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# The impact of cadmium on photosynthetic performance and secondary metabolites in the lichens *Parmelia sulcata, Flavoparmelia caperata* and *Evernia prunastri*

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**Abstract –** Lichens are one of the most common air quality bioindicators. Airborne heavy metal pollution causes various physiological changes in lichens, but sensitivity to metal pollution is species specific. In this research, three lichen species (*Parmelia sulcata*, *Flavoparmelia caperata* and *Evernia prunastri*) were exposed to cadmium (50 mg $L^{-1}$) in laboratory conditions. Photosynthetic efficiency of photosystem II and content of secondary metabolites were determined after one, three and eight days of exposure. In all investigated species treatment of lichen thalli with cadmium significantly changed $F_v/F_m$ and $R_{Fd}$ only after eight days of exposure. Quantification of metabolites showed a decreased content of the medullary depsidones salazinic acid (in *P. sulcata*) and protocetraric acid (in *F. caperata*) but increased content of cortical depside atranorin (in *P. sulcata*) and dibenzofurane usnic acid (in *F. caperata*) after cadmium exposure. However, no changes in secondary metabolites were found in *E. prunastri*. Results show that investigated species are relatively resistant to short-term cadmium-exposure and that secondary metabolites could have an important role in the protection of primary metabolism from negative cadmium impacts, at least in some species.

**Key words**: air pollution, heavy metal, HPLC, photosynthesis

## Introduction

Lichens are perennial, slow-growing organisms that have the ability to take in all necessary nutrients and water directly from the air. Due to the absence of cuticle and roots, they absorb pollutants over their entire surface. Moreover, lichens have efficient mechanisms that take in more nutrients than they need. It has been found that lichens are able to accumulate various metals (cadmium, lead, zinc, etc.) at high levels (Garty 2001, Aprile et al. 2010). Ever since the industrial revolution in the 1800s lichens have been recognized as bioindicators of air quality and, nowadays, they are commonly used to indicate the presence of air pollutants. More recently, lichens have also been used as biomonitors, in experiments measuring the lichen physiological responses to atmospheric pollution over time and providing additional information about the amount and intensity of the exposure (Garty et al. 2003, Bačkor and Loppi 2009, Paoli et al. 2015a). So far, they have been used as biomonitors of atmospheric pollution from different sources (Loppi et al. 2004, Branquino et al. 2008, Lackovičová et al. 2013, Paoli et al. 2015b).

Significant correlations between metal concentrations in the lichen thalli and in their environment have been found in many studies (Garty 2001, Bačkor et al. 2003, Carreras et al. 2005, Dzubaj et al. 2008, Stamenković et al. 2013). However, physiological responses of lichens to metals and their tolerance mechanisms are species-specific, ranging from relative resistance to high sensitivity. Exposure of lichens to metals can affect membrane integrity (Garty et al. 2003, Pisani et al. 2010), chlorophyll content (Bačkor and Zetíková 2003, Carreras and Pignata 2007), photosynthetic performance (Karakoti et al. 2014, Paoli et al. 2015a) and secondary metabolites (Hauck et al. 2013, Gauslaa et al. 2016). Among heavy metals, cadmium (Cd) is considered to be particularly toxic for various lichen species causing adverse physiological changes (Sanità di Toppi et al. 2005). Increased Cd content in the environment can be attributed to human activities such as battery production, industrial metallurgical processes, combustion of fossil fuels and emissions from motor vehicles (Pacyna 1998).

Lichens produce various secondary metabolites that have multiple functions in the interactions of the lichens with the

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environment. Some of them are located in the upper cortex, while most are located in the medulla (Solhaug et al. 2009). These compounds have antiherbivore, antimicrobial and larvicidal effects, and can protect thalli from high UV irradiation and oxidative stress. They might also have important role in metal homeostasis and lichen tolerance to pollution, although the biochemical mechanisms are mostly unknown (Molnár and Farkas 2010). Nevertheless, it is known that lichen substances function *in vitro* as chelators of cations, including heavy metals (Bačkor and Loppi 2009). Few studies have compared metal pollution and secondary compound concentration, and showed different responses. Levels of medullary compounds in *Hypocenomyce scalaris* (lecanoric acid) and *Cladonia furcata* (fumarprotocetraric acid) were increased by metal pollution (Pawlik-Skowrońska and Bačkor 2011). Białonska and Dayan (2005) found that levels of atranorin, a cortical compound, and medullary compounds physodic and hydroxyphysodic acid decreased, while medullary physodalic acid increased after transplantation of *Hypogymnia physodes* to the polluted area. On the other hand, Paoli et al. (2015a) showed that the medullary compound caperatic acid decreased and the cortical compound usnic acid increased in *Flavoparmelia caperata* located close to the landfill.

The aim of this study was to investigate the effects of Cd-exposure on photosynthetic performance and content of secondary metabolites in three widely distributed epiphytic lichen species *Parmelia sulcata*, *Flavoparmelia caperata* and *Evernia prunastri* in a short-term laboratory experiment.

## Materials and methods

### Lichen material

The foliose lichens *Parmelia sulcata* Taylor (Fig. 1A) and *Flavoparmelia caperata* (L.) Hale (Fig. 1B), and the fruticose lichen *Evernia prunastri* (L.) Ach. (Fig. 1C) were collected from old branches of *Quercus* sp. in Maksimir Park (45°49'45"N, 16°01'17"E, 160 m above sea level) in Zagreb in October, 2014. Lichens were placed on several layers of filter paper wetted with distilled water, and then acclimated in a growth chamber for a week. For each species individual thalli were sprayed with 5 mL of Cd solution in a concentration of 50 mg $L^{-1}$, which should cause toxic effects in a short time. Lichens sprayed only with distilled water were used as control. All lichens were kept in the growth chamber under fluorescent light (60 µmol photons $m^{-2}$ $s^{-1}$, photoperiod of 16 h day/8 h night) at 22 ± 2 °C. Chlorophyll fluorescence and secondary metabolite content were determined after one, three and eight days of Cd-exposure. All treatments were done in triplicate.

### Chlorophyll fluorescence parameters

Light-induced chlorophyll fluorescence parameters at continuous saturating white light were measured using a chlorophyll fluorometer (Qubit Systems Inc., Canada) in 30 min pre-darkened thalli. Low intensity red light was used to determine the minimal fluorescence level ($F_0$), and then continuous saturating light ca. 1500 µmol $m^{-2}$ $s^{-1}$ was applied. Upon irradiation, the fluorescence increased from $F_0$ to the maximum $F_m$ and then declined to steady state fluorescence ($F_s$) during seven minutes. Maximum quantum yield of PSII ($F_v/F_m$), a widely used indicator of photosynthetic efficiency of photosystem II, was calculated as $(F_m - F_0)/F_m$ (Maxwell and Johnson 2000). Additionally, the fluorescence decrease ratio ($R_{Fd}$), a parameter directly related to the rate of photosynthesis (Lichtenthaler et al. 2005), was calculated as $(F_m - F_s)/F_s$, according to Lichtenthaler et al. (2005).

### High-performance liquid chromatography

Lichen secondary metabolites were extracted from lyophilised thalli (18 mg) which were suspended in 1.5 mL acetone and incubated for 1 h at 4 °C. The samples were centrifuged for 15 min at 4 °C and 15 000 g. The supernatant was separated with a pipette into a separate tube and centrifuged again for 30 min at 4 °C and 30 000 g. The supernatants were then transferred with a pipette into dark vial glass bottles.

Secondary metabolites were analysed by high-performance liquid chromatography (HPLC) using a Perkin Elmer Series 200 system with a UV/VIS diode-array detector. Analytes were separated on a reverse-phase C18 Brownlee Speri-5 ODS column (5 µm, 250 × 4.6 mm, Perkin Elmer, USA) with pre-column (5 × 4.6 mm). The elution program was modified according to Feige et al. (1993). The mobile phase consisted of 1% (v/v) phosphoric acid (A) and 100% methanol (B). For *P. sulcata* the elution program was: 0.6 min equilibration with 30% B, 11 min linear gradient from 30% to 70% B, 4 min linear gradient from 70% to 100% B,


**Fig. 1.** Lichens used in the experiment: (A) *Parmelia sulcata*, (B) *Flavoparmelia caperata* and (C) *Evernia prunastri*.

10 min isocratic with 100% B and 6 min re-equilibration with 30% B. For *E. prunastri* and *F. caperata* the linear gradient from 70% to 100% B was 8 min instead of 4 min. The flow rate was 0.8 mL $min^{-1}$, and elution was monitored at 245 nm. The identification of lichen metabolites was made by comparing retention times in combination with UV spectral data with known chromatographic data (Feige et al. 1993, Huneck and Yoshimura 1996). Quantification was performed using calibration curves of individual compounds isolated from authentic-source lichens and the results were expressed as mg per gram of dry weight (mg $g^{-1}_{DW}$).

### Statistical analysis

Results were shown as average ± standard error. Determination of photochemical efficiency and quantification of secondary compounds content was performed in triplicate. For processing data Microsoft Excel 2010 and Statistica 10 (StatSoft Inc., SAD) were used. The results were compared by analysis of variance (ANOVA) and post hoc Tukey's test. Differences between means were considered statistically significant at $p \leq 0.05$.

## Results

### Fluorescence parameters

In control lichen species the values of $F_v/F_m$ parameter were around 0.625 during the experiment, except in *F. caperata* which had a slightly lower value (0.56) at the end of the experiment. However, the difference was not significant ($p > 0.05$) compared to the values measured on other days (Fig. 2). Lichens treated with Cd did not show significant differences ($p > 0.05$) from corresponding controls after one and three days of exposure, while after eight days of exposure the $F_v/F_m$ values in all species were significantly lower than the values in untreated lichens measured on the same day ($p < 0.01$) and those measured in Cd-treated lichens on the other days ($p \leq 0.01$).

In untreated lichens, the highest $R_{Fd}$ value (~ 2.4) was measured in *F. caperata*, but it decreased significantly ($p \leq 0.05$) to 1.5 after eight days of experiment (Fig. 3). In the other two species, the $R_{Fd}$ values were lower (1.4 for *E. prunastri* and 1.7 for *P. sulcata*) and no significant change ($p < 0.05$) in values was observed during the course of the

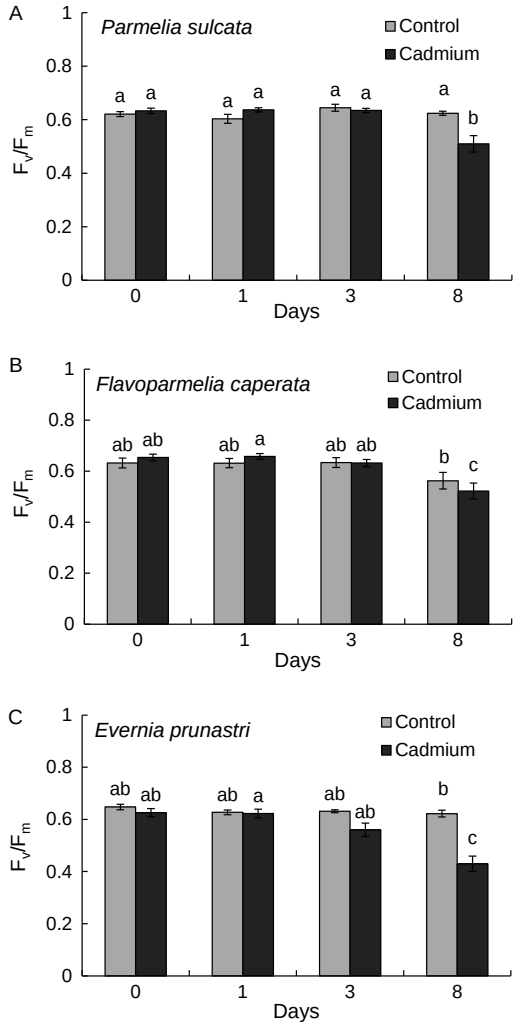


**Fig. 2.** Maximum efficiency of PSII ($F_v/F_m$) in lichens *Parmelia sulcata* (A), *Flavoparmelia caperata* (B) and *Evernia prunastri* (C) before (0 day), and after one, three and eight days of the exposure to cadmium in a concentration of 50 mg $L^{-1}$. Different letters above the bars denote significantly different results ($p \leq 0.05$).

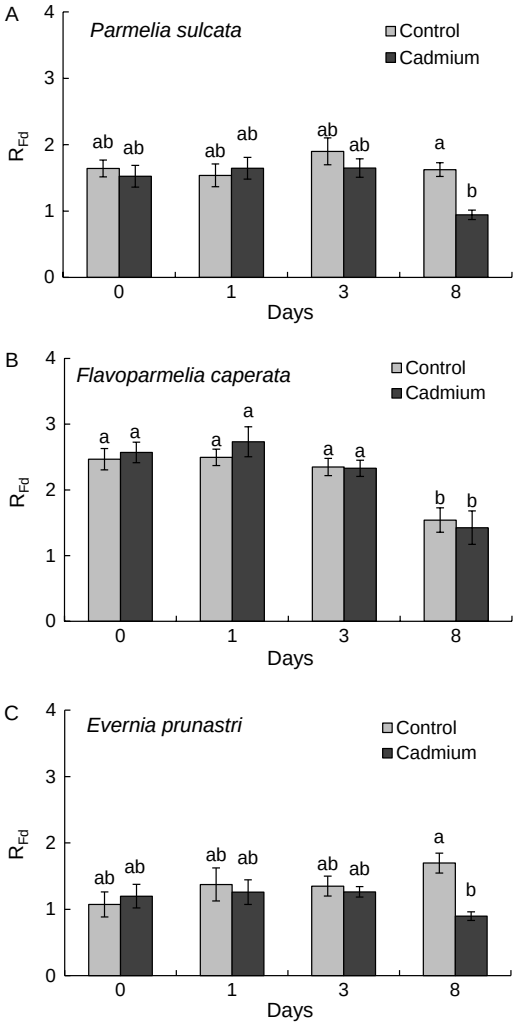


**Fig. 3.** Fluorescence decrease ratio ($R_{Fd}$) in lichens *Parmelia sulcata* (A), *Flavoparmelia caperata* (B) and *Evernia prunastri* (C) before (0 day), and after one, three and eight days of the exposure to cadmium in a concentration of 50 mg $L^{-1}$. Different letters above the bars denote significantly different results ($p \leq 0.05$).

experiment. In lichens treated with Cd, the $R_{Fd}$ values were not significantly different ($p > 0.05$) from those of unexposed controls after one and three days of exposure. However, the values significantly decreased in *E. prunastri* ($p = 0.03$) and *P. sulcata* ($p = 0.05$) eight days after Cd-exposure compared to the corresponding control values measured on the same day. In *F. caperata*, the $R_{Fd}$ of Cd-treated thalli was significantly lower ($p < 0.01$) than $R_{Fd}$ values obtained for treated lichens earlier in the experiment, but it was not significantly different ($p > 0.05$) from the control value measured on the same day.

**Secondary metabolites**

In *P. sulcata* acetone extracts we successfully separated two secondary metabolites and identified them according to their retention times and UV spectral data (Fig. 4). The major metabolite was depsidone salazinic acid (peak at 24 min) while the minor metabolite was depside atranorin (peak at 36 min). In untreated samples, the content of salazinic acid slightly decreased during the exposure experiment, from 2.04 to 1.76 mg $g^{-1}_{DW}$, while the content of atranorin did not significantly change, with values around 0.3 mg $g^{-1}_{DW}$ (Tab. 1). In Cd-treated *P. sulcata*, the content of salazinic acid was lower than in the corresponding control, but significantly ($p < 0.01$) only after three days of exposure when it decreased to 1.10 mg $g^{-1}_{DW}$. In contrast, the content of atranorin was significantly higher ($p < 0.01$) than in the corresponding controls after one and eight days of Cd-exposure, amounting 0.58 and 0.62 mg $g^{-1}_{DW}$, respectively.

In *F. caperata* samples, HPLC analysis revealed depsidone protocetraric acid (peak at 20 min), as a major metabolite, and dibenzofurane usnic acid (peak at 35–36 min), as a minor metabolite (Fig. 5). In control lichens, the contents of metabolites slightly increased during the experiment, from 0.77 to 0.99 mg $g^{-1}_{DW}$ for protocetraric acid and from 0.22 to 0.52 mg $g^{-1}_{DW}$ for usnic acid (Tab. 1). In Cd-treated lichens, the content of protocetraric acid was slightly higher

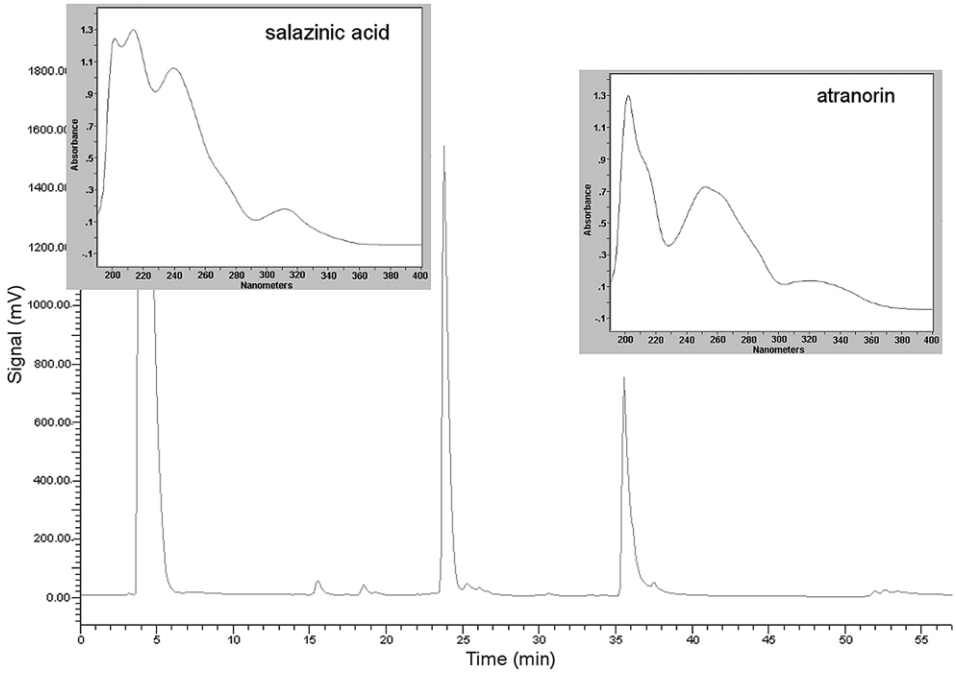


**Fig. 4.** Chromatogram of the acetone extract of *Parmelia sulcata* at 245 nm. Identified peaks: acetone (5 min), salazinic acid (24 min) and atranorin (36 min). UV spectral data of metabolites quantified in the experiment are also shown.

**Tab. 1.** Content of secondary metabolites quantified in untreated (control) and cadmium-treated lichens *Parmelia sulcata*, *Flavoparmelia caperata* and *Evernia prunastri* after one, three and eight days of exposure. Different letters in superscript denote significantly different results ($p \leq 0.05$). DW – dry weight.

| Lichen metabolites (mg $g^{-1}_{DW}$) | | *Parmelia sulcata* | | *Flavoparmelia caperata* | | *Evernia prunastri* | |
|---|---|---|---|---|---|---|---|
| Treatment | Days | salazinic acid | atranorin | protocetraric acid | usnic acid | evernic acid | atranorin |
| Control | 1 | 2.04±0.01$^{a}$ | 0.27±0.06$^{b}$ | 0.77±0.09$^{b}$ | 0.22±0.00$^{b}$ | 1.08±0.09$^{a}$ | 2.11±0.07$^{a}$ |
| | 3 | 1.95±0.23$^{a}$ | 0.35±0.07$^{b}$ | 0.97±0.09$^{ab}$ | 0.53±0.02$^{ab}$ | 1.16±0.10$^{a}$ | 2.03±0.31$^{a}$ |
| | 8 | 1.76±0.07$^{a}$ | 0.26±0.03$^{b}$ | 0.99±0.02$^{a}$ | 0.52±0.05$^{ab}$ | 1.07±0.11$^{a}$ | 2.02±0.24$^{a}$ |
| Cadmium | 1 | 1.73±0.03$^{a}$ | 0.58±0.02$^{a}$ | 0.92±0.01$^{ab}$ | 0.29±0.04$^{b}$ | 1.05±0.06$^{a}$ | 2.01±0.11$^{a}$ |
| | 3 | 1.10±0.18$^{b}$ | 0.35±0.08$^{b}$ | 0.80±0.02$^{ab}$ | 0.42±0.00$^{ab}$ | 1.15±0.11$^{a}$ | 2.14±0.04$^{a}$ |
| | 8 | 1.57±0.22$^{ab}$ | 0.62±0.06$^{a}$ | 0.76±0.10$^{b}$ | 0.75±0.19$^{a}$ | 1.10±0.12$^{a}$ | 1.89±0.09$^{a}$ |

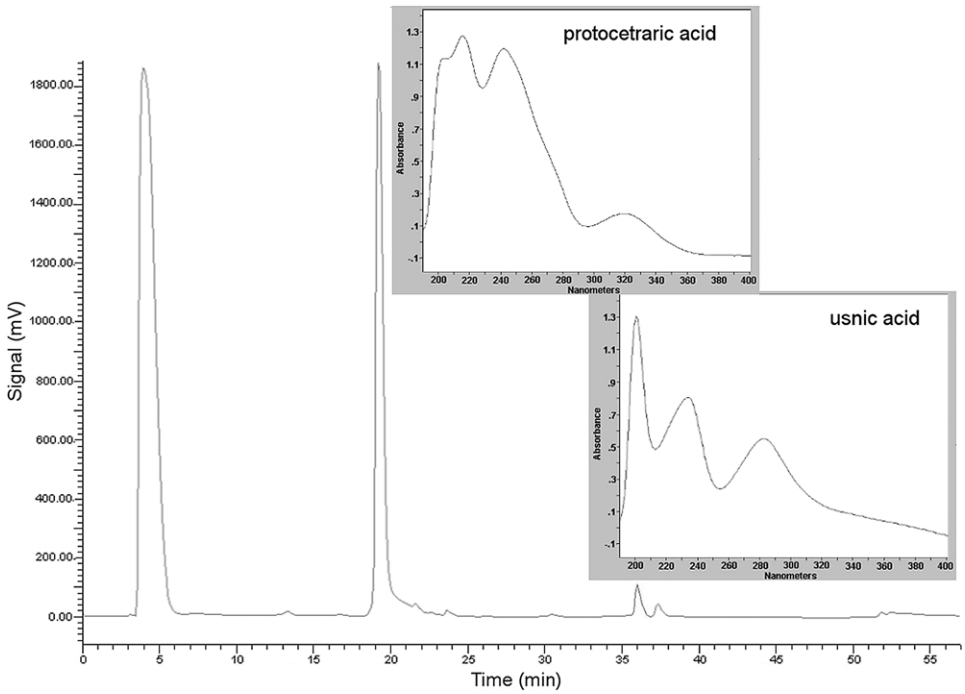


**Fig. 5.** Chromatogram of the acetone extract of *Flavoparmelia caperata* at 245 nm. Identified peaks: acetone (5 min), protocetraric acid (19.5 min) and usnic acid (36 min). UV spectral data of metabolites quantified in the experiment are also shown.

(0.92 mg $g^{-1}_{DW}$) than in the corresponding control after one day of Cd-exposure. However, it decreased to 0.80 mg $g^{-1}_{DW}$ after three days of Cd-exposure and then to 0.76 mg $g^{-1}_{DW}$ after eight days of exposure, which was significantly lower ($p < 0.05$) than in corresponding control. Content of usnic acid in Cd-treated *F. caperata* was mostly similar to control values after one and three days of exposure, but the value increased significantly ($p < 0.01$) up to 0.75 mg $g^{-1}_{DW}$ after eight days of exposure.

In samples of *E. prunastri* we found depsides evernic acid (peak at 31 min) and atranorin (peak at 36–37 min), as major metabolites, and chloroatranorin (peak at 37–38 min) as a minor metabolite (Fig. 6). We further quantified only the two major metabolites. In control samples, the contents of evernic acid and atranorin did not change during experimental period, with the values amounting 1.10 for evernic acid and 2.05 mg $g^{-1}_{DW}$ for atranorin (Tab. 1). Treatment with Cd did not cause any significant change in the content of the investigated metabolites.

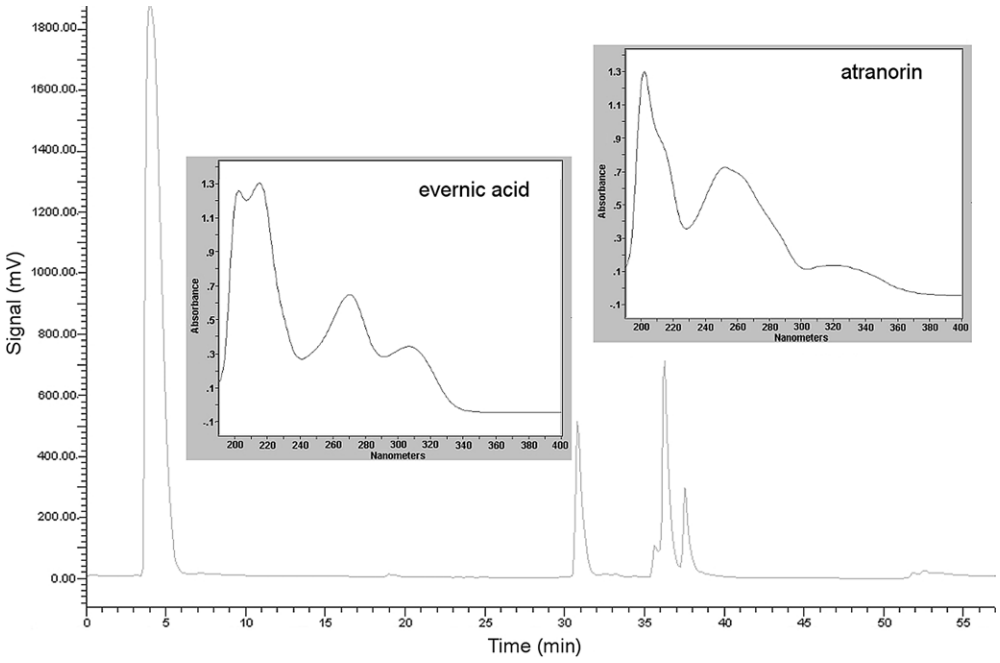


**Fig. 6.** Chromatogram of the acetone extract of *Evernia prunastri* at 245 nm. Identified peaks: acetone (5 min), evernic acid (31 min), usnic acid (35.5 min), atranorin (36.5 min) and chloroatranorin (37.5 min). UV spectral data of metabolites quantified in the experiment are also shown.

## Discussion

Lichens, due to their morphology and physiology, receive all nutrients from the atmosphere, including heavy metals. In this study, the effects of short-term Cd exposure on photosynthetic performance and secondary metabolites content were studied using the epiphytic lichen species *P. sulcata*, *E. prunastri* and *F. caperata*. These lichen species had already been used as bioindicators and/or biomonitors of atmospheric pollution from different sources (Loppi et al. 2004, Lackovičová et al. 2013, Stamenković et al. 2013).

In the last 20 years, chlorophyll fluorescence measurements were successfully employed in various lichen studies investigating heavy metal pollution (Bačkor et al 2010, Karakoti et al. 2014, Paoli et al. 2015a). The maximum quantum yield of PSII ($F_v/F_m$), besides being an important indicator of photosynthetic efficiency, can indicate the vitality of a lichen photobiont (Paoli et al. 2015a). In all three lichen species investigated in our study, the $F_v/F_m$ values of untreated thalli were mostly in accordance with values characteristic for lichens (0.63–0.76), which are lower than those found in plants (0.74–0.83) (Jensen 2002). In Cd-treated thalli significantly lower $F_v/F_m$ values, indicating damage of the photosynthetic apparatus, were observed. However, a decrease was observed only eight days after the exposure suggesting the relative resistance of the lichen photobiont to short-term exposure to high Cd concentration. Bačkor et al. (2010) also reported relatively low toxicity of Cd, compared to other metals, e.g. Cu in lichens *Peltigera rufescens* and *Cladina arbuscula* 24 h after the exposure. Karakoti et al. (2014) showed that the thallus of lichen *Pyxine cocoes*, which contained different amounts of various metals did not show a decrease in $F_v/F_m$. In contrast, a decrease of $F_v/F_m$ value was observed in the epiphytic fruticose lichen *Ramalina lacera* containing higher amounts of Ba, Ni, S, V and Zn after exposure to anthropogenic pollution (Garty et al. 2000). It seems that sensitivity of photosynthetic efficiency to different metals varies between different lichen species. A delay in the Cd-effect on photosynthetic performance observed in our study might suggest that metal toxicity effect could depend on time of exposure. It has been found that prolonged exposure to lead (Pb) leads to an additional decrease of $F_v/F_m$ in *Flavoparmelia caperata* (Garty 2002). In our study, we also employed a fluorescence decrease ratio ($R_{Fd}$), a parameter which, when measured at saturation irradiance is directly correlated to the net $CO_2$ assimilation rate (Lichtenthaler et al. 2005). The $R_{Fd}$ values in control lichens were mostly similar to $R_{Fd}$ values found in lichen *Anaptychia ciliaris* (Valladares et al. 1995). In Cd-treated lichen species, $R_{Fd}$ showed the same trend as $F_v/F_m$ parameter, confirming tolerance of a photosynthetic process to Cd in the investigated species. Interestingly, prolonged time in laboratory conditions caused a decrease of both, $F_v/F_m$ and especially $R_{Fd}$ in untreated thalli of *F. caperata*, suggesting that this species is sensitive to the environmental conditions that are not natural to it.

Most secondary compounds combinations in lichens are species-specific and therefore are widely used in lichen taxonomy and systematics (Molnár and Farkas 2010). In lichen species analysed in this study almost all lichen substances specific for particular species (Nash et al. 2002) were successfully detected: salazinic acid and atranorin in *P. sulcata*; evernic acid, atranorin and chloroatranorin in *E. prunastri*; and protocetraric acid and usnic acid in *F. caperata*. We did not find only two compounds, consalazinic acid, a minor metabolite in *P. sulcata* and caperatic acid, a minor metabolite in *F. caperata*. Caperatic acid is an aliphatic acid which is not detectable by the HPLC method used in this study. Lichen metabolites play an important role in tolerance of lichens to metal pollution (Bačkor and Loppi 2009). Some reports say that compounds located in the medulla (e.g. depsidones) might be chelators of cations (Solhaug et al. 2009). In this study, we found that exposure to Cd in foliose lichens decreased content of the medullary depsidones salazinic acid (in *P. sulcata*) and protocetraric acid (in *F. caperata*), but increased the content of cortical depside atranorin (in *P. sulcata*) and dibenzofurane usnic acid (in *F. caperata*), whereas metabolites (all depsides) of fruticose lichen *E. prunastri* did not change after Cd treatment. These results are in accordance with Lackovičová et al. (2013) and Paoli (2015a) suggestion that the ratio between cortical and medullary secondary metabolites can increase in lichen samples in polluted environment. However, there are some opposite findings that report increased medullary compounds and decreased cortical compounds content after heavy metal exposure. For example, Białonska and Dayan (2005) found increased levels of medullary depsidone physodalic acid but decreased levels of cortical depside atranorin in *Hypogymnia physodes* transplanted to industrial areas with high emissions of heavy metals. Also, Pawlik-Skowrońska and Bačkor (2011) obtained higher amounts of the medullary compounds, i.e. depside lecanoric acid in *Hypocenomyce scalaris* and depsidone fumarprotocetraric acid in *Cladonia furcata* at a mining site polluted with Pb and Zn. Results of several studies suggested that lichen metabolites control metal homeostasis by promoting the uptake of certain metal cations and/or reducing the adsorption of others that could possibly be toxic (Molnár and Farkas 2010). For example, physodalic acid from *Hypogymnia physdodes*, increase the $Fe^{2+}$ uptake and decrease the uptake of $Cu^{2+}$, $Mn^{2+}$ and $Na^{+}$ (Hauck and Huneck 2007, Hauck 2008). Dibenzofurane usnic acid and divaricatic acid were both found to increase the intracellular uptake of $Cu^{2+}$ in *Evernia mesomorpha* and *Ramalina menziesii* (Hauck et al. 2009), but reduce the $Mn^{2+}$ uptake. Recently, UV spectroscopic studies and X-ray diffraction analyses showed that the complexation of metal ions with lichen substances is widespread (Bačkor and Fahselt 2004, Hauck et al. 2009). The most recent study by Gauslaa et al. (2016) reported that medullary metabolites in fruticose (*Ramalina farinacea*, *Usnea dasypoga*) were reduced in polluted sites, but were not in foliose lichens (*Parmelia sulcata*, *Lobaria pulmonaria*) whereas cortical metabolites did not change in any species. Moreover, *L. pulmonaria* experienced strong reduction in viability in polluted sites despite increased content of medullary compound stictic acid which has a possibility of heavy metal chelating. All these results taken together might suggest that the function of lichen secondary

metabolites in metal homeostasis depend on several factors including chemical structure of compounds (depsides/depsidones/dibenzofuranes), their location (cortex/medulla) as well as lichen form of growth (foliose/fruticose) and type of metal. Moreover, Valencia-Islas et al. (2007) suggested that increased content of cortical usnic acid in *Ramalina asahinae* could contribute to the antioxidant protection against air pollution. It seems that although our knowledge about the importance of lichen metabolites has increased in the last few years, their biological roles and interactions have not yet been entirely understood.

In conclusion, our results show that the investigated species are relatively resistant to short-term Cd exposure and that secondary metabolites might have an important role in protection against negative Cd impacts, at least in some species.

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