have been demonstrated to be useful to interpret the results of epidemiological studies (Gailey and Lloyd, 1993). However, this methodology is still open to several criticisms. In 2002, a multidisciplinary study on the use of lichen and moss bags was designed by the authors, with three main aims:

1. To achieve a better knowledge on major- and trace-element uptake and accumulation mechanisms in lichen and moss species used for transplants, by evaluating physiological, chemical, histochemical and ultrastructural changes after exposure in different environmental and climatic conditions
2. To characterise trace metal uptake in variously treated biological vs. synthetic materials
3. To verify possible relationships between the chemical composition of exposed materials and that of PM_{10} collected in the same site, since epidemiological and laboratory studies have indicated that the fraction of atmospheric particulates which can be inhaled (PM_{10}) and associated transition metals are among the factors responsible for the development of cardio-pulmonary diseases (Costa and Dreher, 1997)

As target species, the fruticose lichen *Pseudevernia furfuracea* (L.) Zopf var. *furfuracea* and the pleurocarpous moss *Hypnum cupressiforme* Hedw. were chosen. Both organisms have been employed in many biomonitoring studies (Bari et al., 2001; Vingiani et al., 2004), and their physiology is relatively well-known (Aro et al., 1984; Kranner et al., 2003; Scheidegger et al., 1995; Türk, 1983).

The results will be discussed in a series of contributions: those concerning point (1) are presented in this paper (vitality assessment), and by Rinino et al. (2005) (histochemistry); those related to points (2) and (3) are discussed by Adamo et al. (2007). Further data, concerning the effects of the exposure time, will be presented in a forthcoming paper.

In this contribution, in particular, data concerning biomonitors vitality before and after exposure are presented with two main goals: (1) to identify the most predictive method for a rapid, low cost estimation of vitality among those recommended in studies of this type (see, e.g., Silberstein et al., 1996; Garty, 2002); (2) to evaluate the effects of known concentrations of air pollutants under different climatic conditions. The results will be used to evaluate the contribution and the role of active vs. passive metal uptake (Adamo et al., 2007).

2. Material and methods

2.1. Sampling and pre-treatments of materials

Samples were collected the same day in two areas of NE Italy far from pollution sources. The lichen was collected from the lower branches of isolated Larch (*Larix decidua* Mill.) trees near Sauris di Sotto (Udine) at 1500 m a.s.l. and the moss from calcareous rock outcrops of the northern slope of a dolina near Basovizza (Trieste) at 400 m a.s.l.

The material (ca. 1 kg for each species) was transferred to the laboratory inside plastic bags, where it was carefully cleaned from debris and dead or senescent parts, and left to dry out in dim light. A mixture of moderately isidiate, 28.1 ± 4.0 mm long with a biomass of 33 ± 18 mg ($n = 50$) lobes of *P. furfuracea* was prepared, avoiding those with fruiting bodies (apothecia), infected by lichenicolous fungi, covered by epiphytic algae, or over-isidiate. Only the terminal, green part of each moss shoot was sampled, each being 22.1 ± 6.9 mm long, and weighed ca. 5.2 ± 3.6 mg ($n = 50$).

Lichen and moss material was re-hydrated leaving it overnight in a humid chamber, and washed four times with 10 litres distilled water per 100 g dry weight; after each washing, lasting respectively 20, 15, 10 and 5 min, the material was gently hand centrifuged. The washed material was left to dry out, carefully mixed, divided in aliquots of ca. 500 mg, and stored in petri dishes at −32 °C until use.

2.2. Bag preparation, exposure and collection

Sub-spherical bags, containing 500 mg of lichen and moss material were prepared with a commercial nylon mosquito net, with mesh of ca. 2 mm, cut in pieces of ca. 12 × 12 cm, and closed with a nylon thread. The bags were put in a closed dryer for at least 24 h, weighed, and suspended on latticeworks (Fig. 1). The samples were left for 6 weeks on the roof of an automated air pollution monitoring station located in the industrial zone of Trieste, and outside the main railway station at Naples. The main sources of air pollution are vehicular traffic, shipyards, and metallurgical plants (Adamo et al., 2003; Castello et al., 1995).

During exposure period (Trieste: 1 March 2003–11 April 2003; Naples: 14 April 2003–29 May 2003), the main meteorological parameters and concentrations of conventional air pollutants were recorded, and PM_{10} samples were collected (Table 1).

Both cities are located on the sea, but whereas Trieste has a sub-Mediterranean climate, with a strong influence of dry, continental ENE winds (“bora”) (Stravisi, 1980), Naples has a typical Mediterranean climate, with a prolonged dry season and mild winter, and mainly westerly, humid winds blowing from the Tyrrhenian Sea (SE to SW) (UNESCO-FAO, 1963).

2.3. Vitality assessment

Vitality was assessed, before and after exposure, using two sets of samples for each species. The first set consisted of randomly selected aliquots taken from four bags or from the reference material preserved in petri dishes; these aliquots were employed for transmission electron microscopy (TEM) observations, study of K cellular location, and for the analysis of C, N, and S content. The second set consisted of the entire content of four other bags that were used for photosynthetic pigments content, CO_2 gas exchange, and chlorophyll fluorescence.

2.3.1. TEM observations

Lichen and moss samples were rehydrated in a humid chamber for 48 h. After fixation with 3% glutaraldehyde at room temperature and post-fixation in 2% OsO_4 in 0.1 M phosphate buffer (pH 6.8) at 4 °C, they were dehydrated with ethanol and embedded in Spurr’s epoxy resin. Ultra-thin sections (60 nm) were cut with a diamond knife on a Supernova microtome and sequentially stained at room temperature with 2% uranyl acetate (aqueous) for 5 min and lead citrate for 10 min. Ultrastructural observations were made with a Philips EM208S transmission electron microscope (TEM) operated at 80 kV and equipped with Mega View digital camera.

2.3.2. Cellular location of potassium (K)

The sequential elution technique (Brown and Brown, 1991; Brown and Wells, 1988) was used to determine the cellular location of K in triplicates (30–80 mg each) taken from pre- and post-exposed bags. Lichen and moss samples were stored for 24 h in a glass-lidded tray lined with wet paper, to reactivate the physiological activity and reduce membrane permeability (Buck and Brown, 1979). Extracellular K ions either bound or unbound to the outside surface of lichen and moss cells were recovered by shaking the material in 10 and 5 ml of 20 mM $NiCl_2$ respectively for 40 and 30 min. The samples were then dried out overnight at 80 °C to rupture cell membranes without causing alteration to the distribution of elements (Branquinho, 1997), and weighed. The soluble

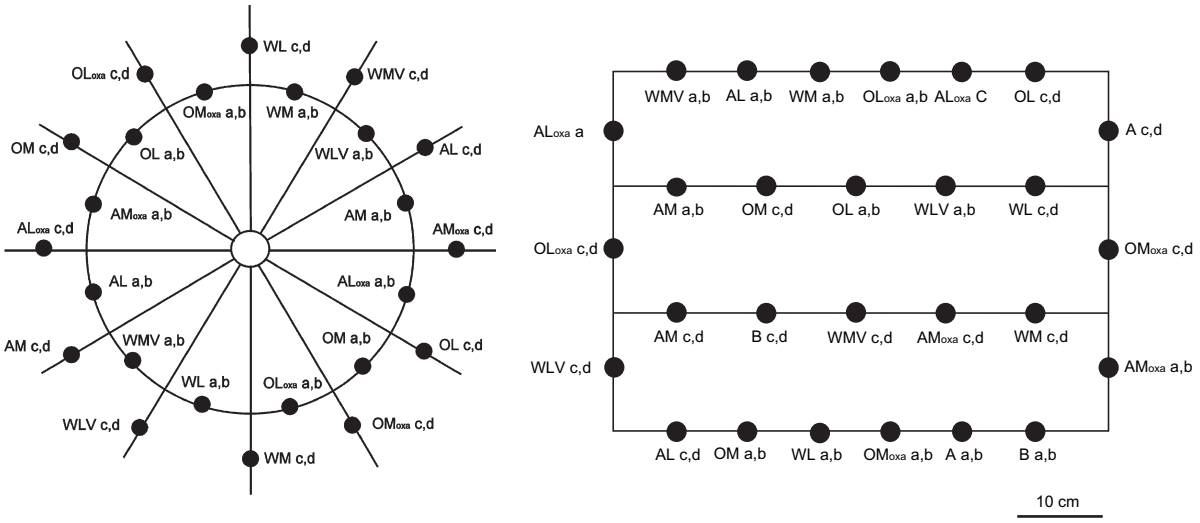

Fig. 1. Arrangement of suspended bags on the two latticeworks used in Trieste (left), and Naples (right), top view. For each position, letters identify samples on the first (a, c) and the second (b, d) latticework, located at c. 3 m from each other. WLV, WMV: water washed lichen/moss for vitality assessment; WL, WM: water washed lichen/moss; OL, OM: oven dried lichen/moss; AL, AM: acid washed lichen/moss; OL_{oxa}, OM_{oxa}: oven dried, NH_4-oxalate treated lichen/moss; AL_{oxa}, AM_{oxa}: acid washed, NH_4-oxalate treated lichen/moss; A, B: synthetic filters A and B. WLV, WMV, WL, and WM were used for this study. Data concerning the others, plus WL and WM are commented upon by Adamo et al. (2006).

intracellular fraction was obtained by shaking the samples in 10 ml of cold 65% HNO_3 for 60 min. The particulate fraction was assessed treating the residue in 7 ml of boiling 65% HNO_3 with successive addition of 3 ml of H_2O_2.

Each fraction was analysed by atomic absorption spectrophotometry (Perkin Elmer Analyst 700) using an air/acetylene flame and with addition of 1 g L^{-1} of $LaCl_3$ to samples and standards, as suppressants of refractory compounds formation.

2.3.3. Carbon, nitrogen and sulphur total content

C, N and S total content was measured according to the flash combustion procedure with a Fisons 1108 Elemental Analyser in triplicates of ca. 10 mg each taken from four bags before and after exposure. In order to avoid H_2O-SO_2 signals overlapping, a trap system of $Mg(ClO_4)_2$ was employed to remove the excess of H_2O from the gases produced with combustion. Accuracy and recovery of elements were checked analysing a sample of sulphanilamide as standard after eight lichen or moss samples.

2.3.4. Photosynthetic pigments

Analysis of photosynthetic pigments was carried out before and after exposure taking 70 mg of material from each bag. These subsamples were left for 10 min over silica and divided in two aliquots of 35 mg each. To remove the lichen substances that can damage chlorophylls (Brown and Hooker, 1977), the lichen material was washed with aliquots of 4 ml pure acetone until the solution did not turn yellow after the addition of a drop of conc. KOH. Lobes and cauloids were cut in small fragments with scissors, and homogenised in a potter in 8 ml DMSO under dim, green light (after adding a small quantity of PVPi for lichen material); tubes were left overnight at room temperature in the dark, and centrifuged at 5000 rpm for 10 min. The supernatant was recovered, the pellet re-suspended in 2 ml DMSO, centrifuged as above, and supernatant mixed with the previous one. The absorbance before and after acidification with 50 μl HCl 1 N for 10 ml sol. was measured with a spectrophotometer Perkin–Elmer model 554, after checking turbidity ($A_{750} < 0.010$). Chlorophylls and total carotenoids estimation was carried out using the equations of Pfeifhofer et al. (2002), whereas the ratio OD_{435}/OD_{415} was used as an estimation of chlorophyll degradation (Ronen and Galun, 1984).

2.3.5. CO_2 gas exchanges

Samples of ca. 430 mg each (i.e. the content of a bag minus the material used for the first chlorophylls assay) were re-hydrated for 18 h in the dark in a closed chamber filled with wet paper, gently sprayed with distilled water and blotted with absorbent paper. The lichen lobes and the moss shoots of each sample were positioned in a petri dish, covered with a thin mesh to avoid the curling of material, and introduced in the cuvette of a LI-COR 6200 (LI-COR, Lincoln, NE, USA) operating in closed circuit. CO_2 exchange rates were measured at 17 ± 1 °C (thallus temperature) and 360 ± 10 ppm CO_2. Optimal conditions for light and thallus water content were determined in a preliminary series of measurements: in *Pseudevernia furfuracea* CO_2 gas exchange was thus measured at 270 μmol photons m^{-2} s^{-1}, at decreasing thallus water content (140–80% RWC); in *Hypnum cupressiforme* at 100 μmol photons m^{-2} s^{-1}, and 310–190% RWC. Alternate series of three measurements of net photosynthesis (Ph_n) and dark respiration (R_d) were taken. At the end each sample was re-hydrated, and used for a second series of measurements. The two triplets corresponding to the two optimal RWC were used for calculating the mean value ± S.D. Gross photosynthesis (Ph_g) was calculated as the sum of Ph_n and $|R_d|$.

2.3.6. Chlorophyll fluorescence

Chlorophyll fluorescence measurements were performed in light and dark-adapted samples immediately after the CO_2 gas exchange measurements. Samples were re-hydrated to obtain high RWCs, transported in a refrigerant room at 18 °C, positioned on wet filter paper, and exposed to the same light intensity used for the CO_2 gas exchange measurements (light source: LEUCI mod. HLI-T250W lamp). After 10 min, light fluorescence measurements were performed. Thereafter samples were re-hydrated and placed in a darkened humid chamber. After 90 min, dark fluorescence measurements were performed.

Twelve measurements were taken for each sample. In *P. furfuracea* the measurements were carried out always in the same position on four lobes, previously selected and marked, of each sample. For each lobe three fluorescence measurements were taken on fixed positions (0.8, 1.6 and 2.4 cm from tip). In *H. cupressiforme*, owing to the small size of shoots and leaflets, measurements were taken randomly on the parts where the fluorescence signal was higher than the resolution level for the selected setting. This caused the exclusion of the most severely damaged portions, and thus an overestimation of the mean values of the moss samples.

Chlorophyll fluorescence measurements were carried out with portable pulse-amplitude modulated fluorescence equipment (MINI-PAM, Walz GmbH, Effeltrich, Germany), using an apposite distance clip. The samples were exposed to a weak, not actinic measuring beam, in order to calculate the minimum fluorescence yield (F_o). Flashes of 0.8 s duration and 8 000 μmol photons m^{-2} s^{-1} allowed the determination of the maximum fluorescence yield of dark (F_m) and light (F_m') adapted samples. F_v/F_m and $\Delta F/F_m'$ parameters were calculated according to Schreiber and Bilger (1993).

Table 1
Main parameters recorded by the automated monitoring stations used for sample exposure (A, C) or other stations present in the surrounding areas (B, D–F), and technical data of analytical instrumentation

Parameters analysed	Trieste	Naples
Climatic data		
Temperature (°C)	A[1]	C[9], E[15]
Pressure (hPa)	A[1]	C[9]
Wind direction (°N)	B[7]	C[9]
Wind speed (m s^{-1})	A[1]	C[9]
Rainfall (mm)	A[1]	F[16]
Solar radiation (W m^{-2})	B[8]	C[9]
Pollutants		
SO_2 (μg m^{-3})	A[2]	D[10]
NO_X (μg m^{-3})	A[3]	C[11]
NO_2 (μg m^{-3})	A[3]	C[11]
NO (μg m^{-3})	A[3]	C[11]
CO (mg m^{-3})	A[4]	C[12]
PM_{10} (μg m^{-3})	A[5,6]	C[13,14]

A: Data from TS02CARP—ARPA Friuli Venezia Giulia (http://www.arpa.fvg.it/Aria-Radia/Tutela-Qua/RETE-DI-RI/CENTRALINE/VIA-CARPIN/index.htm). [1](hourly mean values): meteorological sensors Micros. [2]SO_2 (hourly mean values): UV Fluorescent Sulfur Dioxide Analyzer AF21M Environnement S.A. [3]NO_X, NO_2, NO (hourly mean values): Chemiluminescent Nitrogen Oxide Analyzer AC31M Environnement S.A. [4]CO (hourly mean values): Gas Filter Correlation Carbon Monoxide Analyzer CO11M Environnement S.A. [5]PM_{10} (hourly mean values): Ambient Suspended Particulates Beta Gauge Mass Monitor MP101M Environnement S.A. [6]PM_{10} Sampler (daily samples): Automatic Sequential Sampling Station Skypost PM TCR TECORA, equipped with 47-mm glass fibre filter membranes; 58.5 m^3 air day^{-1}. Data acquisition system: Micros Datapro 32.
B: Data from Meteorological station of Trieste—Piazza Hortis (http://www.dst.univ.trieste.it/OM/OM_TS.html). [7](ten-minute mean and maximum values): Anemometer Micros SVDV. [8](ten-minute mean values): Pyranometer CM 11 Kipp and Zonen.
C: data from NA7; D: data from NA1, NA2, NA3—Centro Regionale Inquinamento Atmosferico (CRIA)—ARPA Campania (http://www.arpacampania.it/home.html). [9](hourly mean values): meteorological sensors Lastem. [10]SO_2 (hourly mean values): UV Fluorescent Sulfur Dioxide Analyzer AF21M Environnement S.A. [11]NO_X, NO_2, NO (hourly mean values): Chemiluminescent Nitrogen Oxide Analyzer ML9841B Monitor Europe. [12]CO (hourly mean values): Gas Filter Correlation Carbon Monoxide Analyzer ML9830B Monitor Europe [13]PM_{10} (hourly mean values): Ambient Suspended Particulates PM_{10} Light Scattering UNITEC LSPM10. [14]PM_{10} Sampler (daily samples): Automatic Sequential Sampling Station Skypost PM TCR TECORA, equipped with 47-mm cellulose filter membranes; 55.8 m^3 air day^{-1}. Data acquisition system: EDA2000 ORION s.r.l.
E: Data from EuroMETEO (http://www.eurometeo.com/italian/home). [15](hourly mean values): meteorological sensors Micros.
F: data from Servizio Idrografico e Mareografico dello Stato. [16](daily mean values): meteorological sensors Lastem.

3. Results

3.1. Weather conditions and concentrations of gaseous pollutants during exposure

The main climatic parameters measured during the exposures are reported in Fig. 2. In Trieste rainfall events were concentrated in the last two weeks, in Naples they were more evenly distributed, but the total amount was lower (38.2 vs. 56.6 mm). Temperature ranged between 6.4 and 12.7 °C (mean minimum and maximum values) in Trieste, and between 15 and 25 °C in Naples. Winds were noticeably more intense in Trieste than in Naples, with a mean hourly speed of 2.5 vs. 1.5 m s^{-1}, and absolute maxima, respectively, of 10.0 and 4.7 m s^{-1}.

Concentrations of conventional air pollutants and PM_{10} recorded by the two automatic devices are reported in Fig. 3. In Trieste in windy days (Fig. 2A,C) pollutant concentrations typically decreased (compare Figs. 2 and 3), probably because winds coming from relatively unpolluted regions diluted pollutants; this did not occur in Naples, where windy events were relatively more constant and of low intensity (Fig. 2B,D).

Total average of maximum daily values of NO_2, CO, PM_{10} and total average of daily mean values of all four parameters were always lower in Trieste (Table 2). With the exception of PM_{10}, all the other parameters were considerably lower than the limits foreseen by the most recent EU directives. The total average of daily mean values of sulphur dioxide, the most harmful myco- and phytotoxic pollutant (Seaward, 1993; Winner, 1988), was always considerably lower than the tolerance threshold of the two species, estimated to be about 60 μg m^{-3} (*P. furfuracea*, see Hawskworth and Rose, 1970), and 40 μg m^{-3} (*H. cupressiforme*, see Gilbert, 1970).

3.2. TEM observations

After exposure the two species showed several ultrastructural modifications, particularly spectacular in the moss.

In pre-exposure leaflets of *Hypnum cupressiforme* both cytoplasm organisation and organelle ultrastructure were well preserved, with plasma membranes appressed to the thick cell walls (Fig. 4A), vacuoles with an electron-dense cell sap present together with spherical mitochondria, and grana, stroma-thylakoids and plastoglobules of chloroplasts clearly distinguishable (Fig. 4C). After exposure, most of the cells appeared strongly plasmolysed and with other serious stress marks (Fig. 4B). Some cells were almost completely empty because protoplasm was condensed at the proximal and distal ends of cells. Few and small, very electron-dense vacuoles were observed, while cytoplasm, and endo-membrane systems were almost completely lacking and the typical ultrastructure of the chloroplast was lost. However, the plasma membranes appeared to be still entire (Fig. 4D).

In *Pseudevernia furfuracea*, on the contrary, the ultrastructural traits of the cells were well preserved in post-exposed material. The photobiont cells retained a large chloroplast with a central pyrenoid surrounded by numerous plastoglobules and a peripheral band of grana- and stroma-thylakoids (Fig. 4E,F). The only significant ultrastructural modification was the presence of concentric bodies, visible in the cytoplasm of exposed mycobiont cells (Fig. 4H).

3.3. Cellular location of potassium

The K distribution among different fractions (extracellular, intracellular and particulate matter) of pre- and post exposure samples is reported in Table 3. The two species had similar intracellular K content before exposure. Both of them suffered

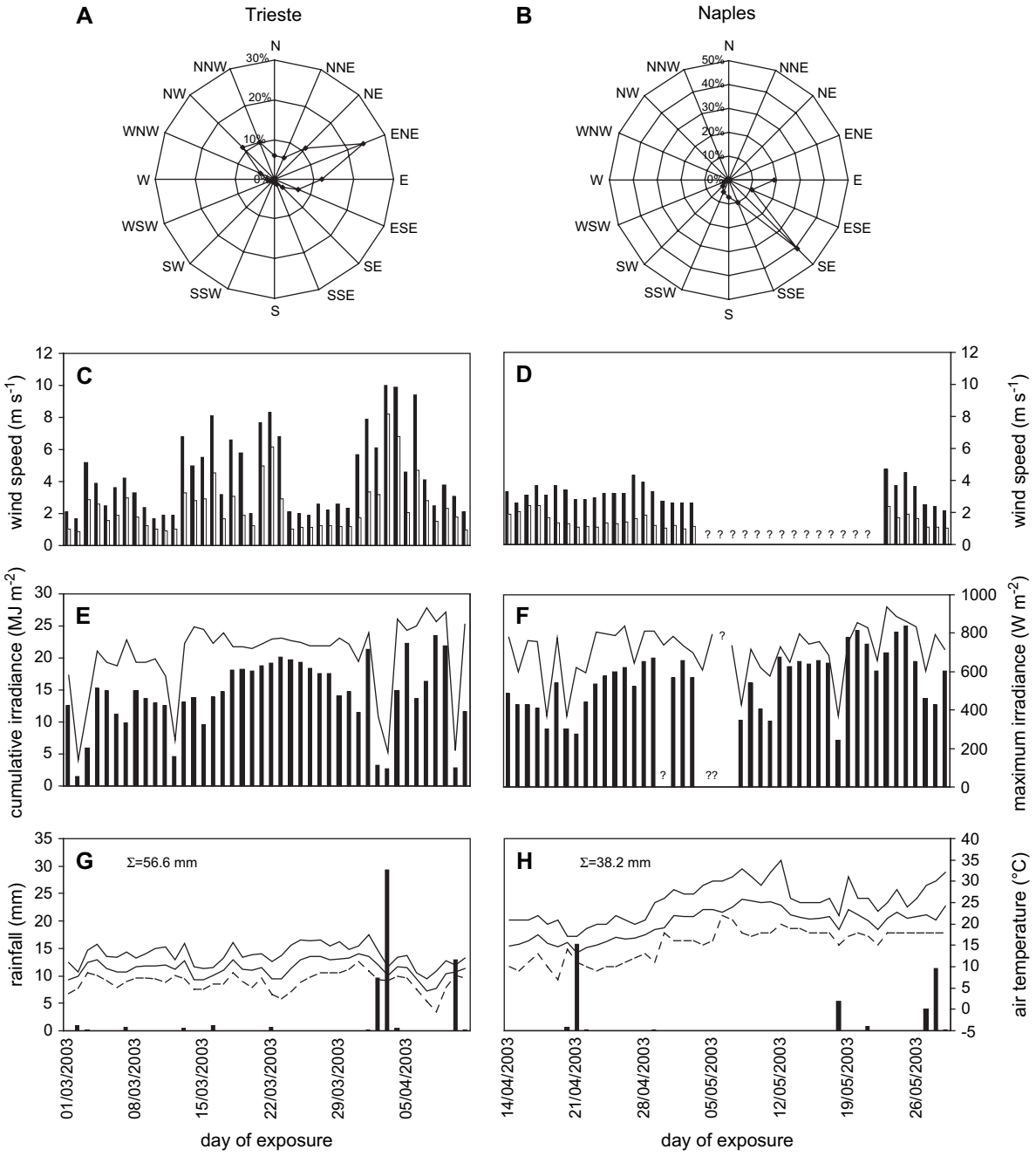

Fig. 2. Climatic conditions in Trieste (left column, A,C,E,G) and Naples (right column, B,D,F,H) during sample exposure: per cent frequency of winds (hourly mean) (A,B); wind speed: daily maximum of hourly means (black bars), and daily average of hourly means (white bars) (m s^{-1}, C,D); daily cumulative global irradiance (bars; MJ m^{-2}), and maximum daily global irradiance (continuous line; W m^{-2}) (E,F); daily rainfall (bars, mm), minimum, mean, and maximum air daily temperature (dashed, thick, and thin line, respectively; °C), and total rainfall during exposure (Σ; mm) (G,H).

a subsequent decrease with exposure, which ranged from ca. 30% to 50%. Apparently, the leakage was more pronounced in the moss samples exposed in Trieste than in those exposed in Naples, whereas the reverse was true for the lichen. However, it must be underlined that this leakage was associated with a concomitant change in K distribution between all cell compartments: in both cities extracellular and particulate K amounts (cold $NiCl_2$ and boiling HNO_3 fractions) significantly increased, apart from the moss in Trieste. This change was more evident in the Naples samples, probably because they entrapped more soil particles. Soils in the Naples area are notoriously rich in K (Imperato et al., 2003). In contrast, it was not possible to observe marked differences between intra- and extracellular K distribution in lichen and moss species in either sites after exposure.

3.4. Carbon, nitrogen and sulphur total content

Both species had similar C, N, and S concentrations before exposure (Table 4). However, a marked difference was

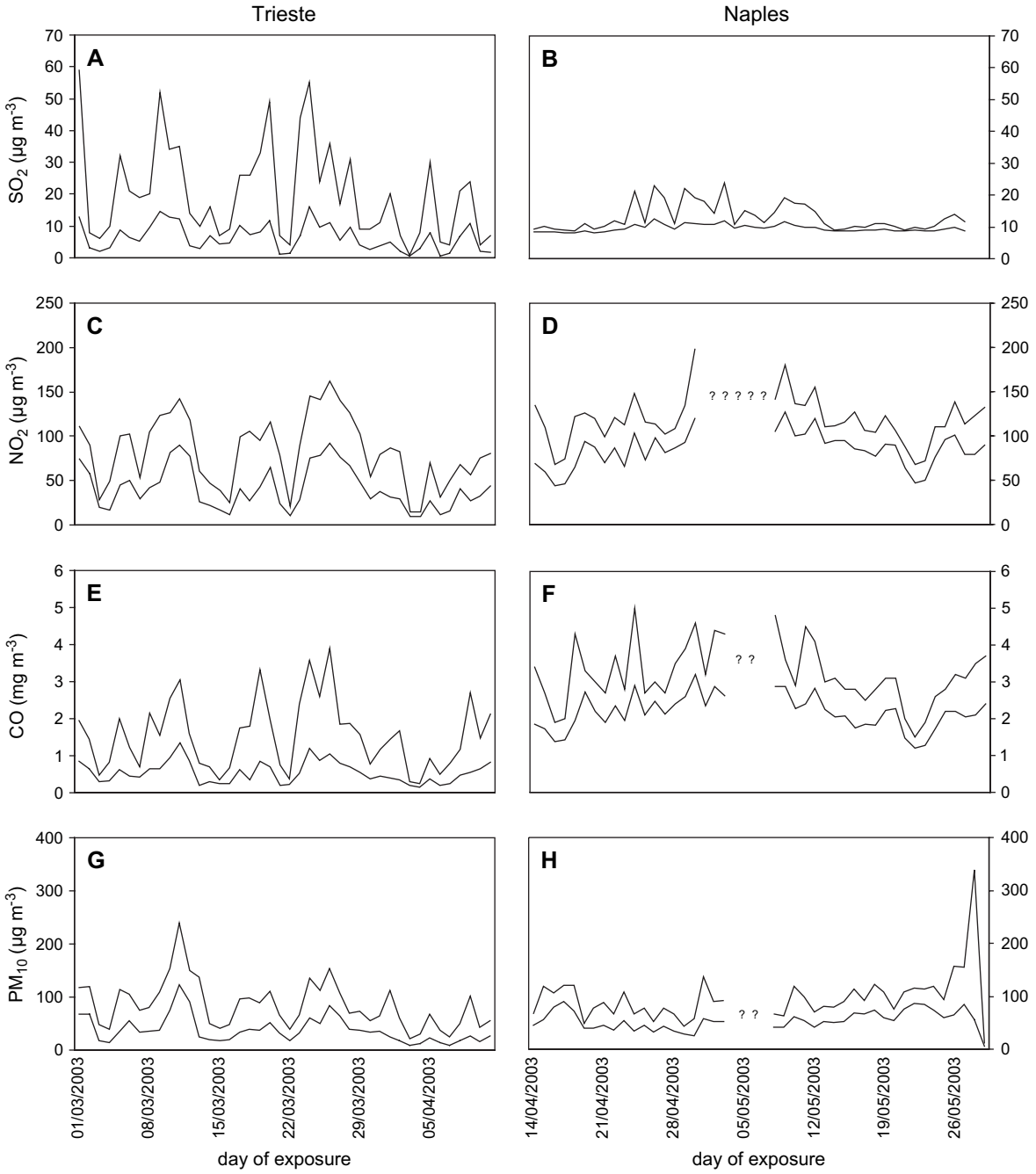

Fig. 3. Air concentration of pollutants in Trieste (left column, A,C,E,G) and Naples (right column, B,D,F,H) during sample exposure: SO_2 (A,B); NO_2 (C,D); CO (E,F); PM_{10} (G,H). Thick line: daily average of hourly mean values; thin line: daily maximum of hourly mean values.

observed after exposure, suggesting that the two cryptogams differ significantly in their capacity to accumulate and retain these elements from the atmosphere (Table 4). Significant increase in C and N content was observed in the lichen exposed in both cities, but not in the moss, which retained its original C and N content. However, the N increase in the lichen was higher in Naples (+26%) than in Trieste (+18%), possibly because of the higher NO_2 availability in the former locality (see Fig. 3C,D). A different pattern in the S content was observed in the lichen, with strong decreases (55–58%) in both cities. After exposure, the S content of the moss was higher only in Naples (+29%). This increase was likely related to a more efficient entrapping of sulphate particles derived from human activities, as well as by fumarolic activity and sea aerosols.

3.5. Photosynthetic pigments

Before exposure, the moss had slightly higher Chl_a and total carotenoids contents than the lichen (respectively, 1.86 ± 0.23 and 0.56 ± 0.06 µg mg^{-1} vs. 1.59 ± 0.21 and 0.44 ± 0.05 µg mg^{-1}), and similar contents of Chl_b (Fig. 5).

Table 2
Comparison of mean values (± S.D.) of pollutants recorded during exposure in Trieste and Naples

Pollutants	Total average of daily mean values		Total average of maximum daily values	
	Trieste	Naples	Trieste	Naples
SO_2 ($\mu g\ m^{-3}$)	*6.3 ± 4.2*	*9.5 ± 1.1*	*20.6 ± 15.4*	*13.0 ± 4.3*
NO_2 ($\mu g\ m^{-3}$)	*41.1 ± 24.0*	*84.1 ± 19.9*	*83.5 ± 39.0*	*118.3 ± 26.5*
CO ($mg\ m^{-3}$)	*0.5 ± 0.3*	*2.2 ± 0.5*	*1.6 ± 0.9*	*3.2 ± 0.8*
PM_{10} ($\mu g\ m^{-3}$)	*37.9 ± 24.6*	*53.9 ± 18.3*	85.1 ± 44.2	97.1 ± 48.5

Significantly different (*t*-test, $p < 0.01$) mean values between Trieste and Naples in italics.

This implies a slight different Chl_a/Chl_b ratio in the two species (3.68 ± 0.60 vs. 3.30 ± 0.36). The smaller difference in the ratio OD_{435}/OD_{415} in *P. furfuracea* is probably determined by the presence of melanins in its lower cortex; these pigments are soluble in DMSO, and their partial removal causes the deviation from the canonical value of 1.40 (Ronen and Galun, 1984).

After exposure, photosynthetic pigments were considerably lower in the moss: Chl_{a+b} and total carotenoids respectively decreased by 37 and 39% of their original value. However, in this species the ratio OD_{435}/OD_{415} decreased only slightly (from 1.40 ± 0.03 to 1.34 ± 0.05 in Trieste, and to 1.31 ± 0.02 in Naples; OD_{435}/OD_{415} ° in Fig. 5), suggesting that the metabolic processes involved in the chlorophylls turnover were still normally active. In the lichen, the post-exposure reduction of photosynthetic pigments was limited, being generally less than 9%, and the variation of the ratio OD_{435}/OD_{415} occurred only in the Naples samples.

An interesting pattern was observed in the variation of the ratio OD_{435}/OD_{415} measured in crude extracts after their acidification for the quantification of phaeophytins. Whereas the mean values measured in pre- and post-exposure samples of *P. furfuracea* did not differ significantly (ca. 0.55 ± 0.01; OD_{435}/OD_{415} * in Fig. 5), those from post-exposure samples of *H. cupressiforme* (both cities) were statistically higher than blanks (0.63 ± 0.03 vs. 0.57 ± 0.00 with $p < 0.001$, Mann–Whitney *U*-test); the addition of further aliquots of acid did not modify these values, indicating that transformation of chlorophylls to phaeophytins had already completed.

3.6. CO_2 exchange rates

Before exposure, the two species had similar rates of CO_2 gas exchange, with $Ph_n = 2.49 \pm 0.35$ mg $CO_2\ g^{-1}\ h^{-1}$ (*H. cupressiforme*), and 2.38 ± 0.47 mg $CO_2\ g^{-1}\ h^{-1}$ (*P. furfuracea*), and $R_d = -0.68 \pm 0.16$ and -0.87 ± 0.15 mg $CO_2\ g^{-1}\ h^{-1}$, respectively (Fig. 6). After exposure, Ph_n and Ph_g decreased dramatically in the moss: Ph_n was reduced almost to zero in Naples, whereas the decrease was a little more moderate (ca. 85%) in Trieste. Since R_d did not change significantly, the reduction of Ph_g was lower (76% and 81%, respectively). In the lichen, CO_2 gas exchanges did not change significantly in Trieste; in Naples, on the contrary, a reduction of ca. 34% and 39% was recorded in Ph_g and Ph_n respectively, whereas R_d kept unchanged in both sites.

3.7. Chlorophyll fluorescence

Chlorophyll fluorescence also indicated that the moss suffered the exposure conditions more than the lichen, although this technique revealed that the lichen also suffered some light damage. The two species had rather similar values of F_v/F_m (0.679 ± 0.027 *H. cupressiforme*, 0.653 ± 0.047 *P. furfuracea*; $n = 96$; Fig. 7), but different $\Delta F/F_m'$ values (0.472 ± 0.045 and 0.339 ± 0.064, respectively; $n = 96$; Fig. 7) because of the different light intensity used (see Section 2). The *P. furfuracea* F_v/F_m value lies within those reported by Calatayud et al. (1997) and Vidergar-Gorjup et al. (2001) (ca. 0.730), and Manrique et al. (1993) and Niewiadomska et al. (1998) (ca. 0.610–0.628); unfortunately, the few data available for *H. cupressiforme* (Deltoro et al., 1998) cannot be used for a direct comparison.

After exposure, F_v/F_m and $\Delta F/F_m'$ values decreased dramatically in *H. cupressiforme* (Trieste: 51% and 49%; Naples: 57% and 59%), but considerably less in *P. furfuracea* (Trieste: 94% and 91%; Naples: 78% and 48%). Parameters that were originally similar in the two species (F_v/F_m variation coefficient: 4% and 7%, respectively), grew considerably after exposure, but less in the lichen (+12% in Trieste and +18% in Naples), than in the moss (+25% in Trieste and +28% in Naples).

4. Discussion

4.1. How to estimate vitality?

In this study we used different techniques to characterise sample vitality. The data gathered were mostly rather similar, with some noticeable exceptions. For instance, we observed a marked difference between intra- and extracellular K distribution in each species at each urban site (Table 3). Many investigators have used K cell distribution as a measure of impact of air polluted conditions on membrane integrity, and vitality, of moss and lichen samples (Brown and Brumelis, 1996; Tretiach et al., 1999). Damage to the cell membrane may imply an inefficient functioning of osmotic regulation mechanism that causes a strong K^+ leakage from the cytoplasmatic compartment and an increase of extracellular K (Brown and Brown, 1991). We suppose that the observed discrepancies in Table 3 can be explained by the fact that the moss is more efficient than the lichen in accumulating particulate matter (see Adamo et al., 2007). As we used cold HNO_3 to disrupt cell membranes, the acid probably solubilised, at least partially, the inorganic

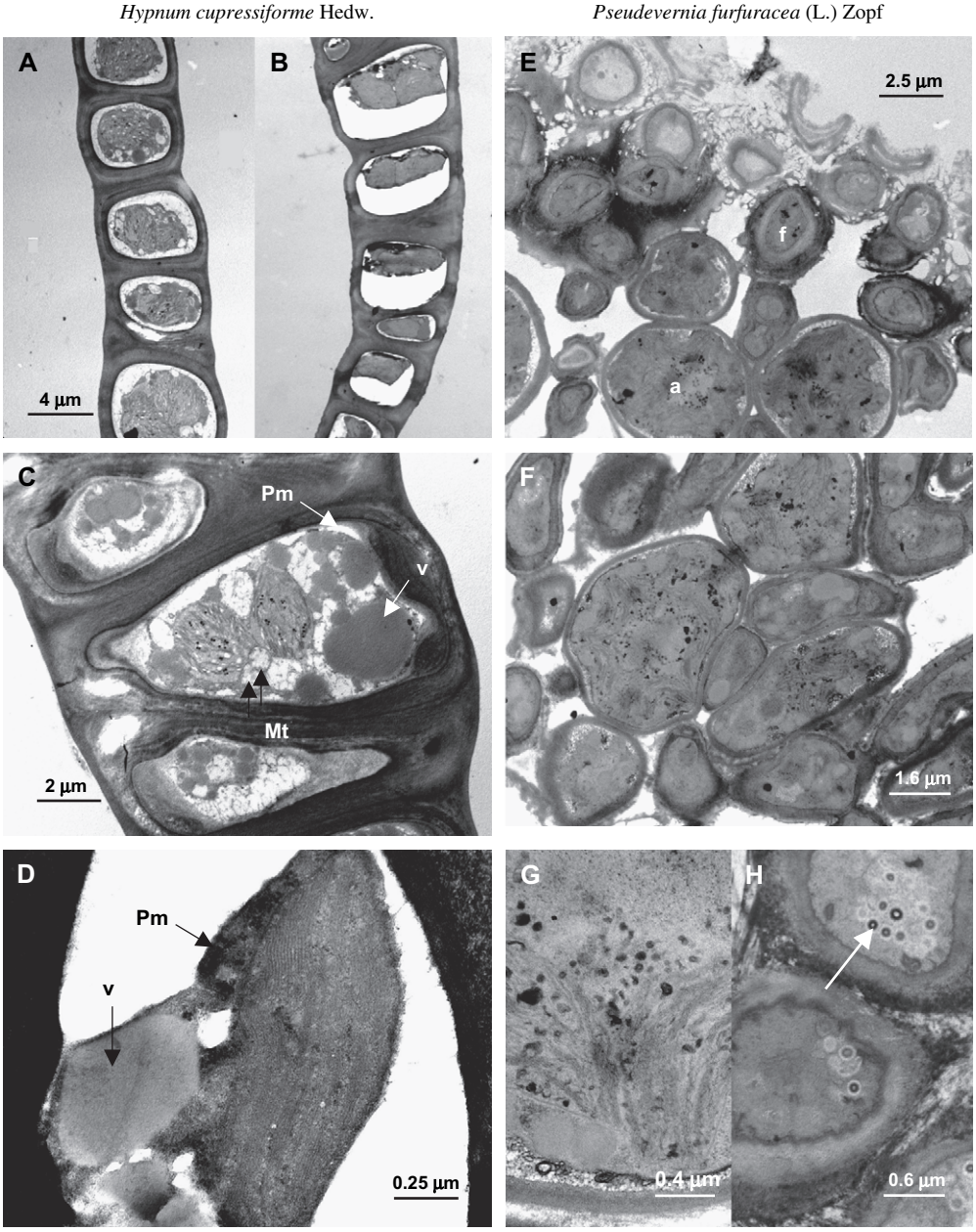

Fig. 4. Cell ultrastructure of *Hypnum cupressiforme* (left column, A–D), and *Pseudevernia furfuracea* (right column, E-H) before (A,C,E,G), and after (B,D,F,H) exposure. TEM, ultrathin sections: Pm, plasma membrane; Mt, mitochondria; v, vacuole; a, cell of the photobiont; f, cell of the mycobiont. The arrow in H shows the presence of concentric bodies in the cytoplasm of a hypha. Further comments in the text.

particulate matter. Being rich in K minerals, dissolved particulate probably contributed to an increase in intracellular K content. This problem might probably be overcome by using cold $NiCl_2$ or EDTA, as opportunely suggested by Brown and Brown (1991), and Branquinho and Brown (1994).

The differences encountered among data sets obtained with the other methods are due to several factors, but the most important is certainly the fact that in most analyses we used subsamples taken from single bags. Within the bag, the material forms a sort of more or less dense ball, and therefore part of the material, placed in the centre of the ball, is relatively protected. If the selected methodology foresees the use of the whole sample, as is the case of the CO_2 gas exchange measurements, this is not a problem, but it may become a source of noise when only small amounts of material are analysed (e.g. in TEM observations; C, N, and S content, chlorophyll fluorescence). For this reason, we regard as most trustworthy the data of CO_2 gas exchange, which show that exposure was more severe in Naples than in Trieste, in good agreement with ultrastructural observations (Fig. 4).

It should be underlined that photosynthesis is extremely sensitive to pollutants and to changes in environmental conditions in general (Larcher, 1995). Research on photosynthesis after transplantation has been limited, but a general reduction, more or less intense, in the gas exchanges has often been documented (Boonpragob and Nash, 1991; Tretiach

Table 3
Potassium mean values (±S.D., μg g^{-1}) and % of cumulative total (in italics), sequentially eluted by cold $NiCl_2$ (extracellular), cold HNO_3 (intracellular) and boiling HNO_3 (particulate matter) in pre- and post-exposed samples of *Hypnum cupressiforme* and *Pseudevernia furfuracea* in Trieste and Naples

	Pre-exposure	Post-exposure	
		Trieste	Naples
Hypnum cupressiforme			
Cold $NiCl_2$ (extracellular)	561±162	716±73	1229±156
	15	*29*	*34*
Cold HNO_3 (intracellular)	2995±373	1566±151	2055±284
	79	*64*	*57*
Boiling HNO_3 (part. matter)	225±47	174±30	336±70
	6	*7*	*9*
Cumulative total	3781	2456	3620
Pseudevernia furfuracea			
Cold $NiCl_2$ (extracellular)	328±91	601±80	523±71
	10	*22*	*25*
Cold HNO_3 (intracellular)	2840±159	1945±191	1305±195
	88	*73*	*61*
Boiling HNO_3 (part. matter)	74±54	135±27	295±184
	2	*5*	*14*
Cumulative total	3242	2681	2123

and Baruffo, 2001), sometimes also in unpolluted areas (Palmqvist and Sundberg, 2000). The low number of studies based on this technique is probably due to the fact that data variability in gas exchange measurements is typically very high, being strongly influenced, for example, by the different ratio of old and young parts (Türk, 1983), development of reproductive structures [in lichens, for instance, that of soredia, see Tretiach and Carpanelli (1992), or isidia, see Tretiach et al. (2005)], and chlorophyll content (Valladares and Sancho, 2000; Valladares et al., 1996). Unfortunately, this noise often obscures effects of the factors that are under investigation. However, CO_2 gas exchange measurements provide an overview of the health condition of the material, and they are very precise if carried out at optimal conditions for light, temperature, and hydration. These optima must be already available from previous studies, or defined in a preliminary phase of the investigation. The weak point is that this technique does not allow one to distinguish specific factors involved in depressing (or increasing) CO_2 gas exchanges.

Table 4
Carbon, nitrogen and sulphur contents in pre-exposure material and per cent variation after exposure in Trieste and Naples

	Ref.	% variation	
		Trieste	Naples
Hypnum cupressiforme			
C (%)	43.47±0.37	2	1
N (mg g^{-1})	15.25±0.28	0	−4
S (mg g^{-1})	2.38±0.20	−58*	29*
Pseudevernia furfuracea			
C (%)	42.19±0.20	6*	6*
N (mg g^{-1})	13.07±0.19	18*	26*
S (mg g^{-1})	2.07±0.20	−58*	−55*

Statistically significant (*t*-test, $p < 0.001$) differences are indicated by an asterisk.

Chlorophyll fluorescence, already used in recent years for studying the effects of gaseous phytotoxic substances (e.g. Garty, 2000; Niewiadomska et al., 1998; Scheidegger and Schroeter, 1995), has been rarely used in studies on the vitality of transplanted lichens and mosses (Grigor'ev and Buchel'nikov, 1997; Kauppi, 1980). With respect to direct measurements of gas exchanges, chlorophyll fluorescence is more feasible, less complex, and rapid, and therefore it is possible to carry out a very high number of non-destructive measurements, which give a very reliable estimation of sample vitality, because they are very efficient in demonstrating small, but significant changes (Jensen, 1994). Nevertheless, in view of the small areas investigated, a high number of measurements are necessary, particularly when the material is potentially heterogeneous.

4.2. A ''still life'' of living and dead samples

The short exposure in the two cities was sufficient to reduce, also considerably, sample vitality of both organisms. The lichen showed a greater resilience, because only some parameters were modified after exposure, whereas in the moss practically all the parameters were reduced, sometimes in an outstanding way, as in the case of net photosynthesis (Fig. 6).

Airborne phytotoxic substances probably played a very marginal role in this modification. Concentrations of the most important gaseous pollutants were relatively low during both exposures (Fig. 3). Although a possible synergistic effect between accumulation of heavy metals and SO_2 has been shown (Miszalski and Niewiadomska, 1993), the data of chlorophyll degradation (Fig. 5) suggest that both organisms did not suffer the toxic effects of acidifying pollutants, or at least they were able to buffer them effectively, as we can hypothesise on the basis of the final value of the ratio OD_{435}/OD_{415} measured after acidification of the extracts.

The differences between the two species can probably be explained in terms of their different ecological requirements. *Pseudevernia furfuracea* is a typical light-demanding lichen, which colonises exposed environments (Wirth, 1995), and show a wide array of physiological, morphological and anatomical features that make it particularly resistant to prolonged desiccation and high light regimes (Rikkinen, 1997). *Hypnum cupressiforme*, on the contrary, is more scio- and hygrophilous: when epiphytic, it typically occurs in the most protected part of the tree canopy, i.e. on lower branches and on the trunk (Diersen, 2001). The exposure in the suspended bags was certainly a drastic change of environment, less traumatic for the lichen, which, particularly in Trieste, showed only minor signs of physiological stress. In the same way, we may explain the noteworthy difference observed in species vitality between the two cities, due to the different environmental conditions (Fig. 2), linked to the climate and to the period of the year when the two exposures occurred. The lower precipitation, and the higher light regime of the exposure period (closer to the summer solstice than in Trieste) can easily explain the more drastic reduction of sample vitality recorded in Naples, particularly evident in *H. cupressiforme*. In mosses, the ability

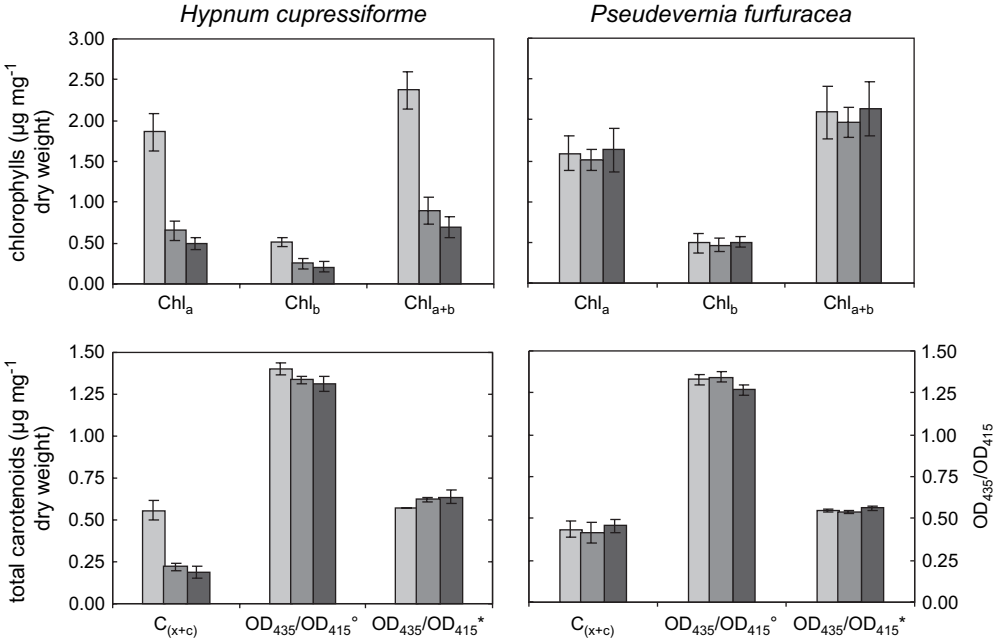

Fig. 5. Photosynthetic pigments content (µg mg^{-1} dry weight) and ratio OD_{435}/OD_{415} (Ronen and Galun, 1984), before (°) and after acidification (*) of crude extracts, in *Hypnum cupressiforme* and *Pseudevernia furfuracea* before (□), and after exposure in Trieste (▨) and Naples (■). Chl_a, Chl_b, Chl_{a+b} = chlorophyll a, b, a + b; $C_{(x+c)}$ = total carotenoids.

to recover to normal conditions upon rehydration following dehydration has been related to the speed and intensity of drying out of tissues (Oliver and Bewley, 1984); indeed, slow drying allows gradual changes in macromolecular configurations, which favour their stability, limit biochemical changes and do not disrupt repair mechanisms. This consideration reinforces the idea that the Naples exposure caused a more intense dehydration from which moss shoots (but not lichen lobes) could not fully recover, because of the deep alterations suffered at the cytological level.

It must be underlined that the two cryptogams differ significantly in their capability of accumulating and retaining atmospheric C, N, and S. The scarce vitality of the moss probably accounts for the retention after exposure of its original C and N content, whereas the lichen clearly reflected NO_2 availability, that was higher in Naples than in Trieste. The sulphur content showed a different pattern, because the lichen suffered a strong decrease (55–58%) in both cities, whereas the increase in the moss samples from Naples which were almost dead (+29%) clearly indicates that this accumulation was likely related to a higher availability of airborne sulphate particles, both of natural (volcanic or marine) and anthropogenic (vehicular traffic), entrapped in the moss shoots.

5. Conclusions

Among the techniques to estimate the vitality of cryptogams, measurements of CO_2 gas exchange must be considered the most suitable if they are carried out under optimal conditions. Unfortunately, this technique is time-consuming, particularly because it is rather difficult to work at constant thallus water contents. From this point of view, chlorophyll

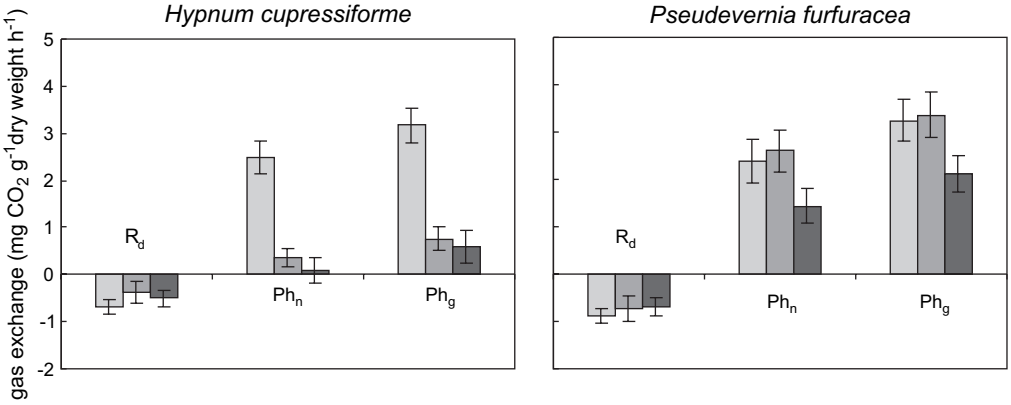

Fig. 6. CO_2 gas exchange (mg CO_2 g^{-1} (dry weight) h^{-1}; mean values ± S.D.) in *Hypnum cupressiforme* and *Pseudevernia furfuracea* before (□), and after exposure in Trieste (▨), and Naples (■). R_d, dark respiration; Ph_n, net photosynthesis; Ph_g, gross photosynthesis.

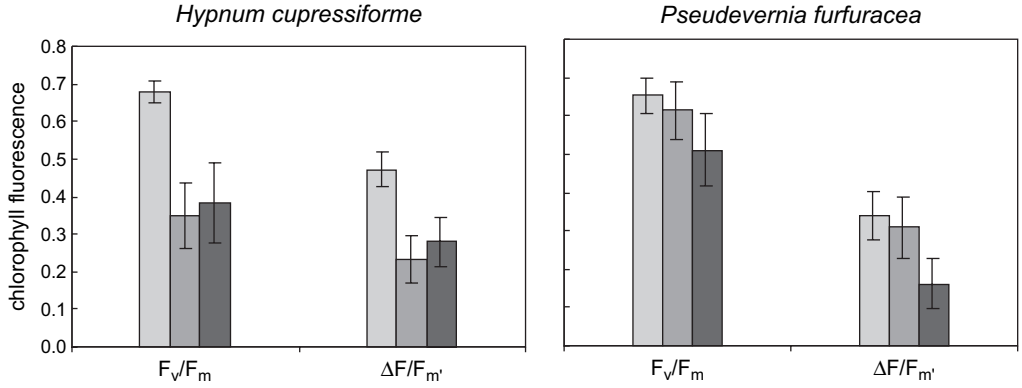

Fig. 7. Chlorophyll fluorescence (mean values ± S.D.) in *Hypnum cupressiforme* and *Pseudevernia furfuracea* before (□), and after exposure in Trieste (■) and Naples (■). F_v/F_m, optimal quantum yield of photosystem II; $\Delta F/F_m'$, effective quantum yield of photosystem II.

fluorescence is more feasible and rapid, but this technique is not without shortcomings, since, for instance, a high number of measuring points are needed, particularly when the material is heterogeneous, as that exposed in suspended bags.

Almost all the techniques used showed that the short exposures were sufficient to cause some damage to both organisms. Under the environmental conditions of both cities the lichen revealed a stronger resilience than the moss, apparently because the two organisms have a different ecophysiology. In both cities the lichen maintained good vitality levels, although some light stress traits were evidenced by TEM analysis and chlorophyll fluorescence. On the contrary, at the end of the Naples exposure, all the moss samples were almost dead, and therefore it can be excluded that they were able to actively accumulate trace metals yielded in solution by rain. In this case, any enrichment in trace metals could solely be related to passive phenomena of superficial adsorption.

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