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Effects of the heavy metals Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} on some physiological parameters of the lichen *Usnea amblyoclada*

Hebe A. Carreras*, María L. Pignata

Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET-UNC. Cátedra de Química General, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Avda. Vélez Sársfield 1611, X5016 GCA, Córdoba, Argentina

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Abstract

The effect of Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} on some physiological parameters of the lichen *Usnea amblyoclada* and the selective uptake of Cu^{2+} and Pb^{2+} was assessed. Fresh thalli were soaked in single or mixed metallic solutions. The concentration of chlorophylls and malondialdehyde; the dry weight/fresh weight ratio as well as the water content and the concentration of Cu, Ni, Pb, and Zn were measured in the treated and control thalli. The exposure to Cu, Ni, and Pb solutions caused several changes on the parameters measured; no differences were found with Zn. A stronger ability for binding Pb^{2+} was also observed. The results suggest that Cu^{2+} was the most harmful cation followed by Pb and Ni. Consequently, the damage observed in *U. amblyoclada* thalli when it is used as a biomonitor in polluted areas is possibly due to the presence of these heavy metals, masking the effect of other gaseous pollutants.

Keywords: Biomonitor; Air pollution; Chemical response; Selective uptake

1. Introduction

Lichens are symbiotic organisms that do not possess roots or waxy cuticles and that mainly depend on an atmospheric input of mineral nutrients. These features, combined with the extraordinary capability of lichens to grow in large geographical areas and to accumulate far more mineral elements than they actually need, place them among the best bioindicators of air pollution. As a result, many studies on heavy metal monitoring in different geographical areas have been undertaken using lichens (Bargagli et al., 1999; Scerbo et al., 1999; Loppi et al., 2002).

The accumulation of heavy metals in lichen thalli is one of the aspects of lichen biology that has been most studied. It usually depends on many factors such as morphology, ion exchange properties, type of reproduction, etc. (Gries, 1996). Therefore, the degree of tolerance to heavy metals is characteristic of each lichen species. A well-documented phenomenon is their ability to tolerate high elemental levels thanks to a number of mechanisms to avoid toxicity, although only a few have been thoughroughly studied for some elements. Although the mechanisms allowing lichens to tolerate metals are still poorly understood, many laboratory studies have established the relative toxicity of heavy metals in lichens and the most common overall toxicity sequence presented is Hg > Cu > Cd > Ag > Pb > Zn (Bačkor and Váczi, 2002).

Lichens are often used to monitor polluted areas in which huge quantities of heavy metals are suspected. However, when the concentration of metals is large enough to become toxic, they themselves cause damage to the lichen thalli. As a result, several physiological mechanisms of response to air pollutants in lichens are altered, and thus change their original sensitivity or tolerance to gaseous compounds like SO_2, NO_x, and O_3.

Usnea amblyoclada has been used as a biomonitor in previous studies in Córdoba, Argentina. Its chemical response to air pollutants has allowed the identification of areas with different levels of pollution and it has proved to be relatively tolerant to the accumulation of heavy metals (Carreras and Pignata, 2002). The main object of the present investigation was to study the effect of solutions

*Corresponding author. Fax: +54 51 4334139.
E-mail address: hcarreras@com.uncor.edu (H.A. Carreras).

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containing Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} on some physiological parameters measured in *U. amblyoclada*. Another aim was to test the selective uptake of some of these cations by soaking lichen thalli successively in Cu^{2+} and Pb^{2+} containing solutions. The results were expected to contribute to a better understanding of the tolerance demonstrated by this species when exposed to a polluted area.

2. Material and methods

2.1. Lichen material and sampling procedure

U. amblyoclada (Müll. Arg.) Zahlbr. is a fruticose green algae lichen with *Trebouxia* sp. as the photobiont. The lichen thalli were collected from Los Gigantes, an area 70 km west of Córdoba city, which has a very low level of pollution. This area was specifically chosen because the species is abundant and therefore the collection of samples causes a very low impact on the natural population density. Great care was taken to collect material under similarly exposed conditions. The basal part of the lichen thalli was detached together with the adhering pieces of rock substrate with a plastic scissor. The thalli were stored in the laboratory at 25 °C under conditions of constant humidity (60%). These thalli corresponded to the baseline material.

2.2. Sample treatment

Even though the immersion of lichens in aqueous metal solutions is not an accurate simulation of field conditions, it will help to understand the selective uptake of heavy metals and the concentration of heavy metals that cause damage to lichen thalli. This information is of great interest because it will allow to confirm whether *U. amblyoclada* can be used as a biomonitor of atmospheric contamination associated to high levels of some heavy metals previously detected in other studies.

The experiments were undertaken 2–3 days after collection. The samples were kept in a laboratory specially conditioned for analyzing trace elements. The solutions were prepared using double-deionized water and their pH values were adjusted to pH 3.5 with H_2SO_4 or HNO_3. All the reagents used were of analytical grade (Merck). In order to study the impact of different concentrations of Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} on some physiological parameters, 2 g of fresh thalli were soaked for 30 min in 100 mL 0.5, 1, 5, and 10 mM $CuSO_4$, $NiSO_4$, $Pb(NO_3)_2$ or $ZnSO_4$ solutions. Control samples of lichen thalli were soaked for 30 min in double-deionized water, the pH value were adjusted to pH 3.5 with H_2SO_4 or HNO_3. In each case, the treated thalli were then thoroughly washed twice with double-deionized water, gently shaken to remove any excess water and allowed to dry at room temperature. The same procedure was also used to assess the effect of SO_4^{2-} and NO_3^- ions, although no significant variations were observed in the parameters measured.

In the second series of experiments, the competitive uptake of Cu^{2+} and Pb^{2+} ions from solutions of different concentrations was tested. The lichen thalli were soaked for 30 min in a combination of Cu-containing solutions (0.05, 0.1, 0.5, and 1 mM) followed by Pb-containing solutions (0.05, 0.1, 0.5, and 1 mM). Control thalli were soaked in double-deionized water at pH 3.5 followed by the Pb^{2+} solutions. This combined treatment was also carried out in reverse: the lichen were soaked first in the Pb^{2+} solutions and then in the Cu^{2+} solutions or in double-deionized water followed by the Cu^{2+} solutions. The samples from the combined experiments were lifted out of the first test solution, rinsed thoroughly in double-distilled water, wiped with filter paper and then soaked again for 30 min in the next test solution. The thalli were then washed twice with double-deionized water and dried at room temperature. Finally, the concentration of Cu^{2+} and Pb^{2+} cations were measured in the lichen thalli of each treatment.

2.3. Physiological determinations

The procedures followed for the quantification of chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*), carotenoids (Carot), malondialdehyde (MDA), dry weight/fresh weight ratio (DW/FW) and the other ratios measured, were as previously described by Carreras et al. (1998) and Carreras and Pignata (2001). All the concentrations were expressed on a fresh weight basis.

Electrical conductivity (EC) is a relative measure of membrane integrity (Garty et al., 2002). Conductivity measurements were performed in lichen thalli previously incubated in a humidity chamber (80%) for 1 h. Whole undamaged lichen thalli were divided into 2 g samples and soaked in 50 mL tridistilled water for 2 h at room temperature. The EC of the water was measured by an electrical conductivity meter with a glass electrode (Oakton WD-35610) and expressed as $mS\,m^{-1}\,mL\,g^{-1}$.

2.4. Metal analysis

Lichen samples (0.5 g) were dried at 60 °C until constant weight and then ashed at 650 °C for 60 min.The ashes were digested using a 5:1 mixture of HCl (18%) and HNO_3 (conc.) at a mild temperature and the solid residue was separated by centrifugation. Finally, 10 ppm of Ge (internal standard) was added and the volume was adjusted to 50 mL with tridistilled water. Aliquots of 5 μL were taken from this solution and dried on an acrylic support. As quality control, blank samples and samples of the standard reference material "Hay IAEA-V-10" were also prepared as described above. The system was calibrated with known concentrations of standard solutions of metals with Ge as an internal standard. Samples were irradiated for 200 s using the total reflection technique (TXRF) at an X-ray fluorescence beamline of the National Synchrotron Light Laboratory (LNLS), Campinas, Brazil. A polychromatic beam of approximately 2 mm wide and 1 mm high was used for excitation. A Si (Li) detector with a resolution of 165 eV at 5.9 keV was used for X-ray detection.

2.5. Statistical analysis

In order to evaluate the concentration of metals incorporated and the effect of the different cation concentrations on the lichen thalli, the results were analyzed using one-way ANOVA. Post-hoc comparisons were obtained using the least significant difference (LSD) test. A P-value < 0.05 was considered significant.

3. Results and discussion

3.1. Incorporation of metals in the lichen thalli

Fig. 1 shows the levels of Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} incorporated by the lichen thalli after being soaked in the different metal solutions. The concentrations of Cu^{2+}, Ni^{2+}, and Pb^{2+} were significantly higher than the levels of heavy metals measured in both the baseline material and the thalli soaked in water. As no significant difference was found between baseline thalli and samples soaked in double-deionized water, only the latter was used as control samples for the statistical analysis. The incorporation rate of Cu^{2+} was similar in thalli soaked in 0.5, 1, and 5 mM solutions, but then it significantly increased with the 10 mM treatment, reaching the highest levels of incorporation. Regarding Ni^{2+}, a slight decrease was found in thalli treated with the 5 mM solution that then increased with the 10 mM solution. The rate of incorporation of Pb^{2+} was comparatively more constant, even though its final concentration in the lichen thalli was not the highest. No

significant variations were found between the original concentration of Zn^{2+} in the baseline material and its levels in thalli treated with the different metal solutions. As observed in a previous study, the levels of Zn in *U. amblyoclada* seem to be naturally high, which could be preventing the uptake of new cations. Chettri et al. (1997) observed no distinct uptake of Zn at low concentrations (10^{-10}–10^{-6} M), but at higher concentrations (10^{-5}–10^{-2} M) Zn uptake was evident in *Cladonia convoluta* and *C. rangiformis* thalli when exposed to single metal solutions.

The differences observed in metal uptake are probably due to the composition and type of cell wall binding sites, leading to a differential absorption and/or cation-exchange. Among these binding sites there are lichen substances suspected of playing a prominent role due to their strong binding ability that could also be implicated in the differential resistance of this lichen species to metal toxicity. Supporting this hypothesis, Bačkor and Fahselt (2004) found crystals of usnic acid, the main lichen substance in *U. amblyoclada*, that contained several heavy metals in lichens grown in a contaminated site. Another fact that could also be influencing the rate of uptake is the size of the different cations, which might affect their ability to reach the binding sites.

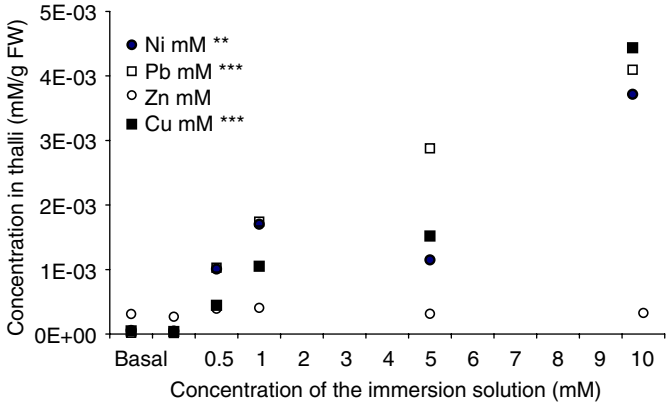

Fig. 1. Concentration of Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in baseline *U. amblyoclada* thalli and in thalli immersed in H_2O, $CuSO_4$, $NiSO_4$, $Pb(NO_3)_2$ or $ZnSO_4$ and results of the ANOVA between different concentrations of each cation (***P value <0.001; **P value <0.01; ns: nonsignificant).

3.2. Effect of Cu^{2+}

Table 1 shows the concentration of physiological parameters measured in lichens treated with the Cu^{2+} solutions. The highest concentration of Chl-*a* was found in lichens treated with water, and its level decreased as the concentration of Cu^{2+} in the solutions increased. The decrease of the Chl-*a* content is usual in plants that have been exposed to copper or other metals and has previously been observed in lichens (Bačkor and Fahselt, 2004). Branquinho et al. (1997) studied the sensitivity of different lichen species to Cu^{2+} and found that thalli of *Usnea* were the most sensitive and even with the lowest concentrations of Cu^{2+} assayed, a complete inhibition of photosystem II was observed. Moreover, Bačkor and Váczi (2002) found that high levels of intracellular Cu degrade Chl-*a*, alter the total carotenoid content and decrease the efficiency of photosystem II in cultures of the lichen photobiont *Trebouxia erici*.

It has been suggested that the Chl-*b*/Chl-*a* ratio is a useful parameter to determine the physiological conditions of lichens and photobionts subjected to air pollution and heavy metals (Chettri et al., 1998). In accordance, Kong et al. (1999) observed that Chl-*a* is more sensitive to oxidative stress than Chl-*b*. The degradation of pigments is usually the result of oxidations induced by contaminants present in the lichen thalli that generate superoxide and other free radicals, which can alter normal metabolic activities like photosynthesis. In this study, the Chl-*b*/Chl-*a* ratio was successfully used to quantify the impact of copper in *U. amblyoclada*. This parameter increased dramatically as did the concentration of Cu^{2+} in the solutions, indicating Chl-*a* degradation. Copper concentrations over 5 mM seem to be particularly toxic, as the values of the Chl-*b*/Chl-*a* ratio were significatively different from all the other treatments. The fact that copper proved to have a damaging effect on the pigment content of *U. amblyoclada* suggests that it was effectively absorbed by the photobiont cell.

The levels of MDA were significantly modified by the Cu^{2+} treatment. Higher levels of MDA were found as the

Table 1
Values of the physiological parameters (mean ± SD) measured in *U. amblyoclada* thalli immersed in H_2O or in different concentrations of $CuSO_4$ solutions and results of the ANOVA between treatments[a]

	Chl-*a* (mg g^{-1} FW)	Carot (mg g^{-1} FW)	Chl-*b*/Chl-*a*	EC (mS m^{-1} mL g^{-1} FW)	DW/FW	MDA (μmol g^{-1} FW)
H_2O	0.206 ± 0.030a	0.067 ± 0.002	0.718 ± 0.033c	2.513 ± 0.071	0.797 ± 0.012	0.188 ± 0.013d
0.5 mM $CuSO_4$	0.168 ± 0.034b	0.080 ± 0.014	0.831 ± 0.042c	3.690 ± 2.709	0.958 ± 0.018	0.211 ± 0.006c
1 mM $CuSO_4$	0.148 ± 0.014b	0.078 ± 0.016	0.874 ± 0.043c	4.563 ± 1.282	0.939 ± 0.009	0.215 ± 0.005c
5 mM $CuSO_4$	0.156 ± 0.044b	0.056 ± 0.020	1.088 ± 0.150b	4.145 ± 1.034	0.887 ± 0.109	0.264 ± 0.009b
10 mM $CuSO_4$	0.118 ± 0.014c	0.036 ± 0.002	1.196 ± 0.011a	4.319 ± 1.319	0.939 ± 0.003	0.318 ± 0.017a
ANOVA (*P*-value)	0.01	NS	0.001	NS	NS	0.000

[a]Mean values in each vertical column followed by the same letter do not differ significantly ($P<0.05$).

concentration of Cu^{2+} increased. Moreover, all treatments with Cu^{2+} had significantly higher concentrations of MDA compared to the water treatment, supporting the idea that this cation is harmful even in the lowest concentrations assayed in this study. Although copper can interfere with a number of physiological processes, the primary site of copper toxicity is probably at the cell membrane (Cabral, 2003). Chettri et al. (1998) presented evidences that the Cu^{2+} ions promote processes of peroxidation in the lipid bilayer of photosynthetic membranes and can also inhibit the mechanisms of defense that destroy peroxide ions. Turton et al. (1997) suggested that the presence of malondialdehyde in biological systems can be related to the peroxidation of unsaturated fatty acids constituting cellular membranes. The consequences of the changes in lipid and protein structure are loss of membrane integrity and selective permeability. Following membrane damage, leakage of electrolytes from the cell can occur (Cuny et al., 2002). Thus, the increase in MDA levels suggests that Cu^{2+} has a damaging effect on the cellular membranes of *U. amblyoclada*.

3.3. Effect of Ni^{2+}

Table 2 shows the physiological parameters measured in lichen thalli treated with Ni^{2+} solutions. It can be observed that the concentration of Chl-*a* decreased significantly as the concentration of Ni^{2+} in the solutions increased. The highest concentration of chlorophyll corresponded to the samples treated with H_2O while the lowest levels corresponded to thalli treated with the highest metal concentration, suggesting that Ni^{2+} has a damaging effect on chlorophyll molecules. It has previously been shown that under acid conditions the main effect of Ni^{2+} on the photobiont is found in chloroplasts and mitochondria, which explains the decrease in the functionality of the photosynthetic system (Roy-Arcand et al., 1989). Our results are also in accordance with Garty et al. (1998) who observed an inverse correlation between the integrity of the chlorophyll content and the concentration of Ni^{2+} in thalli of *R. lacera* transplanted in southeast Israel.

A direct relation was found between the Chl-*b*/Chl-*a* ratio and the concentration of Ni^{2+}, which further supports the hypothesis that Ni^{2+} has a negative effect on chlorophyll content. The lowest Chl-*b*/Chl-*a* ratio corresponded to thalli treated with the 0.5 mM Ni^{2+} solutions, indicating that this cation causes no damage at low concentrations. Significant negative effects on the chlorophyll ratio were only detected with a Ni^{2+} concentration of 10 mM, as the Chl-*b*/Chl-*a* ratio at lower concentrations (1 and 5 mM) was not significantly different compared to thalli treated with water.

Significant variations were also observed in the DW/FW ratio. The highest values corresponded to samples treated with the metal solutions and the lowest value was of the samples soaked in water. These results evidence the damaging effect of Ni^{2+}, in agreement with Chettri and Sawidis (1997) who observed that heavy metals capable of passing through the cell membrane can induce a significant water loss in lichens.

Lichens exposed to heavy metals under controlled conditions undergo a degradation of cell membranes (Hyvärinen et al., 2000, Garty et al., 2003). In agreement, significantly higher values of EC were observed in thalli treated with the highest concentration of the metal. At lower concentrations the values were not significantly different from the specimens treated with water, indicating that the integrity of cell membranes is only significantly altered by high Ni concentrations. This result is in accordance with the effect observed regarding chlorophyll degradation ratio.

3.4. Effect of Pb^{2+}

The physiological parameters quantified in lichens treated with Pb^{2+} solutions are shown in Table 3. As observed with Cu^{2+}, the concentration of Chl-*a* was significantly affected by the treatment with Pb^{2+}. The levels of Chl-*a* decreased as the concentration of the cation increased in the solutions. The lowest levels of this pigment corresponded to the highest concentration of the metal, which can be interpreted as a damaging effect of Pb^{2+} on the content of Chl-*a*. Negative effects of Pb^{2+} have also been found in other lichen species such as *Ramalina lacera* in which a decrease in the level of chlorophylls has been reported in relation to the amount of Pb accumulated in

Table 2
Values of the physiological parameters (mean ± SD) measured in *U. amblyoclada* thalli immersed in H_2O or in different concentrations of $NiSO_4$ solutions and results of the ANOVA between treatments[a]

	Chl-*a* (mg g^{-1} FW)	Carot (mg g^{-1} FW)	Chl-*b*/Chl-*a*	EC (mS m^{-1} mL g^{-1} FW)	DW/FW	MDA (μmol g^{-1} FW)
H_2O	0.206 ± 0.040a	0.067 ± 0.002	0.718 ± 0.033b	2.513 ± 0.071bc	0.797 ± 0.012b	0.188 ± 0.013
0.5 mM Ni SO_4	0.197 ± 0.044a	0.062 ± 0.019	0.245 ± 0.018c	2.136 ± 0.210c	0.868 ± 0.013a	0.151 ± 0.027
1 mM $NiSO_4$	0.106 ± 0.004b	0.055 ± 0.019	0.724 ± 0.183b	2.404 ± 0.283bc	0.889 ± 0.013a	0.157 ± 0.017
5 mM $NiSO_4$	0.101 ± 0.012b	0.065 ± 0.012	0.847 ± 0.108b	2.868 ± 0.187b	0.884 ± 0.010a	0.172 ± 0.002
10 mM $NiSO_4$	0.069 ± 0.021b	0.051 ± 0.022	1.598 ± 0.200a	3.703 ± 0.230a	0.882 ± 0.013a	0.182 ± 0.024
ANOVA (*P*-value)	0.001	NS	0.000	0.004	0.000	NS

[a]Mean values in each vertical column followed by the same letter do not differ significantly ($P<0.05$).

Table 3
Values of the physiological parameters (mean ± SD) measured in *U. amblyoclada* thalli immersed in H_2O or in different concentrations of $Pb(NO_3)_2$ solutions and results of the ANOVA between treatments[a]

	Chl-*a* (mg g^{-1} FW)	Chl-*b*/Chl-*a*	EC (mS m^{-1} mL g^{-1} FW)	DW/FW	MDA (μmol g^{-1} FW)
H_2O	0.206 ± 0.040a	0.718 ± 0.033	2.513 ± 0.071	0.79 ± 0.012c	0.188 ± 0.013
0.5 mM $Pb(NO_3)_2$	0.220 ± 0.036a	1.135 ± 0.444	3.513 ± 0.910	0.934 ± 0.010ab	0.169 ± 0.134
1 mM $Pb(NO_3)_2$	0.161 ± 0.019b	2.274 ± 0.585	3.286 ± 0.390	0.913 ± 0.014b	0.222 ± 0.019
5 mM $Pb(NO_3)_2$	0.154 ± 0.027b	1.766 ± 0.911	4.146 ± 0.553	0.904 ± 0.010b	0.194 ± 0.004
10 mM $Pb(NO_3)_2$	0.154 ± 0.049b	1.742 ± 0.695	3.562 ± 0.429	0.902 ± 0.044b	0.204 ± 0.020
ANOVA (*P*-value)	0.05	NS	NS	0.000	NS

[a]Mean values in each vertical column followed by the same letter do not differ significantly ($P < 0.05$).

Table 4
Values of the physiological parameters (mean ± SD) measured in *U. amblyoclada* thalli immersed in H_2O or in different concentrations of $ZnSO_4$ solutions and results of the ANOVA between treatments[a]

	Chl-*a* (mg g^{-1} FW)	Carot (mg g^{-1} FW)	Chl-*b*/Chl-*a*	EC (mS m^{-1} mL g^{-1} FW)	DW/FW	MDA (μmol g^{-1} FW)
H_2O	0.206 ± 0.040	0.067 ± 0.002	0.718 ± 0.033	2.513 ± 0.071	0.797 ± 0.012	0.188 ± 0.013
0.5 mM $ZnSO_4$	0.269 ± 0.013	0.110 ± 0.018	0.756 ± 0.151	2.351 ± 0.106	0.863 ± 0.108	0.207 ± 0.013
1 mM $ZnSO_4$	0.219 ± 0.054	0.061 ± 0.014	0.910 ± 0.184	2.363 ± 0.343	0.825 ± 0.019	0.194 ± 0.033
5 mM $ZnSO_4$	0.194 ± 0.016	0.068 ± 0.032	1.070 ± 0.231	2.614 ± 0.009	0.816 ± 0.013	0.227 ± 0.025
10 mM $ZnSO_4$	0.203 ± 0.011	0.096 ± 0.009	0.640 ± 0.144	2.860 ± 0.630	0.810 ± 0.020	0.213 ± 0.034
ANOVA (*P*-value)	NS	NS	NS	NS	NS	NS

[a]Mean values in each vertical column followed by the same letter do not differ significantly ($P < 0.05$).

the thalli (Garty et al., 1998). Studies undertaken in other lichen species with *Trebouxia* as a symbiont (the same symbiont as in *U. amblyoclada*), showed that Pb had no significant effect on the photosynthetic process itself (Branquinho et al., 1997). These authors showed that Pb does not act directly on the metabolism of chlorophyll pigments but that it rather alters its synthesis, causing a deficiency in metallic cations essential to the actual composition of the molecules. Thus, Pb^{2+} cations can displace extracellular cations such as Mg^{2+}, causing severe deficiencies in subsequent physiological processes. The fact that no significant difference was observed in the Chl-*b*/Chl-*a* ratio supports the hypothesis that Pb^{2+} does not act directly on the degradation of chlorophyll molecules but instead affects its synthesis.

Regarding the concentration of MDA, no significant differences were found with this metal treatment. A slight increase was observed in the DW/FW ratio of the samples treated with Pb^{2+} compared to samples treated with water, but no differences were observed among samples treated with different Pb^{2+} concentrations. This could be related with the stimulation of certain enzymes that reduce water loss. The stimulation of peroxidase has been observed under conditions of Pb toxicity in leaves of *Glycine max* (Lee et al., 1976) and *Zea mays* (Maier, 1978). The reduction of water loss due to treatment with Pb solutions was also observed in *C. convoluta* (Chettri and Sawidis, 1997).

The fact that no significant differences were observed in both the oxidation products and the Chl-*b*/Chl-*a* ratio and that only a slight increase was found in the DW/FW ratio, suggests that most of the metal cations were bound and rendered inert outside the cell wall as observed by Chettri et al. (1998) in *Cladonia* species. Furthermore, the intracellular metal ions could be captured or form insoluble precipitates and thus are not longer available for the inhibition of cellular processes.

3.5. Effect of Zn^{2+}

Table 4 shows the physiological parameters of lichens treated with different Zn^{2+} solutions. No significant differences were found in the levels of Chl-*a* nor in the Chl-*b*/Chl-*a* ratio. Brown and Beckett (1983) observed that the photosynthetic process of *Cladonia rangiformis* was unaffected by Zn concentrations up to 1 M. Also, some lichen species had been shown to accumulate large quantities of metals (Bačkor and Váczi, 2002). Similarly, the rest of the physiological parameters measured showed no significant differences after the Zn^{2+} treatment. Kauppi et al. (1998) compared the effect of Cu^{2+} and Zn^{2+} in thalli of the *Cladina* genus and reported that the influence of Zn^{2+} was noticeably inferior to that of Cu^{2+}; furthermore, no effects at all were observed in the solutions in which Zn^{2+} was combined with sulfate. These findings are in agreement with the fact that no significant difference was found in the amount of Zn^{2+} incorporated from solutions of different concentrations. Studies on the binding kinetics of Zn^{2+} ions undertaken in a lichen of the *Usnea* genus, *U. florida*, reveal that the binding of this

cation is completely reversible (Wainwright and Beckett, 1975), pH dependent and that it is competitively inhibited by the binding of protons. Wilson and Pyatt (2006) have also suggested that with zinc being an essential element in plant physiology, there should be a mechanism that limits its uptake. These observations help to explain the scarce incorporation of Zn^{2+} in thalli of *U. amblyoclada* soaked in metallic acid solutions. Previous field studies have shown that this species is capable of accumulating Zn and discriminate areas with different deposition patterns (Carreras and Pignata, 2002), suggesting that under laboratory conditions the incorporation of Zn is probably related to the concentration of protons in the metallic solution.

3.6. Uptake of Cu^{2+} and Pb^{2+} ions

In a previous study performed with *U. amblyoclada* different uptake rates were observed for some heavy metals in thalli retrieved from polluted or nonpolluted areas (Carreras et al., 2005). This evidenced the existence of a selective metal uptake, possibly related to the high concentrations of usnic acid deposited on the upper cortex of the thalli, characteristic of the genus *Usnea*. In addition, Chettri et al. (1997) observed in two lichen species that the uptake of metals from a 10 mM mixed metal solution followed the sequence Pb > Cu > Zn. Gailey and Lloyd (1993) reported that metals with a higher affinity for binding sites than divalent cations could saturate the absorption capacity of lichen thalli and thus block the incorporation of other cations. On the other hand, in many lichen species it has been observed that Pb^{2+} is mainly complexed to the fungal cell wall (Sarret et al., 1998). It is reasonable to relate these findings with the fact that no significant detrimental effects on physiological parameters were observed after the incorporation of Pb^{2+} in *U. amblyoclada*, suggesting that these ions are probably tightly bound to binding sites of the mycobiont cell wall forming stable complexes.

The data of the combined treatments with Pb^{2+} and Cu^{2+} solutions are shown in Fig. 2. The levels of Cu^{2+} incorporation in thalli treated with mixed metal solutions were not significantly different compared to thalli treated with deionized water and Cu^{2+}, except for thalli soaked in the more concentrated solutions (0.5 and 1 mM). This result could be due to the fact that at lower concentrations the Cu^{2+} and Pb^{2+} ions are still not competing for binding sites. At higher concentrations their levels of incorporation are lower than those values observed in thalli treated with the single metal solutions (Fig. 1). Similarly, Ekmekyapar et al. (2006) observed that due to the increase in the number of ions competing for the available binding sites and also because of the lack of active sites on lichens at higher metal concentrations, more metallic ions were left unadsorbed in solution at higher concentration levels. When the concentration of ions in the solution increased, the incorporation of Pb^{2+} was significantly higher than that of Cu^{2+} suggesting that Pb^{2+} cations with which the thalli were initially treated are not displaced by Cu^{2+}. These results coincide with those of other lichen species (Chettri et al., 1997) and suggest that Pb^{2+} cations can form a more stable complex in the binding sites of the cell wall thus preventing Cu^{2+} ions from binding.

In thalli soaked first in Cu^{2+} solutions and then in Pb^{2+} solutions (Fig. 3), the levels of Cu^{2+} were significantly lower than those measured in thalli soaked in single-Cu solutions of similar concentration. The highest levels of Cu^{2+} incorporation were observed in thalli treated with 0.5 and 1 mM solution. The remaining concentrations showed

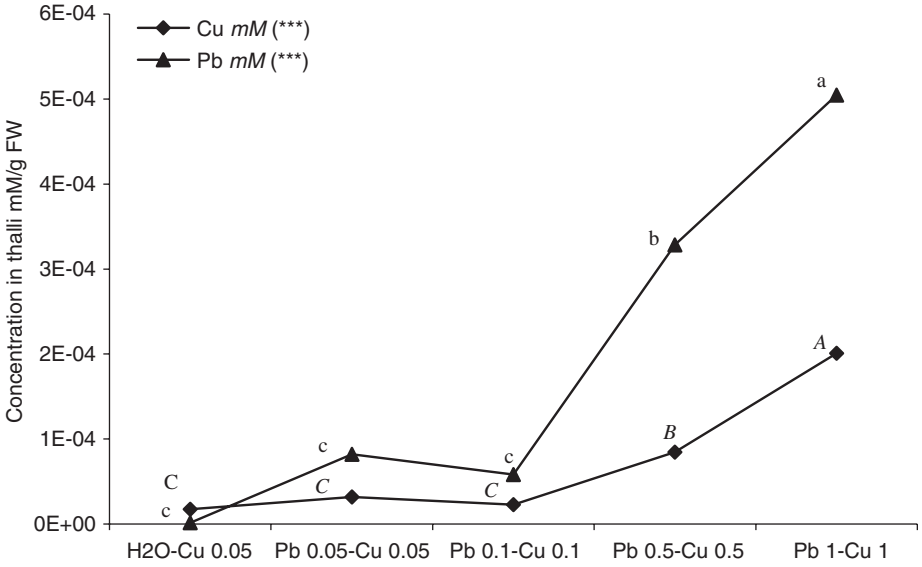

Fig. 2. Competitive uptake of Cu^{2+} and Pb^{2+} by *U. amblyoclada* thalli treated with Pb-containing solutions followed by Cu^{2+}-containing solutions and results of the ANOVA between treatments (***P value < 0.001). Values with the same letter do not differ significantly according to the LSD test ($P < 0.05$).

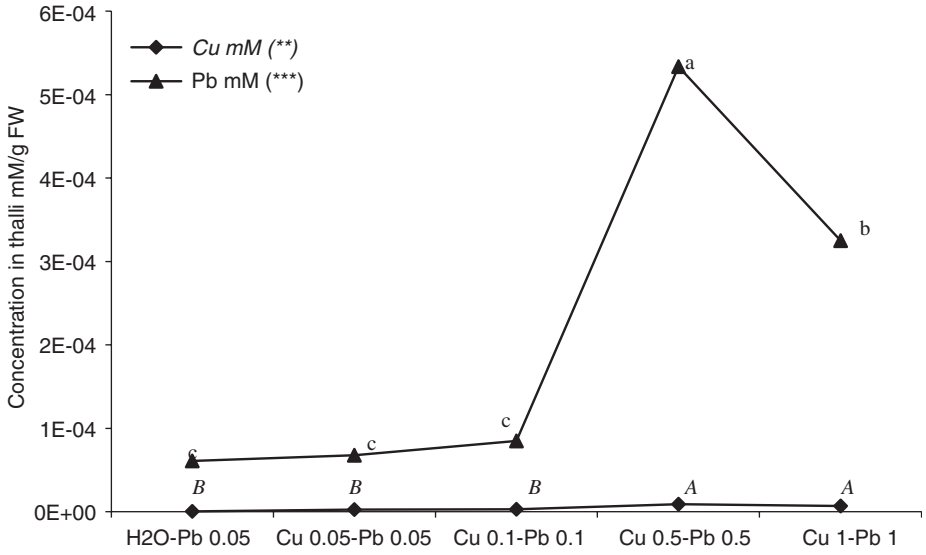

Fig. 3. Competitive uptake of Cu^{2+} and Pb^{2+} by *U. amblyoclada* thalli treated with Cu-containing solutions followed by Pb-containing solutions and results of the ANOVA between treatments (***P value <0.001; **P value <0.01). Values with the same letter do not differ significantly according to the LSD test ($P<0.05$).

no significant differences in Cu^{2+} incorporation with respect to control samples. The fact that *U. amblyoclada* thalli incorporate this element in an increasingly significant way when they are soaked in solutions that contain only Cu^{2+} suggests that these ions were incorporated straight after the initial submersion. However, they were then displaced with the treatment with Pb^{2+} solutions. Concerning the incorporation of Pb^{2+}, although the levels of incorporation were lower than values of thalli treated with the single-metal solutions, the differences were much less than those observed with Cu. No significant differences were found among thalli treated with 0.05 and 0.1 mM mixed-metal solutions and control samples. As previously observed with single-Pb solutions, significant differences of Pb^{2+} incorporation compared to control samples were only observed using treatment solutions of 0.5 mM or higher.

Our findings suggest that *U. amblyoclada* has a stronger ability of binding Pb^{2+} cations, probably as a result of its greater affinity for the lichen cell wall exchange sites and/or the extracellular complexation within the fungal cell wall. The results obtained are probably due to the competition between cations for binding sites, subsequently causing a reduction in the effective concentration of Cu at these sites. These results are in accordance with those of Sarret et al. (1998) who found a similar mechanism of resistance to metallic pollution in *Xanthoria parietina.* Furthermore, these results could partly explain the differences in metal uptake observed in *U. amblyoclada*, indicating that a lower incorporation of some metallic cations could be due to the formation of weak complexes with cell wall ligands that can consequently be easily removed and replaced by cations with a higher affinity for the cell wall binding sites.

4. Conclusions

Concerning the toxicity of the different metals studied in *U. amblyoclada*, the most harmful cation was Cu^{2+}, which probably alters the structure of the cell wall and plasma membrane of the algae causing them to loose their selective permeability. This subsequently allows Cu^{2+} cations to enter the cytoplasm, initiating a series of degenerative processes that probably cause severe alterations to different metabolic pathways such as photosynthesis or oxidative mechanisms. On the other hand, elevated concentrations of Ni^{2+} also cause alterations to the photosynthetic processes taking place in the photobiont. The scarce physiological damage caused by Pb^{2+} ions could be related to a greater affinity of this cation for the binding sites of the cell wall, as evidenced by the results obtained in the experiment of selective Cu^{2+}/Pb^{2+} incorporation. This mechanism of metal immobilization suggests that *U. amblyoclada* is fairly tolerant of high levels of environmental Pb. Finally, the scarce incorporation of Zn^{2+} can be related to the fact that there was no significant effect of this cation on the physiological parameters measured.

Regarding the effects of the cations studied on the physiological parameters measured in *U. amblyoclada*, the following sequence could explain their toxicity: $Cu^{2+} > Pb^{2+} > Ni^{2+} > Zn^{2+}$. This information is particularly important in the selection of suitable adapted lichens for biomonitoring polluted areas. The competitive mechanism of cation uptake in *U. amblyoclada* should be especially taken into consideration when this species is used as a biomonitor to estimate the concentration of some heavy metals in areas where high levels of Pb^{2+} are suspected, which could otherwise lead to an underestimation of other elements present in the environment.

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