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# Nitric oxide affects cadmium-induced changes in the lichen *Ramalina farinacea*

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ABSTRACT

Metabolic responses of epiphytic lichen *Ramalina farinacea* to cadmium (Cd) and/or nitric oxide (NO) scavenger (cPTIO) were studied. Accumulation of Cd and other metallic nutrients was not affected by cPTIO while total and absorbed amounts differed. Cd-induced NO formation was suppressed by cPTIO but ROS signal was synergistically enhanced, confirming that NO is essential to keep ROS under control. This excessive ROS generation could be a reason for depleted amount of all fatty acids, including SFAs, MUFAs and PUFAs. Total content of fatty acids reached 3.89 mg/g DW in control with linoleic (40%), palmitic (24%), oleic (12.8%) and stearic (8%) acids as major compounds: interestingly, shift in relative ratio of saturated (from 40 to 35% of total FAs) versus polyunsaturated fatty acids (from 42 to 48% of total FAs) was observed. Glutathione was suppressed by all treatments but Krebs acids were almost unaffected by cPTIO, indicating no regulatory role of NO in their accumulation. On the contrary, Cd-induced elevation in NO signal was related to increase in ascorbate and proline content while cPTIO suppressed it, indicating a tight relation between NO and these metabolites. Data are compared also with algae and vascular plants to show similarities between various life lineages.

## 1. Introduction

Heavy metals are unavoidable components circulating in the environment owing to natural or anthropogenic factors. Among them, cadmium (Cd) is highly dangerous metal with no known biological function in organisms [1]. Plants relatively readily accumulate Cd as observed in vascular plants and algae [1,2]. Cd accumulation by lichens is also higher compared to e.g. Ni [3,4], making them suitable for physiological studies of Cd toxicity. With respect to specific life of lichens (symbiosis of algae with fungi and often epilithic or epiphytic occurrence), their adaptive mechanisms to heavy metals should differ from vascular plants where root-to-shoot metal translocation may modify toxicity and absolute accumulation.

One of the main sings of excessive accumulation of heavy metals is imbalance in the generation and removal of reactive oxygen species (ROS), leading to so-called oxidative stress. Plants typically respond to Cd by enhanced ROS formation [5,6]. In lichens, monitoring of oxidative stress-related parameters by standard spectrophotometry did not show extensive impact of Cd or Ni [3] but our recent studies provided evidence that fluorescence microscopy is much more sensitive to detect qualitative changes in the ROS formation under Ni, Cr or Cu excess [7,8].

Nitric oxide (NO) is a small gaseous molecule with far-reaching effects in organisms. It has typically protective action under negative impacts including excess of Cd as proven by NO scavengers (cPTIO or PTIO, carboxy/2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and donor (most commonly SNP, sodium nitroprusside): SNP often protects plants while cPTIO had the opposite effect as observed in various species [1,6,9,10]. Owing to reactivity of ROS and NO, their formation may easily be modified by exogenous modulators [6] but the use of some NO modulators may evoke unexpected responses such as altered Cd and thiol accumulation [1]. In lichens, however, the role of

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NO in stress defense was only superficially studied [11].

Plants possess various mechanisms to cope with the oxidative stress, including synthesis of non-enzymatic antioxidants such as ascorbic acid (AsA), thiols and potential metal chelators including organic acids. Not only metal but also given species mainly affect responses of these metabolites to metal excess in plants and lichens [5,7,8,12,13]. Cd excess is known to elevate AsA and to deplete thiols in vascular plants [6] and in some lichens [14] but the involvement of NO in this process and responses of other metabolites in lichens to metal excess are unknown.

We therefore studied the involvement of NO in Cd toxicity using epiphytic lichen *Ramalina farinacea* as some *Ramalina* species may considerably accumulate Cd [4]. NO scavenger cPTIO was used alone and in combination with Cd, allowing comparing single and combined impacts (we also note that PTIO, but not cPTIO may artificially affect accumulation of Cd [1]). We precisely monitored ROS and NO formation with several fluorescence reagents to obtain realistic behavior of their occurrence. In addition to basic antioxidants (AsA, glutathione, and proline), accumulation of Krebs cycle acids and detailed profile of fatty acids in response to Cd and/or NO modulator were performed here for the first time in any lichen species. Data are carefully compared with (only few) available studies involving lichens while comparison with algae and vascular plants allows seeing eventual similarities between distinct life forms in terms of Cd/NO action.

## 2. Materials and methods

### 2.1. Lichen material and exposure conditions

*Ramalina farinacea* (L.) Ach. was collected randomly during September 2017 from the bark of *Quercus* sp. ca. 2 m above ground in the locality Košice-Bankov as reported previously [15]. Macroscopic foreign material was manually removed and samples were prepared and analyzed within 1 week after harvest.

Approximately 0.1 g DW (air-dried) samples were used for analyses. They were exposed to treatments in screw-cap inert plastic tubes (50 mL; Sarstedt, Nümbrecht, Germany) for 24 h in solutions prepared with HEPES buffer (pH 6.5). Cadmium ($Cd^{2+}$) was added in the form of chloride and NO scavenger cPTIO in the final concentrations of 10 or 100 μM (see Supplementary Fig. S1 for responses of NO signal to various Cd and cPTIO concentrations). Control was maintained in HEPES buffer only. Samples were kept in the cultivation room (12 h/12 h, 24/19 °C day/night) at PAR ~30 μmol $m^{-2}$ $s^{-1}$. Absolute dry mass of lichens was determined by weighing the sub-samples dried in an oven at 90 °C. After the incubation period, samples for biochemical analyses were washed thrice with deionised water, carefully dried with filter paper and extracted as mentioned below. Processing of samples for the estimation of metabolites requiring fresh material involved homogenization with cold mortar and pestle with the addition of small amount of inert so-called sea sand (Penta s. r. o., Prague, Czech Republic) to achieve complete thalli disruption.

### 2.2. Fluorescence microscopy

For microscopic analyses, ca. 0.5 cm fragments of thalli were immediately stained and observed. Nitric oxide was comparatively monitored by two staining reagents 2,3-diaminonaphthalene (DAN, $365_{Ex}$/$415_{Em}$ nm) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, $500_{Ex}$/$515_{Em}$, both from Sigma-Aldrich). Stock solution of DAN in 0.62 M HCl was diluted by PBS buffer (50 mM, pH 6.8) to the final concentration of 250 μM and samples were incubated for 60 min at room temperature (RT) and darkness; for DAF-FM DA staining, samples were incubated in a concentration of 50 μM prepared in PBS buffer (50 mM, pH 7.2) for 60 min at RT and darkness [1].

General accumulation of ROS was monitored using CellROX® Deep Red Reagent ($644_{Ex}$/$665_{Em}$ nm, Life Technologies, USA) and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, $502_{Ex}$/$526_{Em}$ nm, Life Technologies, USA): stock solution of CellROX® Deep Red Reagent in DMSO was diluted by PBS buffer (50 mM, pH 6.8) to final concentration of 5 μM and samples were stained for 60 min at 37 °C, while staining solution of DCF-DA was prepared with PBS buffer (50 mM, pH 7.2) in the concentration of 50 μM and samples were incubated in darkness for 30 min at RT as reported earlier [1,5]. DPPP (diphenyl-1-pyrenylphosphine, Life Technologies, USA) is a probe that reacts with hydroperoxides to yield fluorescent product diphenyl-1-pyrenylphosphine oxide ($351_{Ex}$/$380_{Em}$): DPPP was solubilized in DMSO and stored at −20 °C in darkness. Samples were incubated in 50 μM DPPP diluted with PBS buffer (pH 7.2) for 30 min at RT and darkness [5].

Cd was stained using specific reagent Leadmium™ Green AM (Life Technologies, USA) by diluting it in HEPES buffer (50 mM, pH 6.0) to a final dose of 20 μg/mL: samples were incubated for 45 min at 37 °C in darkness [5].

After incubation, samples were always washed three times with respective buffer and observed using fluorescence microscope Axioscop 40 (Carl Zeiss, Germany) equipped with appropriate set of excitation/emission filters.

### 2.3. Assay of thiols, ascorbic acid, Krebs cycle acids and proline

Reduced (GSH) and oxidized (GSSG) glutathione were quantified in homogenates prepared with 0.1 M HCl following the method described by Perez-Rama et al. [16]. The thiol peptides derivatized with monobromobimane were measured using capillary electrophoresis set coupled with diode-array detector (UV-VIS/DAD, 190–600) (Agilent 7100, Agilent Technologies, Santa Clara, CA, USA). Freshly prepared standard solutions (Sigma-Aldrich) were used for the identification and quantification [17].

Krebs cycle acids were monitored in extracts prepared by homogenization of fresh material in deionised water. Samples were centrifuged at 14 000 g for 15 min at 4 °C and quantification was done by capillary electrophoresis [12]. Reduced ascorbic acid was quantified by bathophenanthroline method in homogenates prepared with 0.1 M HCl [18] and proline by the ninhydrin method [19] as described previously.

### 2.4. Quantification of fatty acids

Lichens fatty acid profile has been investigated by using a direct extraction-derivatization technique in which the total lipid are extracted and derivatized to their corresponding methyl esters (FAMEs) at the same time. Briefly, 15 mg DW of samples were placed into screw-top test tube, spiked with 20 μL of the internal standard glyceryl triundecanoate (C11-C11-C11, Merck Life Science, Darmstadt, Germany) at a concentration of 700 mg/L in n-hexane, and subsequently dried under nitrogen flow. FAMEs were obtained by direct derivatization procedure with 500 μL of sodium methylate in methanol (0.5% w/v) and 500 μL of boron trifluoride-methanol solution (14% in methanol), sonicated and then heated at 100 °C. An NaCl saturated solution in distilled water and *n*-hexane was added, shaken on vortex and upper *n*-hexane layer was transferred to vials for gas chromatography (GC).

FAMEs identification was carried out on a GCMS-QP2010 (Shimadzu, Milan, Italy) equipped with a split-splitless injector and an AOC-20i autosampler (see Supplementary Fig. S2 for chromatogram). The chromatographic column was a SLB-IL60i (30 m × 0.25 mm id, 0.20 μm film thickness) column (Merck Life Science). The programmed oven temperature was: from 70 °C to 180 °C (10 min) at 3 °C/min and then up 280 at 3 °C/min. Injector was kept at 280 °C; injection volume: 3.0 μL; injection mode: split 1:10. Helium was used as carrier gas at 30 cm/s linear velocity. MS parameters were as follows: mass range 40–550 amu, scan speed: 3333 amu/s. Ion source temperature: 220 °C, interface temperature: 250 °C. The GCMSsolution software (version 4.41 Shimadzu, Milan, Italy) was used for data collection and handling. Peaks assignment was carried through spectral search of the analytes into the FAMEs dedicate library: LIPIDS library (Shimadzu Europe,

Duisburg, Germany) was mainly used. To support the identification, the system of linear retention indices (LRIs) was used. In particular, a LRIs ± 10 range between experimental (calculated by injection of a C4-C24 even carbon saturated FAMEs standard solution, Merck Life Science) and reference (obtained analyzing FAMEs standard mixtures) LRIs were employed. FAMEs quantification was carried out on a GC-2010 (Shimadzu) equipped with a split-splitless injector (280 °C), an AOC-20i autosampler, and a FID detector. GC column, temperature program, carrier gas and linear velocity were the same as mentioned above for GCMS analysis. The FID temperature was set at 280 °C (sampling rate 40 ms) and gas flows were 40 mL/min for hydrogen, 30 mL/min for make up (nitrogen) and 400 mL/min for air, respectively. Data were collected by LabSolution (version 5.92, Shimadzu). FAMEs have been quantitatively determined using a glyceryl triundecanoate as internal standard as showed in AOCS Official Method Ce1h-05 [20]. This method determines the content and concentration of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) using the theoretical flame ionization correction factors (TCFs). For a major data precision, all samples were derivatized in triplicate.

### 2.5. Assay of cadmium and mineral nutrients

Samples were prepared by mineralization of dry samples in the mixture of concentrated $HNO_3$ and water (5 + 5 mL) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h (complete duration of the mineralization program). Resulting clear solution was placed to inert plastic flasks and diluted to a final volume of 20 mL. All measurements were carried out using an atomic absorption spectrometer (Polarised Zeeman Z – 8200, Hitachi, Tokyo, Japan) as reported previously [1,21]. For the quantification of absorbed minerals, samples were rinsed for 20 min in 5 mM $Na_2$-EDTA to remove surface-bound metals and subsequently in deionised water to remove excess of $Na_2$-EDTA and further processed as described above. Blank (mixture of $HNO_3$ and water) was checked to ensure the correctness of metal quantifications including reference plant material "NCS DC73349 – trace elements in bush branches and leaves".

### 2.6. Statistical analyses

One-way ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the significance of differences ($P < 0.05$) between treatments with 3 independent repetitions for each treatment and parameter. The principal component analysis (PCA) was based on the amount of elements, selected fatty acids and metabolites. The statistical analyses were performed using Statistica software (StatSoft, Inc. 2014, data analysis software system version 12).

## 3. Results and discussion

### 3.1. Cadmium and cPTIO variously regulate ROS/NO balance

Preliminary experiment showed concentration-dependent responses to Cd and/or cPTIO: Cd induced elevation of NO signal and cPTIO reversed it (Supplementary Fig. S1) and higher doses were selected for the subsequent investigation. Lichen samples were stained using two reagents differing in mechanism to detect NO/RNS (DAN and DAF-FM DA): despite sometimes problematic use of cPTIO for quantitative measurement of NO in plants [22], both staining procedures revealed clearly depleted NO signal in response to cPTIO but enhanced in response to Cd alone (Fig. 1A and B). These data confirm microscopic detection of NO to be an effective alternative to quantification requiring sample extraction and thus formation of eventual artifacts. Our data corroborate those from acute Cd excess (short-term exposure) where Cd enhanced NO formation in algae (10 or 100 μM Cd over 24 h [2]) or vascular plants such as chamomile (60 μM Cd over 48 h [1]) and *Arabidopsis* (50 μM Cd over 48 h [6]). At the same time, co-application of cPTIO with Cd suppressed Cd-induced stimulation of NO level in the present study (Fig. 1A and B) and in other species [1,6], indicating that cPTIO works effectively in various species (see also concentration-dependent changes in Fig. S1). Data of NO detection in lichens are rather scarce [11] and our recent studies indicated increase in NO signal in response to Ni (10 or 100 μM Ni over 24 h [7]) but depletion in response to 100 μM Cu (24 h) in two lichens of the genus *Cladonia* [8]. All these findings collectively confirm that NO formation is metal-specific also in lichens.

Owing to complex chemistry between NO and ROS, change in NO content (owing to application of Cd or cPTIO in this case) is expected to evoke some change in the ROS formation. Indeed, Cd induced not only increase in NO level as mentioned above but also elevation of "general" ROS (Fig. 1C and D) and hydroperoxides (Fig. 1E) content as cPTIO alone also did (but with substantially lower intensity). In the Cd + cPTIO treatment, CellROX Deep Red Reagent rather than DCF-DA (Fig. 1C and D) showed additive stimulatory impact on the ROS formation, and hydroperoxides were also elevated (Fig. 1E). Our data are in agreement with the microscopic detection of responses to Cd in vascular plants where Cd strongly enhanced (depending on the applied dose) ROS formation and cPTIO co-application had rather further stimulatory effect [1,6], supporting similar responses in various life forms. It is worth noting that increase in both NO and ROS in response to Cd is rather specific response which has not previously been observed under the excess of other metals in lichens (e.g. 100 μM Cu [8]) or in algae (100 μM Hg but not Pb [23]). The stimulatory impact of cPTIO on the ROS formation observed here may also be indirectly confirmed by studies where it was tested in combination with NO donor (e.g. widely used SNP): in *Brassica juncea*, Cd excess stimulated increase in $H_2O_2$ content which was suppressed by SNP co-application but cPTIO co-application elevated it again to the level of Cd alone [9]. Studies involving other plant species also confirmed negative relation between cPTIO (NO scavenger) and SNP (NO donor) under the excess of Cd in terms of NO/ROS balance [1,6]. Further analyses were performed to detect eventual impact of the altered ROS/NO ratio on the selected metabolites owing to absence of deeper data in lichens or in plants more generally.

### 3.2. Impact of NO on Cd-induced changes of antioxidants and chelators

Thiols are potential chelators of metals and mainly GSH strongly responds to Cd excess [1]. Depletion of GSH evoked by 100 μM Cd (Fig. 2) was also observed in other life forms such as algae (100 μM Cd over 24 h [2]) and vascular species (50 or 100 μM Cd over 48 h [6]) and may be compensated by enhanced synthesis of phytochelatins as observed in response to Cd [1] or Cu [8]. In agreement, even low Cd doses (4.5 and 9 μM over 24 or 48 h) depleted strongly GSH amount in the common lichen *Xanthoria parietina* [14]. Other metals evoke less pronounced changes of GSH content in lichens, e.g. Ni or Cr [7,8] but not Cu [8]. Subsequently, cPTIO had the same impact as Cd alone and did not affect GSH level in combination with Cd (Fig. 2). Less extensive side impacts of cPTIO (in comparison with PTIO) on thiols (alone or in the combination with Cd) have also previously been detected in chamomile (slightly stimulatory [1]) and *Brassica juncea* or *Pisum sativum* (slightly negative [9,10]). It was interesting to find that GSSG (oxidized form of GSH) increased in response to cPTIO but not to Cd (Fig. 2), indicating that depleted NO rather than enhanced ROS levels contribute to these changes. In agreement, Cd evoked depletion of both GSH and GSSG in *Arabidopsis* (100 μM Cd [6]) or algae (100 μM Cd [2]).

Proline is a "stress-related" amino acid which undergoes fluctuation in response to metals [24,25]. Cd had stimulatory but cPTIO inhibitory impact on its accumulation also in Cd + cPTIO treatment (Fig. 2). Besides, a study using Cu-exposed *Chlamydomonas* revealed that Cu-

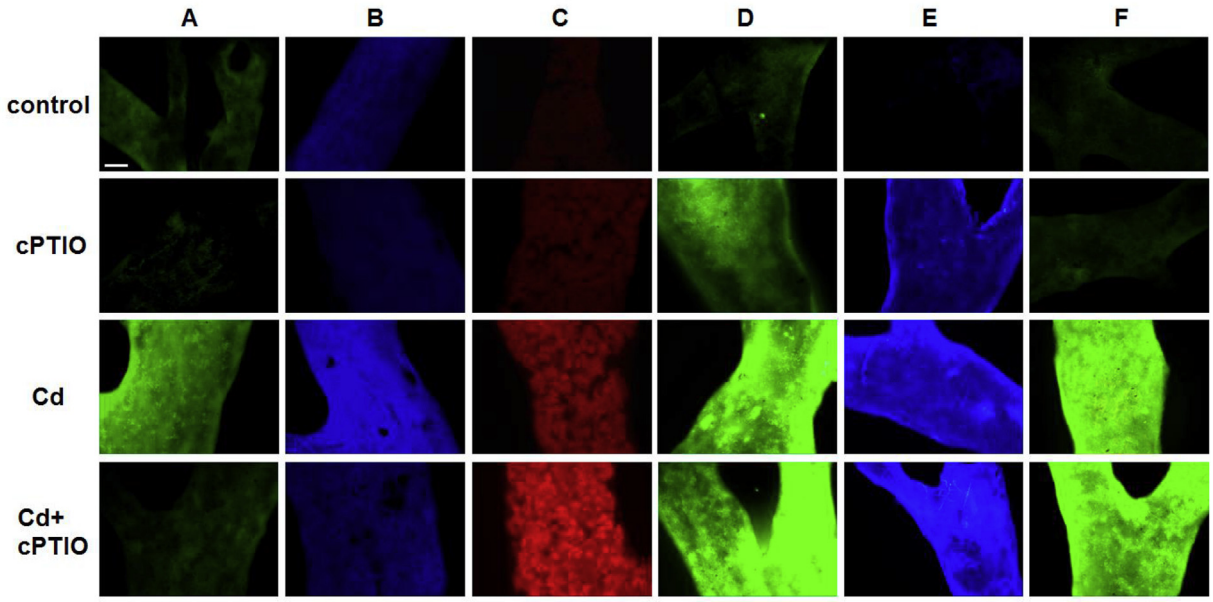


**Fig. 1.** Impact of nitric oxide scavenger (cPTIO), cadmium (Cd) and their combination (Cd + cPTIO) on the formation of nitric oxide (staining reagents 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate for panel A and 2,3-diaminonaphthalene for panel B), reactive oxygen species (staining reagents CellROX Deep Red Reagent for panel C, 2′,7′-dichlorodihydrofluorescein diacetate for panel D and diphenyl-1-pyrenylphosphine for panel E) and cadmium localization (Leadmium™ Green staining reagent, panel F; note that weak signal in control and cPTIO could be a signal of natural Pb in thalli) in the lichen *Ramalina farinacea* after 24 h of exposure (100 µM of each compound was applied). Bar indicates 200 µm. See also Supplementary Fig. S1 for dose-dependent responses of lichen to Cd and cPTIO.

induced proline accumulation was concentration-dependent (stimulation only up to 5 µM Cu) and low NO donor dose (10 µM SNP) enhanced Cu-induced stimulation [24]. The same was observed in freshwater microalga where NO donor SNP reversed negative impact of Cu excess mainly [25]. Unfortunately, we did not find data from lichens reporting the impact of cPTIO on metal-induced proline changes in lichens but it seems that proline amount is tightly regulated with the NO formation (cf. Fig. 1A and B vs. Fig. 2).

Ascorbic acid (AsA) as an essential plant non-enzymatic antioxidant is often affected by metallic treatments in various species. On the contrary to GSH, its accumulation is rather elevated by Cd as observed in the lichen *Xanthoria parietina* [14] or *Arabidopsis* [6] and in the present study (+60%, Fig. 2). Other metals may also elevate AsA in the lichens *Cladonia* such as Ni or Cr, but not Cu [7,8]. Unlike Cd, cPTIO depleted AsA amount and suppressed Cd-induced elevation (Fig. 2). In agreement, similar action of PTIO was observed in chamomile under Cd

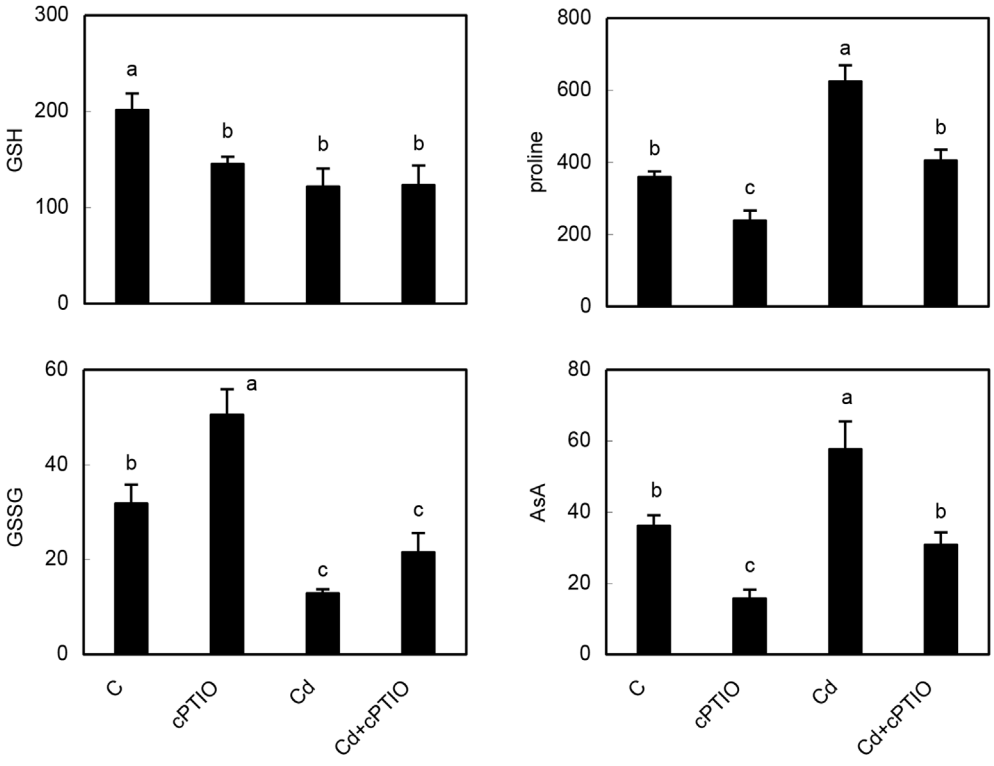


**Fig. 2.** Impact of nitric oxide scavenger (cPTIO), cadmium (Cd) and their combination (Cd + cPTIO) on quantitative changes of reduced (GSH) and oxidized (GSSG) glutathione, ascorbic acid (AsA) and proline in the lichen *Ramalina farinacea* after 24 h of exposure (100 µM of each compound was applied). Unit is µg/g DW for all compounds. Data are means ± SDs ($n = 3$). Values followed by the same letter are not significantly different according to Tukey's test ($P < 0.05$).

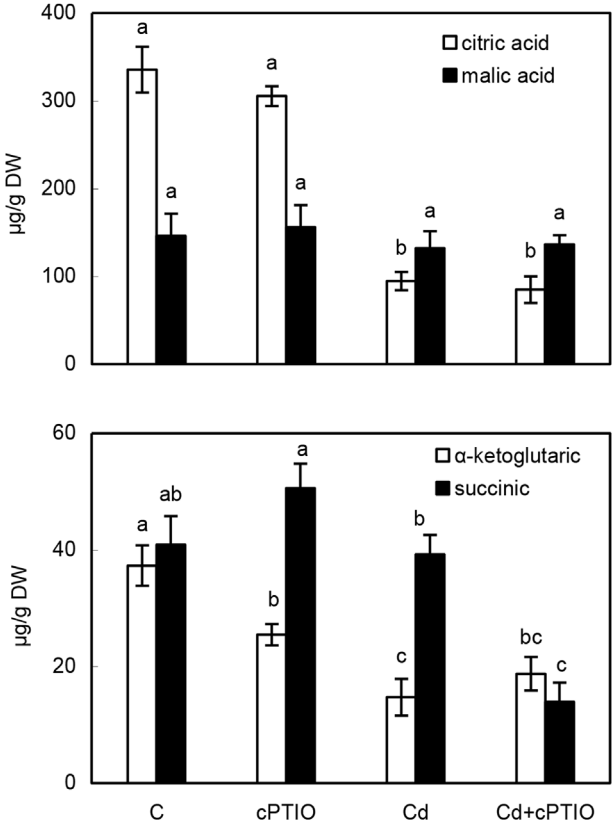


**Fig. 3.** Impact of nitric oxide scavenger (cPTIO), cadmium (Cd) and their combination (Cd + cPTIO) on quantitative changes of Krebs cycle acids in the lichen *Ramalina farinacea* after 24 h of exposure (100 µM of each compound was applied). Data are means ± SDs ($n$ = 3). Values for each acid followed by the same letter(s) are not significantly different according to Tukey's test ($P$ < 0.05).

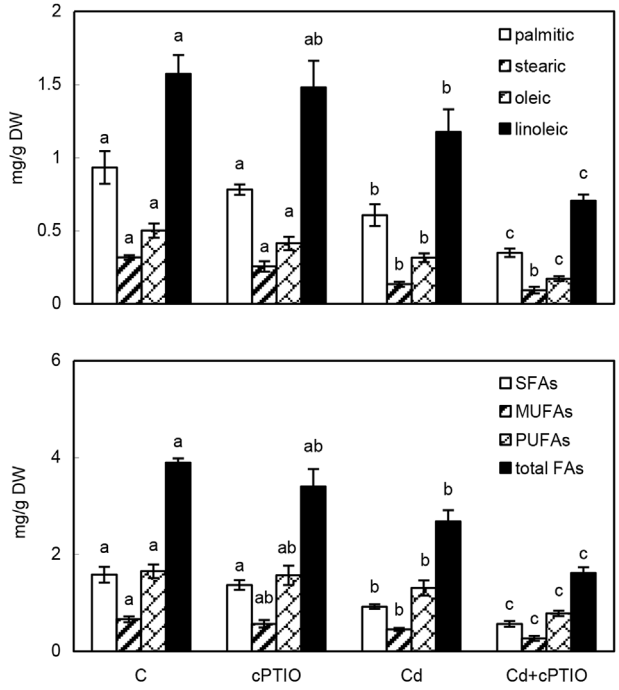


**Fig. 4.** Impact of nitric oxide scavenger (cPTIO), cadmium (Cd) and their combination (Cd + cPTIO) on quantitative changes of individual major fatty acids and fractions of fatty acids (SFAs – saturated fatty acids, MUFAs – monounsaturated fatty acids, PUFAs – polyunsaturated fatty acids, total FAs – total fatty acids) in the lichen *Ramalina farinacea* after 24 h of exposure (100 µM of each compound was applied). Data are means ± SDs ($n$ = 3). Values for individual acids or fractions followed by the same letter(s) are not significantly different according to Tukey's test ($P$ < 0.05). Note that given four acids represent over 85% from total acids and quantitative changes of other acids are shown in Supplementary Table S2.

excess [1] and NO donor (SNP) evoked dose-dependent increase in AsA under Cd excess in microalga [2]. Antioxidative role of AsA has also been confirmed by exogenous treatment where AsA reversed negative impact of Hg including NO and ROS formation [23]. These data confirm the regulatory role of NO in the quantitative changes of AsA. Basal (control) values are similar to those observed in the lichens *Cladonia* [8].

Citric acid was more abundant than malic acid in control thalli, followed by α-ketoglutaric and succinic acids (Fig. 3). These acids were not frequently monitored in lichens and values are similar to those found in epilithic species *Lecanora polytropa* [26] and terrestrial macrolichens [8]. Our present data showed negative impact of Cd excess on Krebs acids while cPTIO augmented negative action of Cd (in Cd + cPTIO treatment) only at the level of succinic acid (Fig. 3). Krebs cycle acids may also serve as chelators of metals in plants [12,27] but we found no lichen data which we could discus in details. However, rather depleted accumulation of the monitored organic acids does not indicate eventual role in metal chelation. In agreement, Ni had negative impact on the amount of malate while citrate and succinate were almost unaffected and α-ketoglutarate trend even differed in two *Cladonia* species [7]. In terms of NO action, it does not seem that it has considerable impact on the accumulation of organic acids and affected the content of α-ketoglutaric acid only (Fig. 3). In vascular plants, for example, NO donor (SNP) had the opposite impact on malate and citrate in the shoots and roots of *Citrus* [28].

### 3.3. Fatty acids are more affected by Cd than by NO

It is rather common in the literature that fatty acid quantification is shown as relative % of total fatty acids (FAs) which is difficult for exact comparison among various biological objects. We therefore mention both absolute (mg/g DW) and relative (% of total FAs) amounts of acids for better clarity. In total, 22 fatty acids including longer chain acids were detected in the lichen *Ramalina farinacea* (Supplementary Table S1) with maximum absolute content of the sum of acids in control to be 3.89 mg/g DW (Fig. 4). This is far lower amount comparing with other life forms such as freshwater microalgae (ca. 70–120 mg/g DW in *Coccomyxa* [29]) while other lichens contain ca. 1–3 mg/g DW of free fatty acids [30].

Palmitic (C16:0), stearic (C18:0), oleic (C18:1n9) and linoleic (C18:2n6) acids were major compounds (Fig. 4; see also Supplementary Fig. S2 for chromatogram), representing over 85% of total fatty acids (and linoleic acid over 40%; see Supplementary Table S2). In agreement with our profile, oleic and palmitic acids were dominant in the lichens *Collema* [30] but, unlike them, we did not detect higher abundance of α-linolenic acid (C18:3n3, ca. 2% from total FAs in control; see Supplementary Table S2). In the photobiont of the lichen *Dictyonema*, palmitic and stearic acids were abundant [31]. Linoleic (17–30% from total FAs) and palmitic (18–27% from total FAs) acids are also dominant in the leaves of vascular plants [32]. MUFAs were less accumulated than PUFAs and SFAs (Fig. 4 and Supplementary Table S2).

Oxidative stress arising from the application of metals may alter quantity of fatty acids (especially polyunsaturated acids), which are prone to oxidation. This fact has been confirmed in the present study and all major acids decreased more in response to Cd than to cPTIO application, being therefore reflected in individual fractions too (Fig. 4). In the combined Cd + cPTIO treatment, major acids and individual fractions showed typically more pronounced depletion than under single treatments. Considering that ROS, but not NO signal,

increased more expressively in Cd + cPTIO, these data indicate negative impact of enhanced oxidative stress on the fatty acids accumulation. In opposite, NO donor (SNP) had negligible impact on fatty acids in ginger leaves [33]. It has recently been found that metallic stress (among other stresses) may evoke formation of nitro-linolenic acid with potential significance in NO metabolism and stress tolerance ([34] and the references therein).

It was interesting to find, if expressed as % of total fatty acids, that all treatments and Cd + cPTIO mostly depleted SFAs (from 40 to 35% of total SFAs) but elevated PUFAs (from 42 to 48%) content (see also trend of minor acids in Supplementary Table S2). This is rather unusual observation because we recently found that even 10 µM Cd depleted % of PUFAs and elevated % of SFAs in the macroalga *Ulva* [35]. However, similarly to our data presented here, ecotypes of Cd hyperaccumulator *Thlaspi caerulescens* revealed depletion of SFAs but elevation of PUFAs with increasing Cd dose which could contribute to defense [32] and similar action could be plausible in lichens.

### 3.4. Changes of Cd accumulation and mineral nutrients content

*Ramalina* accumulated over 1700 µg Cd/g DW and 140 µg Cd/g DW in the total and absorbed fraction, respectively (Fig. 5). It indicates only ca. 8% of Cd to be accumulated inside the thalli. In agreement, earlier study confirmed ca. 10-fold difference between total and absorbed Cd in lichens *Peltigera* and *Cladonia* exposed to 100 µM Cd under similar exposure conditions and total content reached over 1200 µg Cd/g DW ([3], we note unit error in this paper: mmol/g DW should be µmol/g DW, no tissue may contain over 30 mmol Cd/g DW = over 3300 mg Cd/g DW). On the contrary, common lichen *Xanthoria parietina* contained only up to 11 µg Cd/g DW when exposed to 9 µM Cd over 48 h [14]. Co-application of cPTIO did not affect Cd accumulation as proven quantitatively (Fig. 5) and qualitatively (Fig. 1F) which could be, at least partially, a reason why Krebs acids did not differ considerably. In line with these data, PTIO but not cPTIO affected Cd accumulation in chamomile seedlings and therefore cPTIO is more suitable (no side effects) to modulate NO level also in lichens [1].

Other quantified essential metallic nutrients revealed two interesting findings: i) none of the minerals was affected by Cd or cPTIO,

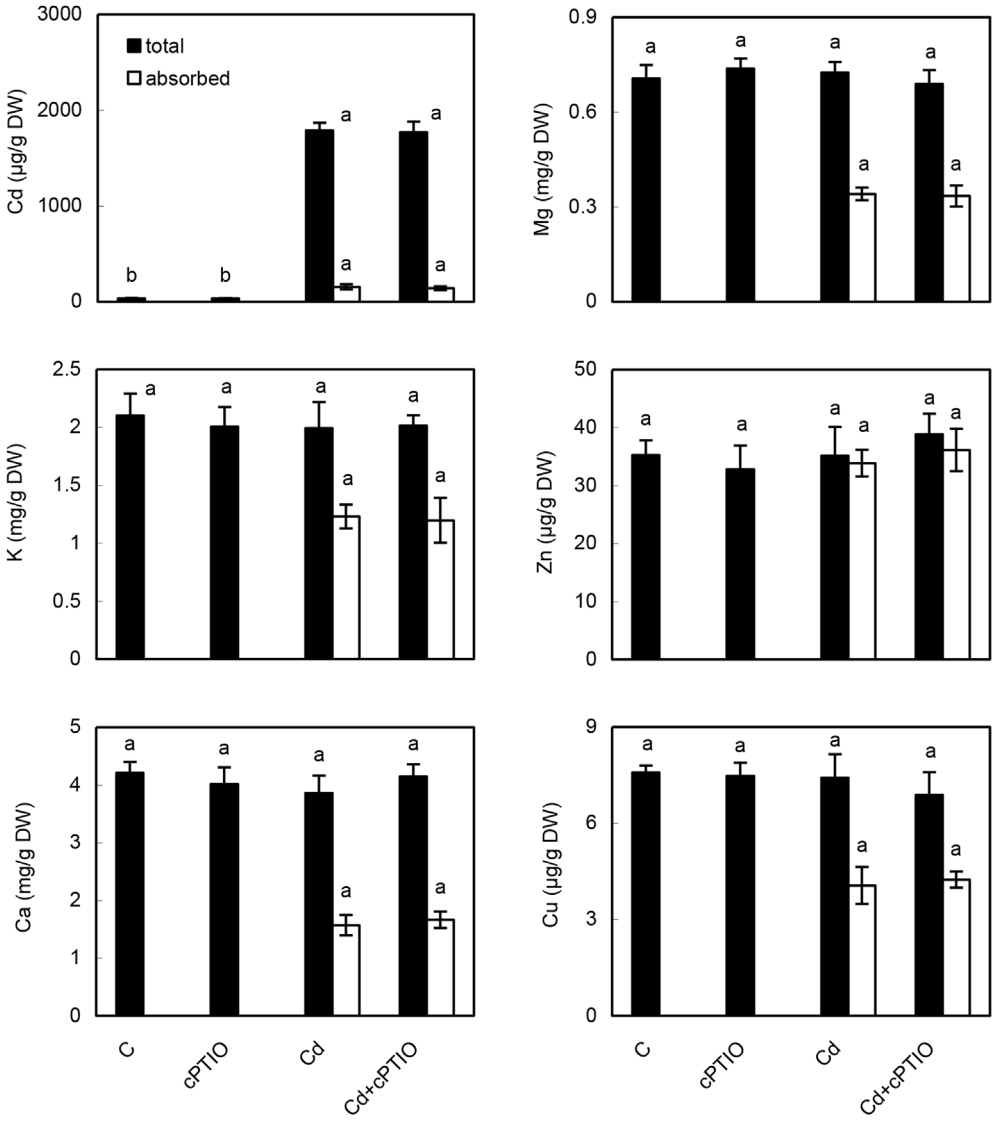


**Fig. 5.** Impact of nitric oxide scavenger (cPTIO), cadmium (Cd) and their combination (Cd + cPTIO) on quantitative changes of cadmium and selected mineral nutrients content in the lichen *Ramalina farinacea* after 24 h of exposure (100 µM of each compound was applied). Data are means ± SDs ($n = 3$). Values for each fractions followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$). Note absence of significant changes for any treatment or nutrient. Absorbed content means that thalli were rinsed with $Na_2$-EDTA to remove surface-bound metals.

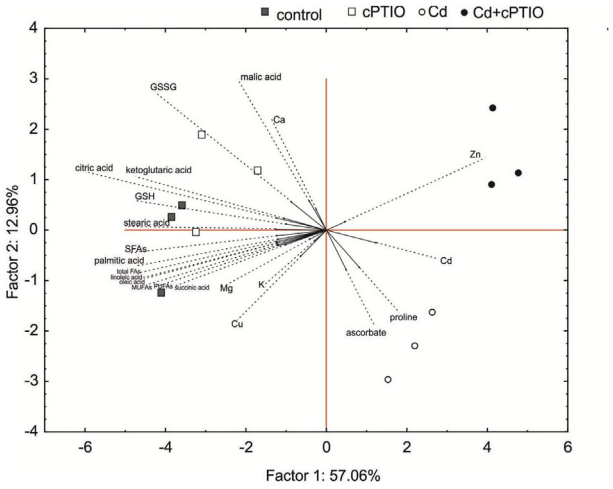


**Fig. 6.** Principal component analysis (PCA) of the amount of elements, selected fatty acids and metabolites in the lichen *Ramalina farinacea* after 24 h of exposure to various treatments.

confirming physiological relevance of the applied Cd dose (Fig. 5) and ii) only amount of Zn did not differ significantly in total and absorbed fraction, indicating that considerable part of other metals (40–60% from total content of K, Ca, Mg, and Cu) is localized on the surface of thalli. This is a natural expectation with respect to epiphytic nature of given lichen species. Only slight significant changes in Zn content have been previously observed in two *Cladonia* species exposed to 100 µM Cu while K and mainly Ca amount were more extensively depleted by 100 µM Cu and/or Cr [8]. It is concluded that NO metabolism modulated by cPTIO application does not evoke extensive changes in metallic nutrients including Cd accumulation and therefore observed changes of metabolites including cross-talk between Cd and NO are free of eventual misinterpretations (which could be evoked by strong shift in nutrient balance).

### 3.5. PCA analysis

The PCA analysis of the given variables showed clear separation of individuals into four groups according to the treatments (Fig. 6). The first factor represented more the 57% of the total variation and strongly negatively depended on the fatty acids, GSH, citric acid, ketoglutaric acid and succinic acid. The factor showed also positive correlation with Cd and Zn. It facilitated separation of Cd-treated samples from both control and cPTIO treatments. In turn, the second factor facilitated separation of Cd-exposed samples from Cd + cPTIO treatment. This factor represented 13% of the total variation and it was positively correlated with GSSG, malic acid and Ca while it was negatively correlated with AsA, proline and Cu.

## 4. Conclusions

Present study confirmed that cPTIO effectively modulates not only NO formation in lichen but also its cross-talk with Cd at the ROS/NO level (i.e. higher ROS but lower NO formation in combined than in single treatments). Cd-induced elevation in NO content was related to increase in ascorbate and proline content while cPTIO suppressed it. Interestingly, glutathione was depleted in all treatments but Krebs acids were almost unaffected by cPTIO, indicating no essential role of NO in their accumulation. On the contrary, fatty acids were depleted the most in combined treatment where the highest ROS formation was visible. However, shift in relative ratio of saturated versus polyunsaturated fatty acids indicates eventual defense mechanisms to Cd-induced oxidative stress.

## Author contribution

Experimental design, collection of samples, spectrophotometry and manuscript preparation (JK and JH), chromatography and mineral analyses (SD), analyses of fatty acids (GM and LM), fluorescence microscopy (PB).

## Disclosure statement

The authors declare that there are no conflicts of interest.

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Sponsors had no involvement in the present study.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.12.001.

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