

Effect of Metal Cations Pb^{2+} , Cu^{2+} , Zn^{2+} , Mg^{2+} and Fe^{2+} on Some Physiological Parameters of Lichen *Parmotrema dilatatum*

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Abstract Lichens are used in air quality biomonitoring. They accumulate the air pollutants including trace metal elements. However, the bioaccumulation of trace metal elements in the thalli of lichens has considerable consequences on their physiology. Indeed, trace metal elements have the ability to induce the production of reactive oxygen species (ROS) which react with several vital biomolecules of the species. This causes serious morphological, metabolic and physiological abnormalities depending on the lichen species. In this study, fresh thalli of *Parmotrema dilatatum* lichen are in contact with a metal salt solution at different concentrations. The content of chlorophyll a, phospholipids, total polyphenols and total proteins were determined before and after treatment with the different metal salt solutions. The chlorophyll a content decreases significantly in the presence of copper and zinc ions while carotenoids degrade significantly in the presence of zinc. Significant degradation of the species' membrane in the presence of Iron and Copper was also observed. These observations suggest the possibility of electrolyte leakage in case of a biomonitoring situation on fixed sites, which are potentially rich in copper and iron. Also lichen *Parmotrema dilatatum* present a great capacity of metal cations detoxification by using proteins in particular in case of lead and copper. A non-protein detoxification response was observed with the carotenoids in presence of zinc.

Keywords: lichens, atmospheric pollution, *Parmotrema dilatatum*, bioaccumulation, metal trace elements

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1. Introduction

Lichens are organisms resulting from a symbiotic association between alga and fungus. They have neither roots nor cuticles and depend mainly on the atmospheric supply of mineral nutrients. Lichens are well known to be reliable sentinels of air pollution effects [1-6], they can accumulate elements at levels exceeding their physiological needs [7]. However, in air pollutants biomonitoring, the specific characteristics of lichens are sometimes limited by their toxico-tolerance. Elements accumulated in biological tissues impact lichen survival differently [8,9,10,11]. The toxicity of metal cations on biological systems lies in their ability to activate reduced forms of oxygen [12,13] leading to increased formation of reactive oxygen species (ROS). These ROS can induce oxidative stress [12,13,14] that can damage the cellular integrity [15], increase the peroxidation of membrane lipids [16] and then can affect the efficacy of the photosynthetic apparatus [17] through photosynthetic pigments degradation [18,19]. To mitigate these adverse effects, lichens have developed a defense mechanism composed of biological

molecules. Physiological detoxification responses are very useful as stress indicators and can be used to detect early signs of altered environmental conditions [20]. Changes in physiological parameters of sensitive species can be used as indicators of environmental stress related to a specific area [21].

In this study, the effects of metals cations on some physiological parameters of lichen *Parmotrema dilatatum* were evaluated. The Effects of pigments, peroxidation of membrane lipids by malondialdehyde (MDA) analysis, total polyphenols and total proteins were determined. The main objective of this research was to understand the effects of these metal cations on physiological parameters of *Parmotrema dilatatum* lichen.

2. Materials and Methods

2.1. Lichen Sampling and Laboratory Treatment

Thalli of the lichen *Parmotrema dilatatum* were collected in the forest reserve of the LAMTO (Figure 1) ecology station (5.02°C West and 6.13°C North).

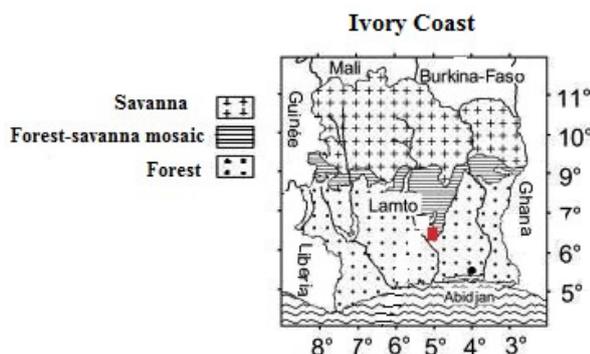


Figure 1. Location of Lamto reserve

This area is a natural park of 2500 ha, with a tropical climate, the average temperature per year is 28.28°C, the annual rainfall value of 1194 mm and the humidity rate is above 58%.

The samples were taken with the adhering pieces of substrate, on adult trees with diameters 20 cm larger. Once the lichens were collected, they were immediately placed in paper envelopes to avoid transpiration of the plant material and transported to the laboratory.

At laboratory, lichens are detached from their substrates and then cleaned manually to remove soil debris, leaves, dust or insects. The thalli were then thoroughly washed with distilled water.

2.2. Determination of Saturation Concentration (Cs)

Stocks of metal salt solution of concentration 1, 2, 6, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 g/L were prepared by dissolving appropriated mass of metal salt ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ and $\text{Pb}(\text{NO}_3)_2$) in distilled water. In a 250 mL beaker, 1g of fresh lichen is placed in contact with 50 mL of metal salt solution and kept stirred at 300 rpm by a vibrating stirrer (Heidolph Vibramax 100) for 24 h. The stressed lichen samples are then filtered under gravity and then dried in an oven at 105°C.

The dried lichen samples are crushed using a mortar and a porcelain pestle. The powder obtained is dried again in the oven at 105°C for 4 h. A quantity of 0.3 g of dry powder is taken and placed in a 50 mL porcelain crucible and then calcined in a muffle furnace at 550°C for 4 h. The ashes obtained are placed in teflon tubes with mixture 5:1 (v/v) of hydrochloric acid (20%) and nitric acid (69%), and then placed in a water bath at 80°C for 1 h. The mineralized sample is collected in a 25 mL volumetric flask and filled with distilled water to the mark. Solutions are analyzed using an Atomic Absorption Spectrophotometer (SpectrAA. 20, Varian, New-York, USA) with automatic output data storage mode.

For each metal, the concentration allowing maximum lichens biosorption was determined. This concentration is called the saturation concentration of the lichens biosorption sites and is noted Cs (g/L).

2.3. Analysis of Physiological Parameters

The effect of the metal cation on the physiological parameters of the species was studied in solutions of the

metal salt in concentrations varying in steps of Cs (1/6, 2/6, 3/6, 4/6, 5/6, 6/6). In a 250 mL beaker, 1g of fresh lichen is placed in contact with 50 mL of a metal solution and kept stirred for 2 h. The lichens samples were then filtered and dried at room temperature for 4 h. A control sample was prepared by placing 1 g of fresh lichen with 50 mL of distilled water, under the same conditions. After drying the sample is quickly ground in porcelain mortar with a pestle and then subjected to the analysis of physiological parameters variability. The optical densities were read using a spectrophotometer (WFJ-752 UV-VIS Spectrophotometer, Beijing China). The contents of chlorophyll a (Chl a), carotenoids (Car), malondialdehyde (MDA), total polyphenols (Phe) and total proteins in the thalli of the specie were then determined.

2.3.1. Analysis of pigments

A quantity of 0.3 g of fresh lichens powder is placed in a centrifuge tube with 0.01 g calcium carbonate (CaCO_3) and then 10 mL of acetone is added. The tubes are centrifuged at 5000 rpm for 30 min. The optical densities were read with the spectrophotometer at 480, 646.6, 663.6 nm. A reading is performed at 750 nm to correct turbidity interference. The contents of chlorophyll a (Chl a) and carotenoids (Car) are determined from Porra [22] equations.

2.3.2. Analysis of lipoperoxides

Lipoperoxides analyses were performed using the Heath and Packer method [23].

A volume of 5 mL of a 10% (m/v) trichloroacetic acid (TCA) solution is added to 0.3 g of lichens powder. The solution is left for incubation during 10 min and then 5 mL 0.5% (m/v) thiobarbituric acid (TBA) is added.

The tubes are carefully closed and placed in water bath at 95°C during 30 min. In the course of this incubation phase, a complex is formed between TBA and aldehydic compounds, essentially malondialdehyde (MDA) complex: TBA-MDA. The reaction is stopped by cooling the tubes in ice. The cooled sample is then centrifuged at 10000 rpm for 10 min. After this centrifugation, the supernatant is recovered for determination of lipoperoxides content, using the spectrophotometer. The optical density of the TBA-MDA complex is read at 532 nm against a blank sample that is performed with the extraction buffer. The optical density is corrected by reading at 600 nm. The concentration of MDA is calculated using its molar extinction coefficient $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$ [24].

2.3.3. Analysis of polyphenols

The polyphenols content was quantified using Swain and Hillis [25] method. This method is based on the use of Folin-Ciocalteu reagent. 0.2 g of lichens powder was introduced in a centrifuge tube and then methanol-water mixture (5 : 2, v/v) was added. The mixture is incubated for 10 minutes, and then centrifuged at 4000 rpm for 10 minutes. 0.1 mL of supernatant solution were taken and placed in a glass test tube with which was previously covered with aluminum foil, and then 0.1 mL of Folin-Ciocalteu reagent (10 times diluted) and 1 mL of sodium carbonate (10%) were added. After 2 h of incubation in darkness and at room temperature, optical density was measured at 760 nm. The concentrations of total polyphenols was calculated from the regression equation of the calibration

range established with gallic acid and is expressed in milligram Gallic Acid Equivalent per grams of lichen (mg GAE/g).

2.3.4. Analysis of Proteins

The proteins content determination is performed according Bradford [26] method. The principle of this method is based on the binding of Coomassie Blue G-250 with peptide bond. The formation of this binding causes change in the maximum of absorption of the dye, from 465 nm to 595 nm. To performed proteins extraction, lichens are ground in a mortar held in ice. The extraction buffer consists of a mixture of potassium phosphate (50 mM, pH = 7), sodium ascorbate (5 mM) and EDTA (0.2 mM). 5 mL of extraction buffer were added to 0.2 g of lichen.

The mixture was then centrifuged at 9000 rpm for 15 min. The recovered supernatant represents the fraction of soluble proteins or crude extract.

0.1 mL of the crude extract was added to 2.5 mL of Bradford reagent. Coloration develops in 5 min. Absorbance is read at 595 nm. The calibration curve is performed with increasing concentrations of bovine serum albumin (BSA). The Results were expressed in μg per g equivalent BSA (μg BSAE/g).

2.4. Data Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the STATISTICA software (version 8.0.360.0). Whenever ANOVA indicated significant effects ($p < 0.05$), a two by two comparison of means by Tukey's HSD (honestly significant difference) test was performed.

3. Results and Discussion

3.1. Saturation Concentration (C_s)

The results of study on effect of the metal initial concentration on the specie biosorption capabilities are presented in the Figure 2. We observe that, for each metal considered, the quantity of biosorbed metal increases with the concentration of the metal salt solution, reaches a maximum and then decreases.

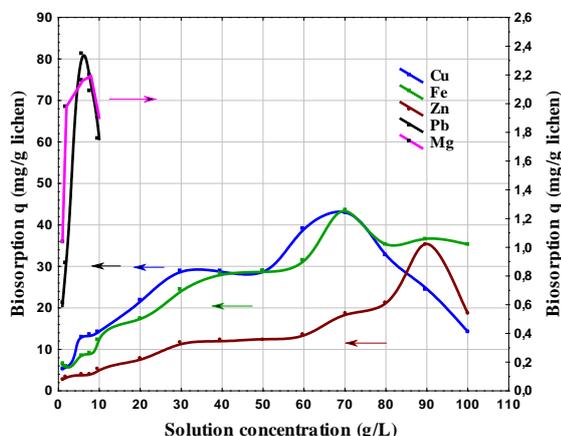


Figure 2. Evolution of the amount of metal biosorbed as a function of the initial concentration of the metal salt solution (CuSO_4 ; MgSO_4 ; FeSO_4 ; ZnSO_4 and $\text{Pb}(\text{NO}_3)_2$). The arrow indicates the axis of reading

The values of the maximum biosorption (q_{max}) as well as the saturation concentration (C_s) of the metallic salt solution are showed in Table 1. The value q_{max} was reached at an initial concentration C_s of metal salt solution.

Table 1. Maximum biosorption of the metal (q_{max}) of metallic cation and initial metallic salt concentration (C_s), leading to saturation of the lichens biosorption sites

Solution	C_s (g/L)	q_{max} (mg/g lichen)
CuSO_4	70	43.049
FeSO_4	70	43.340
MgSO_4	8	2.193
ZnSO_4	90	35.406
$\text{Pb}(\text{NO}_3)_2$	6	81.360

From these results it appears that, the maximum biosorption of each metal is reached (C_s). Similar maximum biosorptions results were found by Wannaz [27] on *T. capillaris*. Also Al-Homaidan [28] observed maximum copper biosorption by *Spirulina platensis* at only 100 mg/L. This suggests that the specie *Parmotrema dilatatum* is highly tolerant to the metal salt solutions in this study.

3.2. Effect on Chlorophyll a (Chl a)

The results of the assessment of metal cations effect s on the content of Chl a are presented in Figure 3. It is observed that the Chl a, gradually decreases with the concentration of the metal ion increases. The lowest levels of Chl a content are observed at the saturation concentration C_s . The results show that copper (Figure 3-d) followed by zinc (Figure 3-c) are the metals cations that degrade significantly Chl a, while magnesium (Figure 3-b) has little effect on the content of Chl a. Fe (II) and Pb (II) ions also contribute to the degradation of Chl a. Copper is an essential trace element found in proteins as a cofactor. It contributes to the functioning of the plant through many biological processes such as photosynthetic electron transport, mitochondrial respiration, cell wall metabolism and some hormones [29]. At high concentrations, copper causes a significant decrease in the content of Chl a, the main constituent of photosystem II. The decrease in Chl a content caused by copper can disrupt or even cause the complete inhibition of photosystem II (PS II) [30,31,32,33]. The disturbance of PS II affects the growth or even the respiration of the lichens [17,29]. Prolonged inhibition of PS II would lead to the systematic death of the species.

Zinc also has a negative impact on the integrity of Chl a. Excess zinc causes chlorosis of the thalli and disrupts photosynthesis. In this study, the evolution of zinc concentration significantly disrupts the Chl a content. However, this disturbance remains lower than that observed in case of copper. This same observation was made by Carreras and his collaborators during a study on *Usnea amblyoclada* [14].

Iron is a trace element necessary for the survival of the species. Its presence in high quantities increases the production of reactive oxygen species by Fenton reactions. These ROS are likely to cause directly or indirectly, the hydrolysis of the phytyl ester and the oxidative opening of the tetrapyrrolic macrocycle [34].

In the case of lead, there is a degradation of Chla, but relatively less than that of copper, this could be due to difficulties of penetration into cells due to its large size (ionic radius of Pb = 119 pm). Studies conducted [35] on *Ramalina lacera* showed significant degradation of Chla in contact with lead. Magnesium has a small negative impact on the Chla content. This could be due to the high involvement of this metal in the many biological processes necessary for the species to function.

3.3. Effect on Carotenoids

Analysis of the results presented in Figure 4 shows that the increase in the concentration of the metal in solution degrades the carotenoids.

The concentration effect is significant for zinc

(Figure 4-c), while moderate degradation was observed with the other metal cations.

When the *Parmotrema dilatatum* species is putted with metal cation solutions of varying concentration up to the saturation concentration Cs, metal cations causes the overproduction of reactive oxygen species (ROS) such as the radical Superoxide and the hydroxide anion. Carotenoids, in particular β- carotenes, are known for their antioxidant character linked to the unsaturation of their carbon chain [36]. This antioxidant character could explain in part their degradation under stress caused by the overproduction of ERO. It should be noted that carotenoids are also photonic collectors in photo-collecting antennas. Their degradation under the effect of zinc cations is likely to cause the disruption of photosynthesis, by partial or complete inhibition of PS II [10,31,32,33].

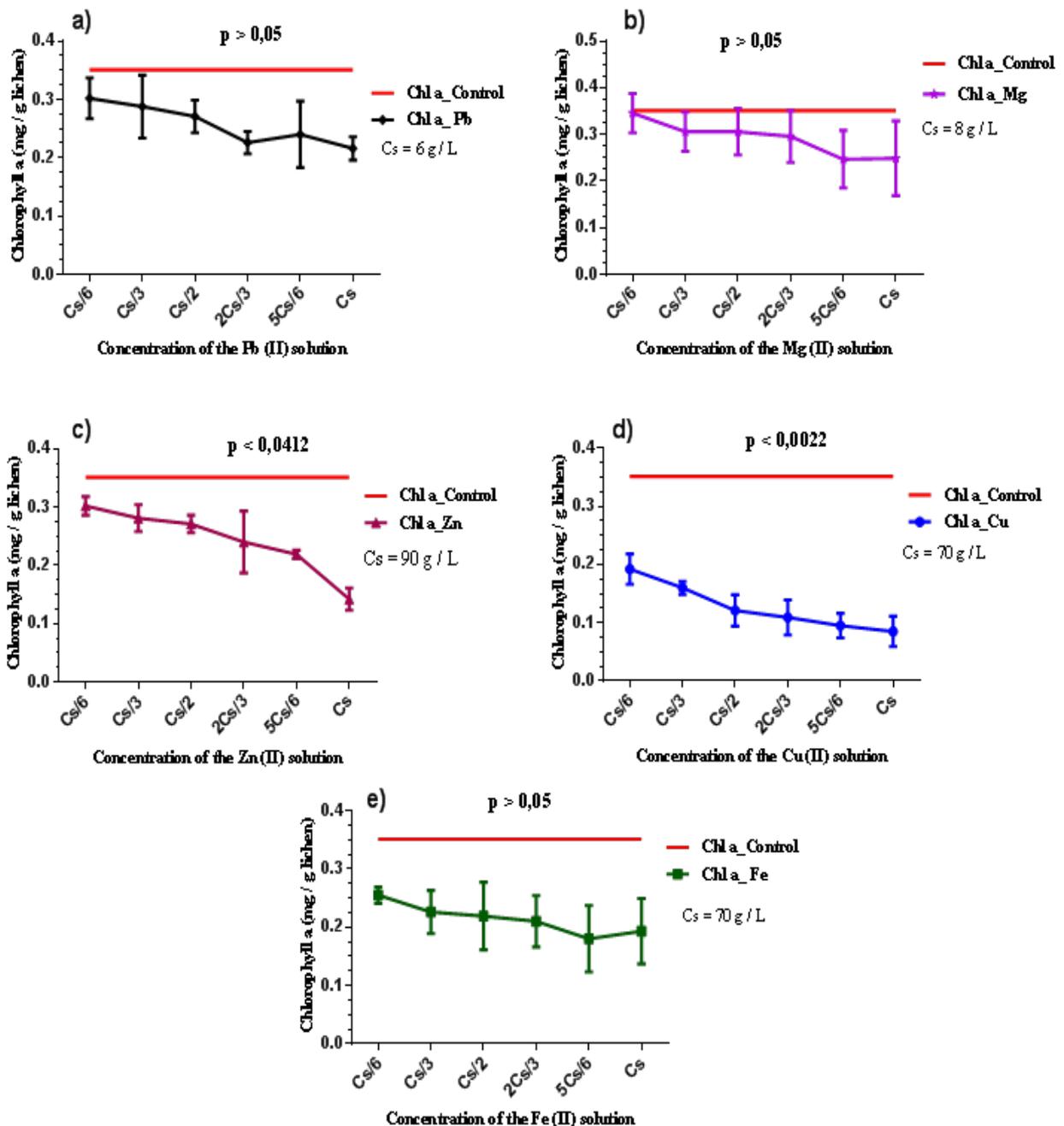


Figure 3. Chlorophyll a content in thalli of *Parmotrema dilatatum* after immersion in different metal salt solution

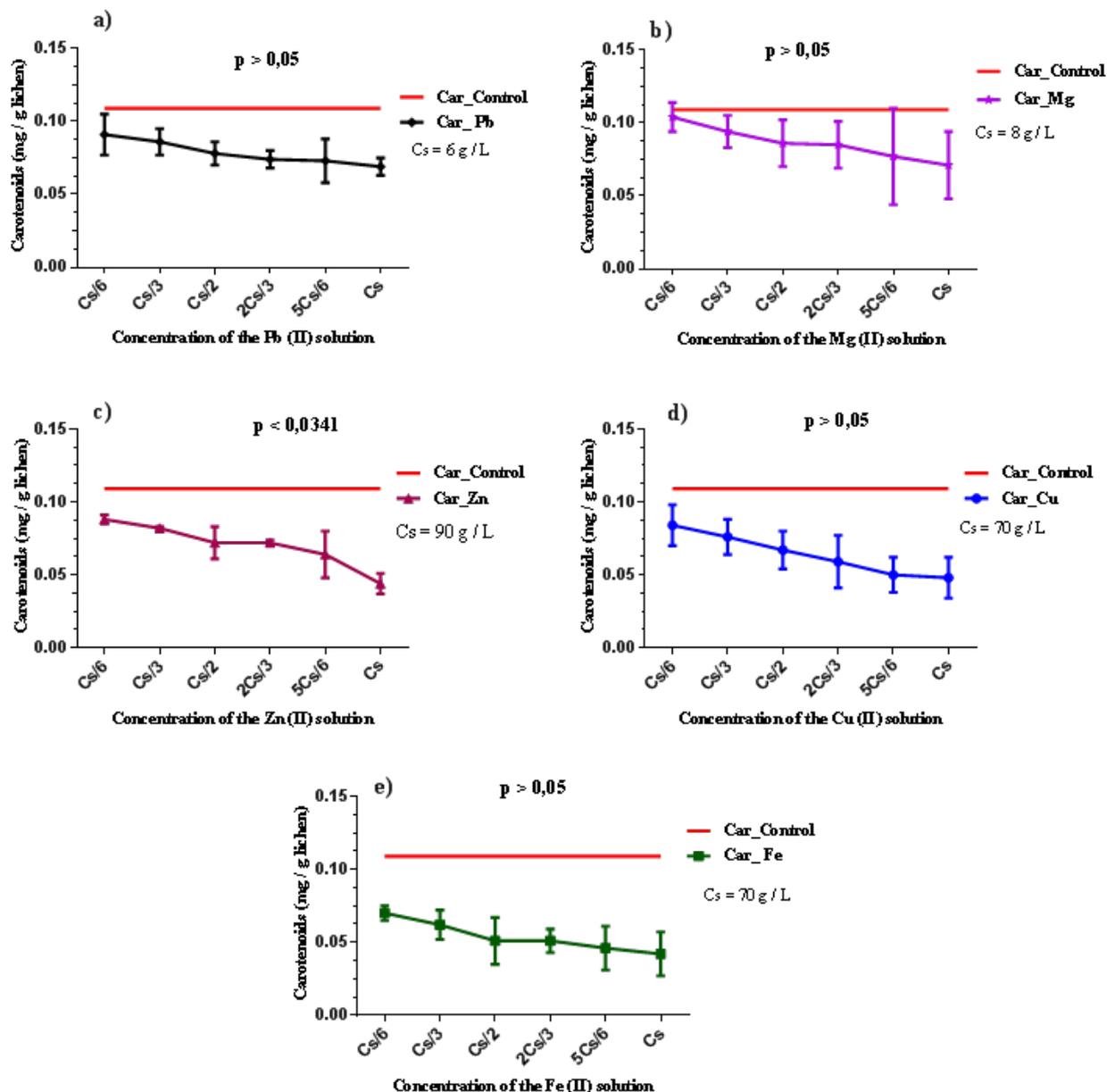


Figure 4. Carotenoids content in thalli of *Parmotrema dilatatum* after immersion in different metal salt solutions

3.4. Effect on the Membrane (MDA Production)

Membrane integrity is essential for trapping of pollutants in particular the metal trace elements. A significant degradation of the latter is likely to cause a loss of electrolytes and consequently a release bioaccumulated pollutants into the surrounding environment [15,37,38].

In this work, we evaluated the membrane through the degradation of its phospholipids and this via one of their degradation residues MDA.

The quantification of MDA provides information on the loss of membrane integrity. The modification of the permeability of the membrane can favor a more or less important release of electrolytes in solution. Relative electrolyte loss is a test commonly used to assess the impact of toxicity.

In addition, integrity measurement is essential to understand the mechanism of trapping metal and organic pollutants. The results of the MDA quantification are illustrated in Figure 5. The analysis of the curves in this figure shows that, more we tend towards the saturation concentration Cs, the greater the MDA production is. The effect of concentration on MDA production is significant for Fe. Magnesium also causes a strong degradation of phospholipids. Iron plays an important role in the production of reactive oxygen species (ROS) and mainly the hydroxide radical, considered very toxic for phospholipid fatty acids [7,39]. The magnesium is nutriment for the species but it's also very harmful to the membrane in high doses. This could be explained not only by the inductive nature of ERO production [40] but also by the slow and intracellular nature of the biosorption of this metal [41]. The lowest impacts on phospholipids are recorded with lead, zinc and copper.

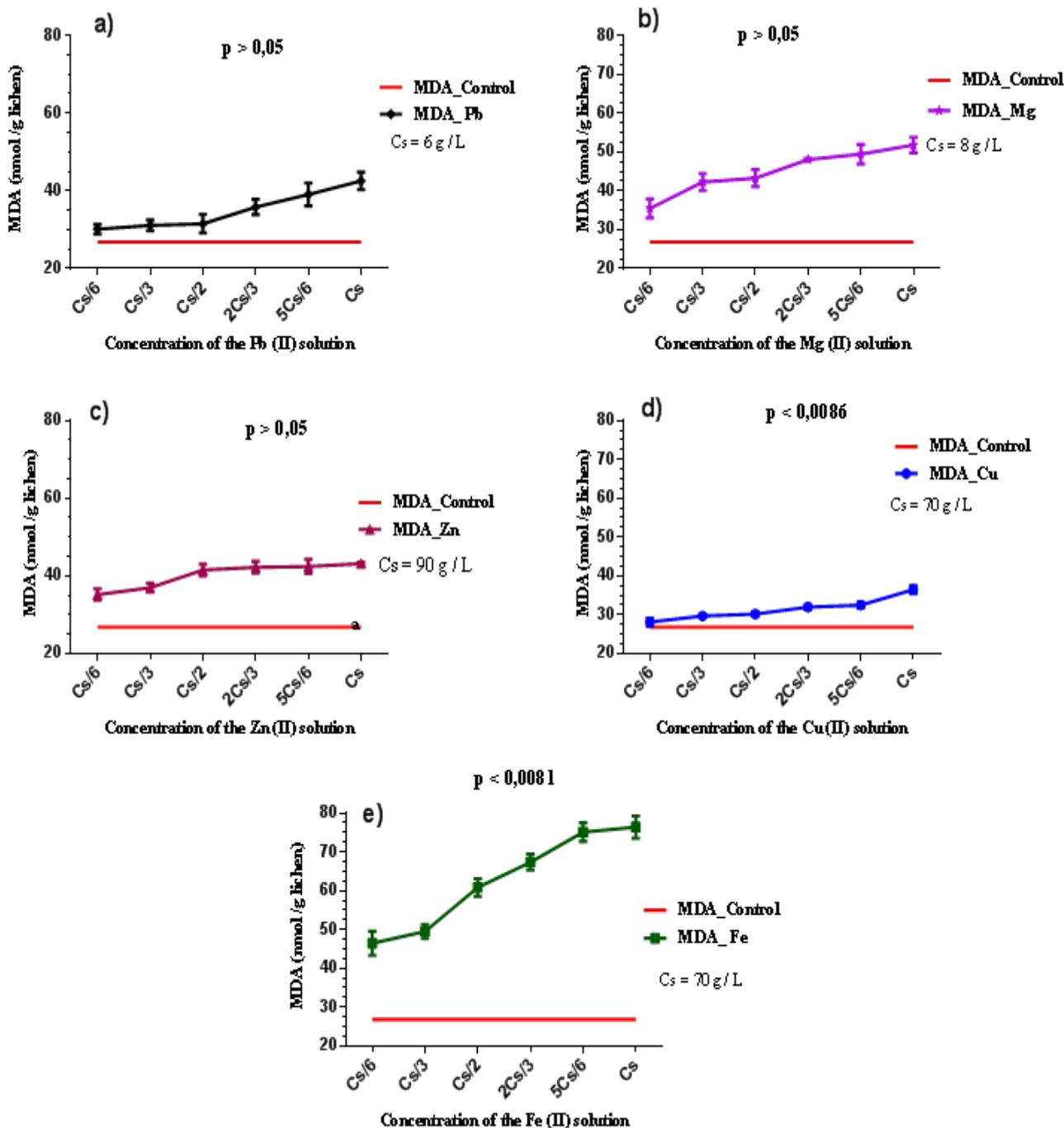


Figure 5. Content of MDA in thalli of *Parmotrema dilatatum* after immersion in different metal salt solutions

3.5. Effect on Total Polyphenols (Phe)

In this study, the effect of metal cations on total polyphenols from lichens secondary metabolism was evaluated. This result shows the decrease in total polyphenol content; this end can be translated as a response to the stress of metal cations.

The study shows that this decrease depends on the nature of the metal in solution.

Results show that total polyphenol concentrations are low for lichens immersed in iron and lead solutions. This can be justified by the different detoxification mechanisms implemented by *Parmotrema dilatatum*. Oligo-element iron has a double character: on the one hand it induce

production of reactive oxygen species (Fenton reaction, Habber Weiss reaction) and on the other hand it penetrates extra and intra cellular environments [42].

Polyphenol concentrations decrease slightly in the presence of copper, magnesium or zinc. This could be due to the intervention of these cations in other biological or enzymatic processes. Indeed, copper is involved in redox reactions in mitochondria, chloroplasts and cytoplasm or in electron transport during respiration [43,44]. Zinc is also essential in low doses to special proteins known as "zinc fingers" that bind to DNA and RNA and contribute to their regulation and stabilization [45]. It is a constituent of various enzymes, such as oxidoreductases, transferases and hydrolases [46].

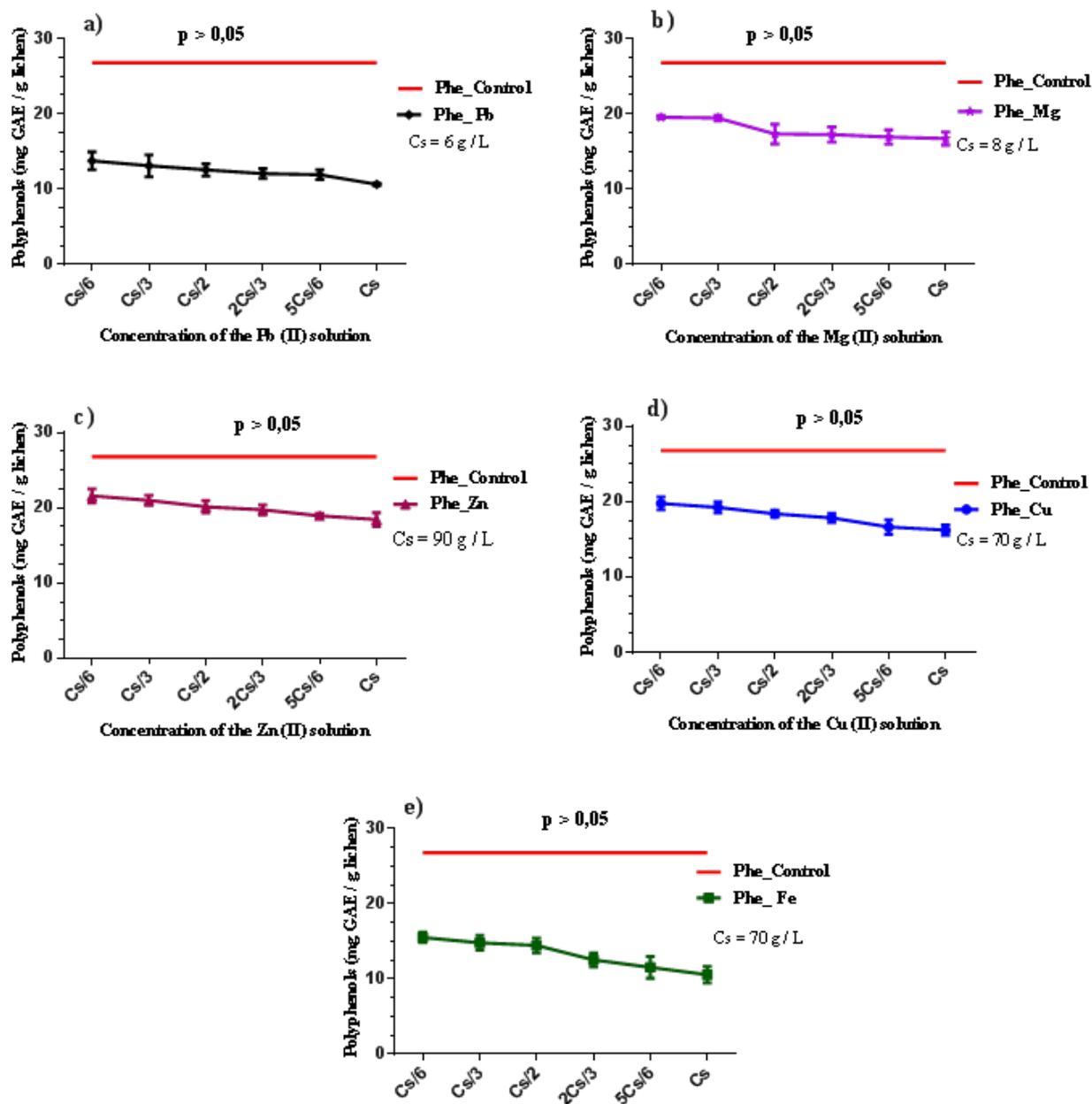


Figure 6. Content of polyphenols in the thalli of *Parmotrema dilatatum* after immersion in different metal salt solutions

3.6. Effect on Total Proteins

Results of total proteins determination in *Parmotrema dilatatum* thalls after contact with metal solutions are shown in Figure 7.

Proteins are part of the various defense systems mobilized by lichens, to cope with oxidative stress, caused by the presence of metal ions. A significant increase in total protein content could reflect a strong detoxification capacity of specie. Analysis of the Figure 6 shows that the presence of metal ions in solution causes an overproduction of proteins. Whatever the metal in solution, the total protein content increases, goes through a maximum, then decreases at high Cs fractions.

Zinc, copper and iron are metalloproteins; they act as cofactors for enzymes. Superoxide dismutases for example exist under several isoforms: Cu/Zn-SOD, Mn-SOD, Fe-SOD and Ni-SOD [47]. The cofactor is a metal or association of metals contained in the active site (hydrophobic well at the center of the protein) that traps

the superoxide anion, in the case of SOD [48,49,50]. The high levels of total protein induced by the presence of zinc and copper are probably due to the synthesis process of antioxidant enzymes. Indeed, the activity of antioxidant enzymes is also a response to the presence of phytotoxic agents [51]. In this study, the ability of phytochelatin to complex metal ions and the detoxification response of enzymatic proteins of *Parmotrema dilatatum* is significant with lead. Indeed lead is involved as an activating agent for phytochelatin [52,53,54], which would explain its role in the significant observed overproduction of protein. Finally, the evaluation of this last physiological parameter of our study indicates the strong detoxification capacity via protein of specie *Parmotrema dilatatum* in stress scenario in metal salt solution. The observed strong detoxification activity via proteins is an important parameter in environmental biomonitoring, particularly in the case of active biomonitoring (transplantation on the species' study sites) over the medium and long term.

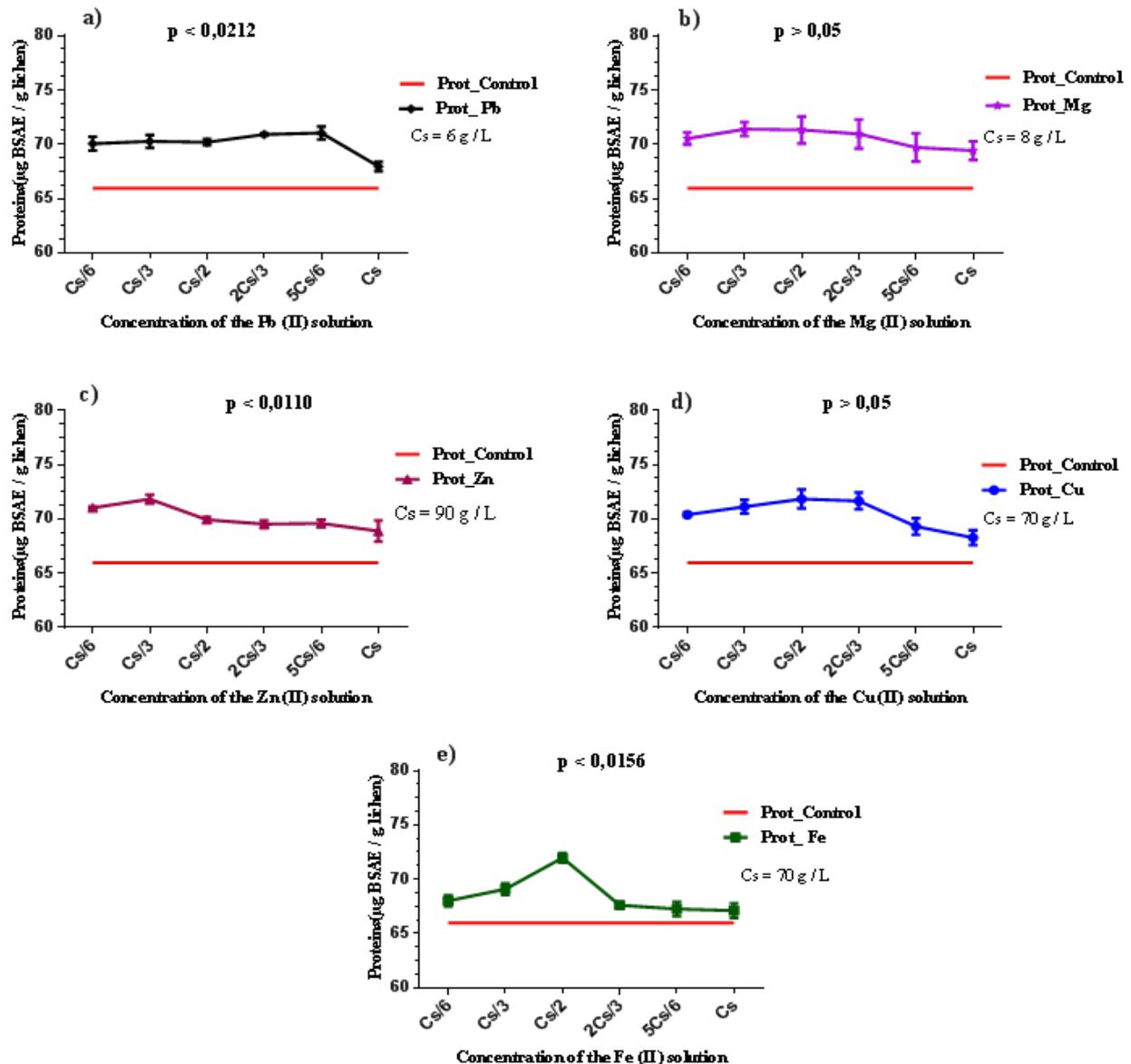


Figure 7. Content of proteins content in the thalli of *Parmotrema dilatatum* after immersion in different metal salt solutions

4. Conclusion

This study shows that the bioaccumulation of metallic elements such as Cu, Zn, Fe, Pb, Mg on lichens has a considerable effect on its physiology. Regarding photosynthetic pigments, our study shows a decrease in levels in the presence of these metal ions. Membrane degradation, decrease in total polyphenols, and overproduction of proteins evaluate the considerable toxicity of metals to lichen *Parmotrema dilatatum*.

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