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Seasonal variations in intracellular trace element content and physiological parameters in the lichen *Evernia prunastri* transplanted to an urban environment

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Abstract – In this study we investigated the seasonal variations in the intracellular content of 14 trace elements (Al, As, Ba, Cd, Ce, Cr, Cu, Fe, Mn, Ni, Pb, Pd, Sb, Zn) and physiological parameters (namely chlorophyll *a*, chlorophyll *b*, ergosterol, photosynthetic efficiency, cell membrane integrity) in the thalli of the lichen *Evernia prunastri* (L.) Ach. exposed to an urban environment (Siena, central Italy). Lichen thalli were collected before each exposure period from an unpolluted area and transplanted to 16 sites; every 3 months the thalli were retrieved and replaced with new ones. Exposed-to-control ratios of trace elements revealed a marked intracellular accumulation of Cd in summer and autumn, and of Sb in autumn and spring, possibly as a result of vehicular traffic pollution. However, considering the low absolute concentrations of these elements, the intracellular fraction of depositions may hardly have caused an impairment of physiological parameters. As a matter of fact, indicators of photobiont vitality (content of chlorophylls *a* and *b* and photosynthetic efficiency) did not show any fluctuation across seasons, while changes in the indicators of mycobiont vitality (cell membrane damage and ergosterol content) overall did reflect some seasonal changes and/or lichen growth.

Keywords: air pollution, antimony, bioaccumulation, biomonitoring, cadmium, epiphytes, heavy metals

Introduction

It is generally accepted that elements accumulated in lichen thalli reflect deposition of elements from the atmosphere. Lacking specialized structures, with metabolism depending on mineral uptake from the atmosphere, lichens are very efficient in trapping trace elements from the surrounding environment, tending to reach an equilibrium between the elemental content in the thallus and the respective environmental levels (Loppi and Paoli 2015). Basically lichens have three uptake mechanisms: 1) particulate trapping onto the thallus surface and/or within intercellular spaces, 2) extracellular cation exchange, and 3) intracellular accumulation through selective uptake mechanisms (Bačkor and Loppi 2009). Element uptake starts with wet or dry depositions on the thallus surface and may continue with the transfer into the thalli according to their chemical binding affinities for the cell wall and plasma membrane components (Bačkor and Loppi 2009). Thus, airborne trace elements mainly occur in particulate material deposited onto the lichen surface or between intercellular spaces; many cations can be trapped by reversible binding to negatively charged anionic sites in the cell wall or the outer layer of the plasma membrane (Bačkor and Loppi 2009). These two fractions represent the extracellular components of the trace element depositions in lichen thalli. Finally, cations may enter and accumulate inside mycobiont and photobiont cells through energy-dependent and plasma membrane controlled systems (Bačkor and Loppi 2009). This fraction represents the intracellular component originating from trace element depositions.

With the transplant technique, healthy lichen thalli are taken from a relatively clean site, transplanted to the study site(s) and their response recorded (Bargagli and Mikhailova 2002). At polluted sites lichens will adapt their metabolism according to the new environment and accumulate airborne elements to levels far above their metabolic requirements (Garty 1993).

However, exposure to a polluted environment can lead to physiological damage to sensitive species as a result of

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pollution intensity, and hence of elemental saturation (Bergamaschi et al. 2007, Bačkor and Loppi 2009, Maslać et al. 2016). According to Godinho et al. (2008), the elemental content of lichen transplants does not unequivocally represent the average or cumulative environmental availability during the exposure period, because it is also dependent on the physiological status of the samples. The same authors suggested that by the introduction of a physiological parameter into a mathematical model, good correlations between total lichen elemental contents and bulk deposition may be obtained. Nevertheless, it should be borne in mind that in terms of pollutants, physiological alterations are mainly caused by the intracellular fractions of these elements (Branquinho et al. 1999). In addition, elemental content and physiological parameters may strongly vary across seasons, due to changes in both chemical and meteorological conditions in the environment to which the lichens are exposed (Malaspina et al. 2014).

Field applications of lichens as bioaccumulators of trace elements have mainly focused on total concentrations, generally disregarding element localization at extracellular or intracellular level. This latter point is very important, since the intracellular fraction of depositions is responsible for the toxic effects at physiological level, and may thus in turn influence total concentrations. The purpose of this study was to investigate the seasonal variation of the intracellular content of selected trace elements in lichen thalli and its interaction with selected physiological parameters, taken as markers of photobiont and mycobiont vitality.

Materials and methods

Experimental design

The lichen *Evernia prunastri* (L.) Ach. is a fruticose (shrub-like) species with high surface-volume ratio and is commonly used in biomonitoring studies of trace element depositions (Ayrault et al. 2007, Loppi et al. 1998). From June 2012 to June 2013, every 3 months, thalli were collected and exposed for 3 months at ca 2 m above ground at 16 sites within the urban area of Siena (Tuscany, Italy). Ten thalli were taken before each exposure and used as control (pre-exposure).

Siena (55,000 inhabitants) is located at an elevation of 322 m a.s.l., the climate is humid sub-Mediterranean with an average annual temperature of 13.9 °C and an annual rainfall of about 850 mm. Lacking industrial activities, the urban area is characterized by limited air contamination, and the main source of atmospheric pollution is from vehicular traffic (Loppi et al. 2002), that is maximal in spring and summer due to massive tourism. Counting workers and tourists, the daily traffic flow in spring is about 26,000 cars: 17,000 enter the town in the morning and 9,000 exit (data of the Municipality of Siena). Meteorological and environmental data for each season during the experiment are summarized in Table 1.

Tab. 1. Meteorological and pollution data within the urban area of Siena (Tuscany, Italy) during the study period (June 2012 – June 2013). PM_{10} – particulated matter less than 10 μm; NO_2 – nitrogen dioxide; SD – standard deviation.

Meteorological parameters	summer	autumn	winter	spring
Rainfall (mm)	206	424	475	247
Number of rainy days	7	38	36	47
Number of stormy days	4	12	7	8
Mean relative humidity (%)	53	76	76	65
Mean temperature (°C)	23	10	7	17
Average (±SD) PM_{10} (μg m^{-3})	30±9	29±10	34±11	29±8
Average (±SD) NO_2 (μg m^{-3})	35±19	32±17	37±18	30±19

Trace elements

Lichen samples were cleaned with nylon tweezers under a binocular microscope to remove extraneous material. Samples were then washed with deionized water to remove elements simply deposited as particles and then washed with Na_2-EDTA-0.2 M to remove the fraction accumulated extracellularly (Branquinho and Brown 1994).

Air dried samples were then homogenized under liquid nitrogen with a ceramic mortar and pestle. About 300 mg of the homogenized material were mineralized with a 6:1 v/v mixture of concentrated HNO_3, H_2O_2 and HF at 280 °C and pressure of 55 bars, in a microwave digestion system (Milestone Ethos 900).

Intracellular trace element (Al, As, Ba, Cd, Ce, Cr, Cu, Fe, Mn, Ni, Pb, Pd, Sb, Zn) concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin- Elmer Sciex 6100) and expressed on a dry weight basis. Analytical quality was checked by the Standard Reference Material IAEA-336 'lichen'. Precision of analysis was estimated by the variation coefficient of four replicates and was found to be within 10% for all elements.

Photosynthetic pigments

About 20 mg of lichen material was dissolved in 1 mL of dimethylformamide and homogenized with Ultraturrax for 1 minute. The homogenate was centrifuged at 10,000 rcf for 10 minutes and 50 μL of the resulting supernatant was analysed by high performance liquid chromatography (HPLC) (Waters LC I Plus) using an Ascentis Supelco C18 column (250×4.6 mm, porosity 5 μm). Concentrations of photosynthetic pigments were determined according to Suzuki et al. (1993), using water/methanol/acetone as mobile phase with a flow rate of 1 mL/minute. Runs were monitored at 440 nm. Quantification of chlorophyll *a* and chlorophyll *b* was performed using calibration curves of standards from Sigma-Aldrich (USA). Three replicates were measured for each sample.

Photosynthetic efficiency

Chlorophyll *a* fluorescence emission was analysed to assess the quantum efficiency of open photosystem II (PSII) reaction centres, a measure of photosynthetic efficiency

(Maxwell and Johnson 2000), using the physiological indicator F_v/F_m. Prior to the measurements, lichen samples were fully hydrated and maintained in a dark chamber (20 °C, relative humidity (RH) 99%) for 24 hours. After the hydration, samples were dark-adapted for 30 minutes and then exposed to light for 1 second with a saturating light pulse (3000 μmol m^{-2} s^{-1}).

Integrity of cell membranes

The plasma membrane integrity can be easily evaluated by placing a piece of lichen thallus in deionized water and measuring the variation in electric conductivity (Marques et al. 2005). Prior to the measurement, lichen thalli were placed in a humid chamber (RH>90%) for 24 hours to stabilize electrolyte leakage (Buck and Brown 1979). About 50 mg of lichen material was rinsed 3 times for 5 seconds in deionized water to remove particles deposited onto the lichen surface (Garty 1993). Samples were then soaked for 1 hour in 50 mL of deionized water and thereafter fully hydrated thalli were placed overnight at –80 °C to generate mechanical breakage of all cell membranes, in order to have a theoretical maximum cell membrane damage. Samples were again soaked in 50 ml of deionized water and the final results were expressed as relative electric conductivity.

Ergosterol

About 20 mg of lichen material was homogenized in 1 mL of absolute ethanol with Ultraturrax for 2 minutes and then centrifuged at 10,000 rcf for 20 minutes. 200 μL of the supernatant was added to 1 mL of a 1 M NaOH solution in 90% methanol and 10% of water and then placed in a thermostatic cell at 37 °C for 2 hours under stirring. For each sample, 2 mL of n-hexane was added to extract ergosterol and the supernatant was processed in a thermostatic centrifuge at room temperature to promote the volatilization of n-hexane. The dried product was then resuspended in 1 mL of absolute ethanol for about 2 minutes in a vortex, to promote the complete resuspension of ergosterol. Samples were analysed by HPLC (Agilent 1100 Series). Ergosterol was separated with an Agilent C18 column (25×4.6 mm, porosity 5 μm) using methanol as mobile phase at the flow rate of 1 mL/minute and then read at 280 nm (Dahlman et al. 2002). The calibration curve was prepared with standard solutions of ergosterol (Sigma-Aldrich, 96%) from 1 to 20 μg/mL in absolute ethanol (HPLC grade).

Statistical analysis

Since the intracellular content of some elements also fluctuated in pre-exposure control samples, for data interpretation the data were normalized by calculating the ratio between the values after each exposure and that prior to each exposure (exposed-to-control ratio, EC ratio) as suggested by Frati et al. (2005).

A one-way ANOVA was run to check for differences in physiological parameters across seasons, using the Tukey test ($p<0.05$) for post hoc comparisons. Correlation analysis was used to find significant relationships between element concentrations and physiological parameters, using the Pearson correlation coefficient ($p<0.05$). Prior to analysis, data not matching a normal distribution (Shapiro-Wilk W test at the 95% confidence interval) were log-transformed.

Results

According to the EC ratios, expressed as % variation (Fig. 1), a significant intracellular uptake (EC ratio>175%, as suggested by Frati et al., 2005) occurred for Cd in summer ($p<0.01$) and autumn ($p<0.05$), and for Sb in autumn and spring ($p<0.05$). To a lesser extent, intracellular uptake (EC ratio about 150%) occurred also for Pb and Zn in autumn and spring.

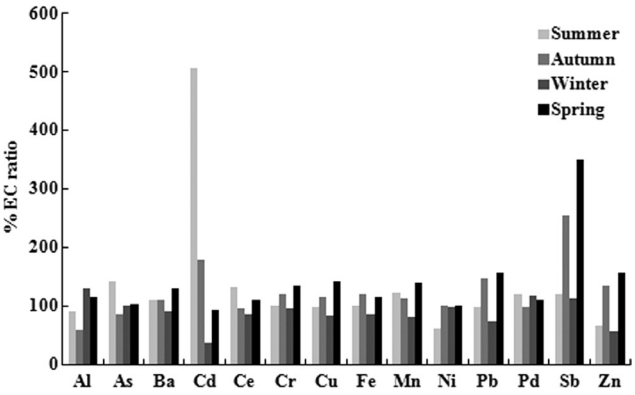

Fig. 1. Intracellular accumulation of metals, calculated as exposed-to-control (EC) ratio, in thalli of the lichen *Evernia prunastri* (L.) Ach. at the end of the four seasonal exposures in the urban environment of Siena.

The values of ecophysiological parameters at the end of each exposure season are shown in Table 2. Markers of photobiont vitality (content of chlorophyll *a* and *b*, photosynthetic efficiency) did not show any seasonal variation, while markers of mycobiont vitality showed seasonal fluctuations: cell membrane damage was higher during dry periods (spring and summer) and lower during humid periods (autumn and winter), while the content of ergosterol was higher in winter. Correlation analysis did not show any significant relationship between intracellular trace elements and physiological parameters (data not shown).

Discussion

A lichen bioaccumulation study by Monaci et al. (1997) revealed that vehicular traffic was the main source of air pollution in the urban area of Siena. A subsequent lichen bioindication study by Loppi et al. (2002) showed a general improvement in air quality over time, and this trend towards the improvement of conditions was confirmed 10 years later by Paoli et al. (2013a). This latter study showed that the diversity of epiphytic lichens increases with distance from traffic emissions, and that traffic is still the most important source of air pollution by heavy metals in the area.

Tab. 2. Mean values (±standard deviations) of physiological parameters in the thalli of the lichen *Evernia prunastri* (L.) Ach. at the end of the four seasonal exposures in the urban environment of Siena. Different letters indicate statistically significant differences ($p<0.05$). F_V/F_M – photosynthetic efficiency; El.C (%) – relative electric conductivity.

	Summer	Autumn	Winter	Spring
Chlorophyll *a* (μg mg^{-1})	1.05±0.19	1.13±0.23	1.18±0.24	1.08±0.23
Chlorophyll *b* (μg mg^{-1})	0.22±0.04	0.25±0.04	0.22±0.05	0.23±0.05
F_v/F_m	0.67±0.05	0.75±0.10	0.78±0.14	0.66±0.05
El.C. (%)	59.6±4.5 (a)	48.9±6.6 (b)	51.7±7.9 (b)	60.9±10.7 (a)
Ergosterol (μg mg^{-1})	0.43±0.10 (b)	0.42±0.10 (b)	0.72±0.15 (a)	0.54±0.15 (ab)

Intracellular element uptake has been calculated according to the EC ratio, since this ratio allows the accumulation degree of elements in transplanted lichens to be defined, corrected for their concentration in control samples (Frati et al. 2005). Our results indicated the intracellular accumulation for Cd and Sb, although in different seasons. In a previous study carried out in Siena, Loppi and Paoli (2015) showed that total element concentrations in the thalli of *E. prunastri* exposed and retrieved monthly reflected the characteristics of bulk deposition, and traffic was identified as the main source of Sb deposition. On the other hand, wear of tires, brakes, engine, and vehicle components is a well known source of Cd and Sb atmospheric pollution (Cadle et al. 1997, Beckerman et al. 2008). However, it should be considered that intracellular concentrations of Cd and Sb measured in the present study (range of seasonal average: Cd=0.03–0.08 μg g^{-1} dw, Sb=0.22–0.46 μg g^{-1} dw) were low and in line with intracellular values reported for lichens from unpolluted areas (Bačkor et al. 2010, Paoli et al. 2013b). Nevertheless, assuming Sb as a main tracer of traffic emissions, positive correlations emerged between Sb and Ba ($r=0.52$, $p<0.05$), Cr ($r=0.65$, $p<0.001$), Cu ($r=0.66$, $p<0.001$), Fe ($r=0.37$, $p<0.05$) and Zn ($r=0.70$, $p<0.001$), suggesting also that the intracellular content of these elements was partially associated with vehicular traffic. The content of trace elements in lichen thalli is greatly influenced by season (Corapi et al. 2014). Rain may change the elemental composition of the particles adsorbed onto the thallus surface through the partial removal of the adhering particles, forming leachates enriched in soluble elements (Lang et al. 1976, Brown and Brown 1991). In fact, rain after dry periods may cause displacement of cations and modifications of the elemental composition of the cell wall (Bargagli 1998, Reis et al. 1999), by removing cations from the thalli or even making them available for intracellular uptake, hence stimulating cellular metabolism (Brown and Brown 1991, Nash and Gries 1995). The uptake of metals can be substantially considered a passive process, in which elements move from more to less concentrated positions, e.g., from extracellular to intracellular spaces in the lichen thallus (Kularatne and de Freitas 2013). Similarly, the loss of metals from the intracellular sites can be considered the consequence of the balance between the concentrations at intracellular and extracellular binding sites, and at once, between the whole lichen and the surrounding environment (Wolterbeek et al. 2003).

Changes in ecophysiological parameters of transplanted lichen samples depend especially on the different levels of pollutants (Godinho et al. 2004, Ra et al. 2005, Lackovičová et al. 2013), but might also be influenced by the different environmental and microclimatic conditions at the exposure sites (Rydzak 1968). Cell membrane damage was higher during dry periods (spring and summer) and lower during humid periods (autumn and winter). Although seasons with the highest cell membrane damage correspond with the periods of heaviest traffic, and accumulation of heavy metals is known to cause cell membrane damage (Garty et al. 1993, 1998; Paoli et al. 2011), no correlation emerged with trace elements accumulated intracellularly, and it is thus possible that a limited water availability, or a combination of air pollution and unfavourable water conditions, may have negatively affected this parameter. Paoli et al. (2013b) showed that Sb accumulated at intracellular level caused deleterious effects on membrane integrity in the lichen *Xanthoria parietina*, but these Sb values were much higher than those measured in the present study. Similarly, Bačkor et al. (2010) found that intracellular Cd accumulation at values similar to those found in the present study hardly caused cell membrane damage in the lichens *Peltigera rufescens* and *Cladina arbuscula* subsp. *mitis*.

The content of ergosterol, the principal sterol of the plasma membrane of fungi, was significantly higher in winter than in the other seasons, reflecting the amount of metabolically active cells in the mycobiont, and hence, mycelial growth (Sung et al. 1995). This is consistent with much higher total lipid content in winter found in the lichen *X. parietina* (Piervittori et al. 1995). Interestingly, in winter, intracellular accumulation of heavy metals was limited, and probably ergosterol increased in response to meteorological and environmental conditions favourable for lichen metabolism.

Conclusions

Exposed-to-control ratios of trace elements revealed intracellular accumulation of Cd and Sb in varying seasons, possibly as a result of vehicle traffic pollution. However, considering the low absolute concentrations of these elements, the intracellular fraction of deposition can hardly have caused any impairment of physiological parameters. As a matter of fact, indicators of photobiont vitality (content of chlorophylls *a* and *b*, and photosynthetic efficiency)

did not show any fluctuation across seasons, while changes in the indicators of mycobiont vitality (cell membrane damage and ergosterol content) overall did reflect some seasonal changes and/or lichen growth.

Further investigation is necessary to shed light on the role of intracellular trace element content in lichens, in order to allow comparison of these data with other biomonitoring studies and get an insight into the ecophysiological effects of air pollution.

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