Environmental and Sustainability Indicators 16 (2022) 100211

Contents lists available at ScienceDirect

Environmental and Sustainability Indicators

journal homepage: www.sciencedirect.com/journal/environmental-and-sustainability-indicators

Use of lichen species for air pollution biomonitoring: Case of Dar-Chichou forest (Cap-Bon, North-East Tunisia)

Sonia Dhaouadi [a,b,*], Noureddine Khalloufi [a], Khaoula Ayati [a], Nesrine Ayeb [a], Mustapha Béjaoui [a,**]

[a] University of Carthage, Faculty of Sciences of Bizerte, LR01ES14 Environmental Biomonitoring Laboratory, 7021, Jarzouna, Tunisia

[b] University of Carthage, Higher Institute of Sciences and Technologies of the Environment, Borj-Cédria, Tunisia

ARTICLE INFO

Keywords:
Air quality
Biomonitoring
MTE
Biomarkers
Lichen species

ABSTRACT

In the present work, we investigated the bio-uptake of 4 MTE (Ni, Cd, Pb, Zn) in the thalli of some species of lichens near a road crossing the Dar-Chichou forest (NE- Tunisia). In the absence of previous studies on the lichenic heritage of this forest, the objectives of this work are.

first of all to identify for the first time the lichen species present in the forest, to determine the impact of this road pollution on certain physiological parameters of these pioneer species, such as the chlorophyll *a and b* levels, the content of carotenoids, theperoxidation (H2O2) and the ipoperoxidation (MDA) and on the other hand, to show the bioindicatory and bioaccumulative importance of these lichens in front of certain trace metals (Pb, Cd, Zn and Ni). In this study, 23 lichen species with different thallus was detected. The amounts of MTE in thalli of several lichen species were determined using the flame atomic absorption spectrometry. Significant positive correlations between the production of H_2O_2 and the MDA content and on the MTE content (Ni, Pb, Zn) indicating a defense against oxidative stress. The presence of *Evernia prunastri*, *Xanthoria parietina*, *Physcia adscendens* and *Cladonia stellaris*, made it possible to classify the air quality of the Dar-Chichou Forest. *P. adscendens* present the weakest reaction against oxidative stress (H_2O_2) and seemed the most resistant compared to the two other species. This study was conducted as part of a comprehensive approach combining chemical and biological parameters to assess the effect of road traffic on air quality. It provides the first database in Tunisia referring to MTE contamination and their probable biological effects. This study fills a necessary gap in the literature.

1. Introduction

Air pollution by Metallic Trace Elements (MTE) has now become a public health problem that mobilizes the scientific community around the issue of remediation. MTEs are given special attention not only because of the risks they may pose to human health but also because of the dangers associated with their persistence on ecosystems. This form of air pollution can be studied by several methods, pertinent among which is the bioindication by lichens.

Indeed, lichens have long been defined as a permanent monitoring system for assessing air pollution (Conti and Cecchetti, 2001; Pignata et al., 2007; Conti et al., 2016). They are particularly well-suited organisms for the study of gaseous and particulate air pollutants. They owe this efficiency to their anatomical particularity (vegetative structure in the form of a thallus resulting in a very high surface/volume ratio, absence of waxy cuticle, stomata and conductive vessels, presence of a cortex rich in mucilages and often porous structure) and to their physiological characteristics. They are therefore sensitive to the fallout of pollutants present in the atmosphere (Bieri, 2017).

The response of lichens to air pollution has been studied for decades, by using their diversity, presence or absence in an ecosystem (Conti et al., 2016), but also by using their physiological responses through measurable parameters such as the production of photosynthetic pigments, H_2O_2 content, MDA and other parameters (Conti and Cecchetti, 2001). For this, the researchers adopted one of two methods. the first uses indigenous species, naturally present in the study area (Augusto et al., 2007; Blasco et al., 2008). In this case, certain species such as *Xanthoria parietina* accurately reflect the chemical composition of the air (Scerbo et al., 1999, 2002; Cuny et al., 2004). the second method uses transplanted species (Frati et al., 2007; Bergamaschi et al., 2007;

* Corresponding author. University of Carthage, Faculty of Sciences of Bizerte, LR01ES14 Environmental Biomonitoring Laboratory, 7021, Jarzouna, Tunisia.
** Corresponding author.
E-mail addresses: sndhaouadi@gmail.com (S. Dhaouadi), mustapha.bejaoui.fsb@gmail.co (M. Béjaoui).

https://doi.org/10.1016/j.indic.2022.100211
Received 11 July 2022; Received in revised form 6 October 2022; Accepted 27 October 2022
Available online 28 October 2022

Pacheco et al., 2008; Paoli et al., 2013; Demiray et al., 2012; Guttova et al., 2011; Oztetik and Cicek, 2011a). In this case, changes in the physiology and chemical composition of these lichens provide information on the concentration of inorganic and organic contaminants in the air (Demiray et al., 2012; Guttova et al., 2011; Oztetik and Cicek, 2011a; Giordano et al., 2005).

In both cases, significant negative correlations were recorded between MTE concentrations and certain physiological parameters (Nannoni et al., 2015). In our present work, the forest of Dar-Chichou in the Cap-Bon, a Tunisian place under scrutiny in this study, is an artificial forest created in 1972 (Brun, 2007), subject to intense road traffic through the C26 road that crosses it. Indeed, the daily flow of vehicles is 6472 (Anonymous, 2021).

In the absence of previous studies on the lichenic heritage of this forest, the objectives of this work are to identify for the first time the lichen species present in the forest, to determine the impact of this road pollution on certain physiological parameters of these pioneer species, and to show the bioindicatory and bioaccumulative importance of these lichens in front of certain trace metals (Pb, Cd, Zn and Ni).

This study would also make it possible to determine the quality of the forest atmosphere via bioindication. Indeed, it has been shown that the concentrations of bioaccumulated trace elements MTE in the thalli are directly correlated with those of the environment [(Anderson et al., 1978; Herzig and Urech, 1991; Sloof and Wolterbeek, 1991; Joyce et al., 1991; Herzig and Market, 1993; Bari et al., 1998; SenIsil Ezgi Eryilmaz and Ozakca, 2014); (Nannoni et al., 2015)].

2. Materials and methods

2.1. Study area and sampling sites

The forest of Dar-Chichou is an artificial forest that spreads over an area of 5000 ha. Located in the north-east of Tunisia, it covers the eastern part of the peninsula of Cap-Bon from the coast of Sidi Daoued to Oued el Gsab near Hammam Laghzaz. It is bounded to the northwest by the Mediterranean and to the east by the Sidi Abderrahmen Mountain range.

The southern boundary is formed by Jebel Korbous and Jebel Sidi Abid to the north. The climate is a Mediterranean one belonging to the sub-humid bioclimatic stage with mild winter (Abazar, 2015). At the time of its establishment, the main species that make up afforestation were in the following proportions: 29% *Pinus pinea* L, 9% *Pinus halipensis* mill, 9% Resinous mixture, 8% *Eucalyptus* sp, 15% *Acacia cyanophylla* Lindl and 30% miscellaneous. The forest is crossed by the C26 road which generates a fairly intense road traffic. The study sites (Fig. S1) were chosen on the basis of their distribution on both sides of the C26 road that crosses them.

Samples were all taken on the same day (April 21, 2019). Lichen species are harvested with a stainless steel knife and stored in plastic vials previously labelled and then brought back to the laboratory. They are then identified by observation with a binocular magnifying glass by referring to guides and determination keys based on morphological criteria (Clauzade and Roux, 1985; Tiévant, 2001; Van Haluwyn and Lerond, 1993; Van Haluwyn and Asta, 2009; Van Haluwyn et al., 2012) and on the study of colored reactions (Gavériaux, 2003).

2.2. Lichen analysis

2.2.1. Metals uptake in the lichen's thallus

The lichens were carefully washed in deionized water (Leblond, 2004). They were sorted under a binocular magnifying glass to remove all traces of foreign matter (dust, leaves, soil, wood chips) (Vitali et al., 2019). Samples taken underwent dehydration at 105 °C for 72 h, then grinding using a ball mill for 15 min at a frequency of 25 Hz until obtaining a homogeneous and very fine powder. After sieving, we add 300 mg of dry matter of the grind, a volume of 7.5 ml of nitric acid at 65% and 2.5 ml of hydrochloric acid at 37%. Mineralization is done using a digester (Kjel Digester K-446/K-449) for 20 min at 180 °C. It is finished when the solution becomes clear.

After decantation, the filtration is carried out using ash filter paper in sterile vials to obtain clearer and more transparent solutions. Concentrations of specific elements in lichen samples were determined by the flame atomic absorption spectrometry using the Varian 280 Rapid Sequential Atomic Absorption Spectrometer (Varian, Australia) for Zn and the Varian Zeeman 280 Atomic Absorption Spectrometer using the Graphite Tube 120 Atomizer (Varian, Australia) for the Pb, Cd and Ni.

2.2.2. Photosynthetic pigments (Chl a, Chl b and carotenoids) measurement

The extraction of photosynthetic pigments is done through the presence of 80% acetone according to the method of Lichtenthaler and Welburn (1983). The amount of 300 mg of fresh material (sorted under a binocular magnifying glass) is cut into thin strips in the presence of 5 ml of 80% acetone. The mixture is maintained for 72 h in total darkness and at 4 °C. The extract obtained is used for the determination of chlorophylls *a and b* as well as carotenoids. The determination of photosynthetic pigment content in thalli is done from the simultaneous measurement of the optical density at three different wave lengths: 470 nm, 646.8 nm and 663.2 nm. The pigment contents of the leaves are then determined by reference to the following formulas (Junglee et al., 2014):

$$\text{Chl } a\ (\mu\text{g ml}^{-1}) = (12.7 * \text{DO663.2}) - (2.69 * \text{D} \qquad \text{O646.8})$$

$$\text{Chl } b\ (\mu\text{g}^{-1}\text{ml}) = (22.9 * \text{DO646.8}) - (4.68 * \text{D} \qquad \text{O663.2})$$

$$\text{Carotenoids } (\mu\text{g}^{-1}\text{ml}) = (5 * \text{DO470}) + (2846 * \text{DO663.2}) - (14{,}876 * \text{DO646.8})$$

2.2.3. Hydrogen peroxide (H_2O_2) measurement

The H_2O_2 amount was determined using the method described by Junglee et al. (2014). The procedure is to grind 0.15 g of fresh material in the presence of 2 ml of trichloroacetic acid (TCA) (0.1%). The homogenate was centrifuged at 12,000 rpm for 30 min at 4 °C. Then 0.5 ml of supernatant is mixed with 0.5 ml of phosphate buffer 10 mM (PH 7) and 1 ml of KI (1M). The determination of H_2O_2 is based on measurement by spectrophotometer at 390 nm. The concentration of H_2O_2 is calculated using the extraction coefficient at 390 nm (ε = 10 mM^{-1}. Cm^{-1}) and the contents are finally expressed in μMol g^{-1} FW (Draper and Hadley, 1990).

2.2.4. Malondialdehyde MDA dosage

The MDA dosing reaction is based on the formation in an acidic and hot medium between the MDA and two TBA molecules Draper and Hadley method (Van Haluwyn and Lerond, 1986). MDA was extracted from fresh plant material (0.15g) crushed in the presence of 2 ml of TCA 0.1% (w/v). The homogenate obtained was centrifuged at 12,000 rpm for 30 min. To 1 ml of supernatant is added 1 ml of 0.5% thiobarbituric acid (TBA), prepared in 20% TCA. The mixture was incubated at 90 °C for 30 min, then cooled in ice for 10 min and centrifugated at 12,000 rpm for 10 min at 4 °C. Absorbance was read at 532 nm and MDA content was calculated using the extinction coefficient 155 mM-1. Cm-1). Lipid peroxidation was expressed in nMol MDA g^{-1} FW.

2.3. Data interpretation and statistical analysis

In order to determine the state of air quality, we used the floristic approach that uses lichen in its entirety. We have chosen, among the floristic methods, the qualitative one that is based on lichen associations (Van Haluwyn and Lerond, 1986).

The MTE measurements were compared with the Nimis and Bargagli scale (Nimis et al., 1999). It is composed of 7 classes, ranging from a natural environment ("naturalness") to a polluted environment ("alteration") (Table S1) This scale is based on data obtained over several years and on different taxa of cortical lichens including *X. parietina*.

Results are expressed of box plots as mean ± 2 standard error (2SE) and statistical analyses were performed using Statistica 8 for Windows. Structure of the data was first tested in order to assess normality (Shapiro-Wilk test) and equality of variance (Bartlett test). Variation of MTE contents and biomarkers reactions for all samples were tested using one way ANOVA. Whenever the ANOVA detected significant differences, post-hoc comparisons were made using Tukey's HSD test. A probability level of less than 0.05 was considered significant (95% confidence interval).

Principal Component Analysis (PCA): an ordination method for analyzing any table of statistical data representing n individuals described by p quantitative variables (Nimis et al., 1999; Celeux et al., 1989). PCA was performed on the mean values of all the measurements taken (H_2O_2, MDA), the levels of bioaccumulated heavy metals and the 23 lichenic species analyzed (Table 1). Data processing was performed using XLSTAT software 2020 version; after $y = \log (x + 1)$ transformation.

3. Results

Lichen surveys carried out at the six sites in the study area show an overall diversity of 23 species (appendix). Indeed, depending on the type of thallus, the species identified can be divided into 4 categories: 5 crustose species, 5 fruticose, 2 foliose and 11 squamous species.

The specific wealth recorded in the sites located north of the C26 road (Nd1, Nd2 and Nd3) is greater than that of the sites located in the south (Sd1, Sd2 and Sd3) regardless of the distance from this road. There are 9 species in Nd1 against 3 in Sd1; 12 in Nd2 against 6 in Sd2 and 12 in Nd3 against 8 in Sd3 (Table S2).

The most frequently encountered species are *Xanthoria p*arietina, *Physcia adscendens* foliaceous species often present in highly anthropized and disturbed environments, and *Cladonia stellaris* (in all study sites except Sd1).

3.1. Metals bio-uptake

The spatial distribution of the specific accumulation of MTEs (Pb, Ni, Cd and Zn) (Fig. S2) has made it possible to note that the species X.pa, P. ad and C.st are almost present in all the sites prospected and are the most accumulative. However, *Ramalina canariensis* (R.ca) shows high levels of Ni in Sd1 (83.13 μg g^{-1} DW), Sd2 (82.9 μg g^{-1} DW) and Nd2 (31.1 μg g^{-1} DW); in Pb in Sd1 (32.3 μg g^{-1} DW) and Sd3 (26.5 μg g^{-1} DW) and in Zn in Sd1 (55.8 μg g^{-1} DW) and Nd2 (31.2 μg g^{-1} DW) (Fig. S2) as for species with restricted distribution, the accumulation of MTEs is different depending on the species and the site where it is recorded. Indeed, for the species *Pertusaria leioplaca* (P.le), *Amandinea punctata* (Am.pu), *Cladonia foliacea* (C.fol) harvested only in the Nd1 site, the accumulation of metals is very low. While for *Trapelia glebulosa* (Tr.gle), Zn (98.32 μg g^{-1} DW) and Pb (98.36 μg g^{-1} DW) reach high levels.

Table 1
Codes of parameters used on PCA's.

Parameter	PCA Code
Hydrogen peroxide	H2O2
malondialdehyde	MDA
Chlorophyl a	Chl *a*
Chlorophyl	Chl *b*
Carotenoids	Carot
Nickel	Ni
Cadmium	Cd
Plomb	Pb
Zinc	Zn
Nd1, Nd2, Nd3	North-Sites of the road
Sd1, Sd2, Sd3	South-Sites of the road
Colored marks and abreviation	Species names

In the Nd2 site, A.cu, is characterized by low accumulation for the 4 metals. In Nd3, *Cladonia foliacea endiviifolia* (C.Fo.en) is the most accumulating species of Ni, Pb and Zn (respectively contents: 198.33, 158.33 and 242.9 μg g^{-1} DW) while *Cladonia firma* (C.fim) and *Cladonia ramulosa* (C.ram) are the poorest. In Sd2, *Evernia prunastri* E.pr has a very low accumulation power for all MTEs. Finally, in the site Sd3 *Cladonia ciliata* (C.ci) has a considerable accumulation power for Pb.

The species X.pa is one of the 12 recommended lichen species in the NF X43-904 standard as frequently used in bioaccumulation studies of MTEs. These species can thus be used to identify the threshold of naturalness of the ecosystem of Dar-Chichou according to the scale of Nimis and Bargagli (Nimis et al., 1999). According to this scale, the state of naturalness or deterioration in this forest differs according to the accumulated metal. Indeed, concerning the Cd, all the recorded values (0.33–0.89 μg g^{-1} DW) are below the threshold of naturalness (below the limit of class 4 "low naturality/alteration" indicated by a green line in Fig. 2). For the Pb, all the values are between the threshold of naturalness class 3 "*middle naturality*" and the threshold of alteration (class 6 "*high alteration*" indicated by a red line in Fig. 1, with the exception of the maximum value (111.283 μg g^{-1} DW) which slightly exceeds the alteration threshold. Concerning Zn, the median value is below the threshold of naturalness, but some values far exceed the threshold of alteration. Finally, all values of Ni are above the level of alteration and fit into the class 7, indicating a "*very high alteration*" by this metal.

3.2. Principal components analysis (PCA)

To better understand the relationships that exist between the levels of MTE in the different lichen species studied and their biochemical responses (photosynthetic activities and responses to stress), a principal component analysis has been necessary. In this PCA, the first two main axes PC1 and PC2, having an eigen value greater than 2 and extracting 62.15% of the global variance, are then suitable to explain the distribution of the various parameters and sites studied (Table 1) (Fig. 2).

The PC1 axis, which extracts 39.28% of the variance, makes it possible to identify on the positive side the three metals bioaccumulated (Ni, Pb, Zn) by the different species as well as the marker of oxidative stress (H_2O_2) and the marker of lipid peroxidation (MDA). This grouping on the same side of the axis is justified by positive and significant correlations of each of these metal pollution parameters (Ni, Pb, Zn) and biomarkers (H_2O_2, MDA). The PC2 axis which extracts 22.87% of the total variance makes it possible to identify on its positive pole an increasing gradient of the chlorophyll parameters (Chl *a* and Chl *b*).

This factorial plane, PC1-PC2, also makes it possible to identify the characteristic species of the different sites in relation to their bioaccumulative properties and their biochemical reactions against oxidative stress and lipid peroxidation. Indeed, the PC1 axis makes it possible to distinguish 09 species among the 23 species analyzed and to divide them into two opposite groups, the first grouping together 03 species (P. ad, C.st, C.fo.en), located on the positive side of the axis and the second, containing 06 species (P.le, C.gr, Am.pu, C.ch (*Cladonia chlorophaea*), C. ca (*Cladonia carneola*), C.ce (*Cladonia cervicornis*) on the negative side of the axis. In relation to their distributions and the relative contributions of the sites, the first group is associated with an increasing gradient of bioaccumulated Ni, Pb and Zn and a strong antioxidant (H_2O_2) and lipoperoxidase (MDA) reaction (Table 2), then that the second is rather associated with a lower bioaccumulation of metals and less efficient antioxidant and lipoperoxidase reactions. As for the axis PC2, it makes it possible to identify two groups; the first, on the positive side, contains 05 species; (R.ca, E.pr) from the southern sites and R.po (*Ramalina pollinaria*), A.cu (*Alyxoria culmigena*), R.la (*Ramalina lacera*) from the northern sites characterized by a more advanced photosynthetic capacity materialized by Chl *a* rate and higher Chl *b*. The second group, on the negative side, with 05 species (Xpa, Sp.cr, Tr.gle, C.ran (*Cladonia rangiformis*), C.ci) exhibiting the weakest photosynthetic capacities (Table 2).

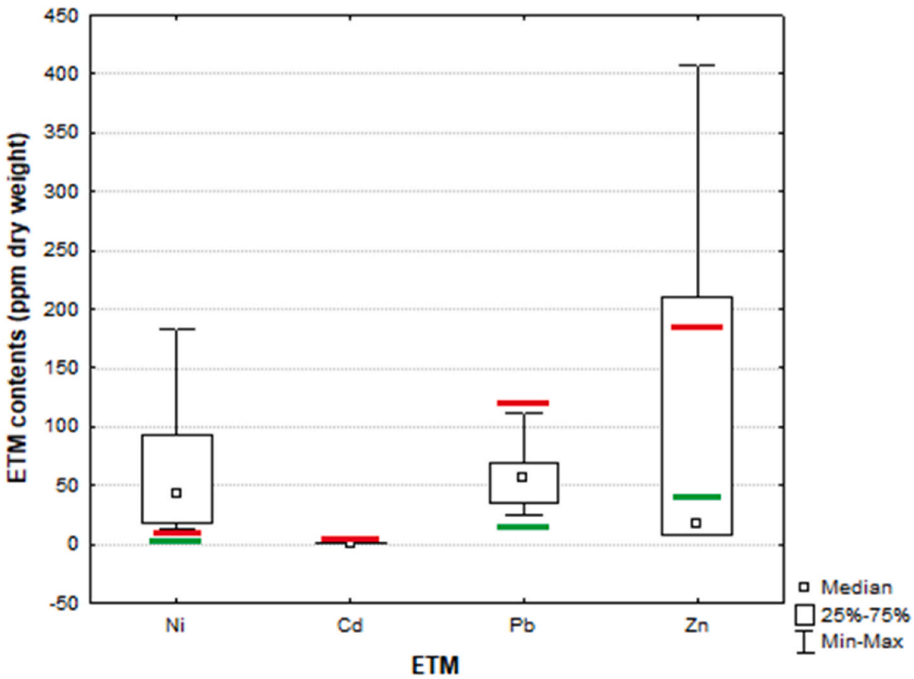

Fig. 1. Boxplot of MTE concentration in thallus of *Xanthoria parietina* in all sites prospected. (***Red line: max; Green line: min***). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

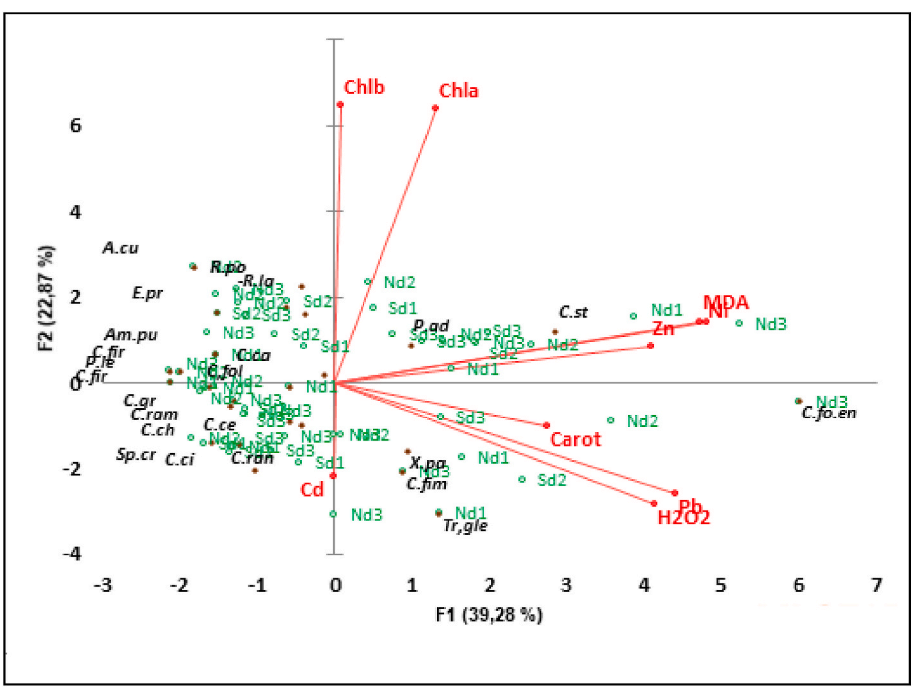

Fig. 2. Principal Component Analysis of biomarkers (H_2O_2, MDA), levels of chlorophylliens pigments (Chl *a*, Chl *b*, Carotenoids) and levels of Ni, Cd, Pb, Zn accumulated in the various lichenic species collected in Dar Chichou Forest around the C26 road at Avril 2019. X.pa: *Xanthoria parietina*, P.le: *Pertusaria leioplaca*, (C.gr) *Cladonia grayi,* (Am.pu) *Amandinea punctata,* (C.gr) *Cladonia grayi* (C.fol) *Cladonia foliacea,* (Sp.cr), *Sporodophoron cretaceum*, (P.ad) *Physcia adscendens*, (C.st) *Cladonia stellaris*, (R.la) *Ramalina lacera* (A.cu) *Alyxoria culmigena*, (C.fim) *Cladonia firma*, (C.ch) *Cladonia chlorophaea*, (R.po) *Ramalina pollinaria*, (R.ca) *Ramalina canariensis*, (C.ran) *Cladonia rangiformis*, (C.ce) *Cladonia cervicornis*, (C.fir) *Cladonia fimbriata*, (C.fo.en) *Cladonia foliacea endiviifolia*, (C.ram) *Cladonia ramulosa*, (C.ca) *Cladonia carneola*, (E.pr) *Evernia prunastri* and (C.ci) *Cladonia ciliata*.

Table 2
Coefficients in the linear combinations of variables making up PC's axes. (Hydrogene Peroxyde H_2O_2, Malondialdehyde: MDA, Chl *a*: chlorophylle a, Chl *b*: chlorophylle b and Carotenoid: Carot).

	Chl *a*	Chl *b*	Carot	H_2O_2	MDA	Ni	Cd	Pb	Zn
PC1	0,057	0000	0,246	**0563**	**0,733**	**0755**	0,000	**0634**	**0,548**
PC2	**0,776**	**0799**	0,020	0152	0,039	0039	0,090	0130	0,014

The study of the correlations between the various parameters measured for the entire lichen biocenosis of Dar-Chichou, shows significant positive correlations between, on the one hand, the production of H_2O_2 and the MDA content and on the other hand the Ni, Pb and Zn contents. The production of H_2O_2 is correlated with the production of MAD. For photosynthetic pigments, only carotenoids are positively correlated with the increase in Ni, Pb and Zn contents and it seems that Ni only among the 4 metals studied influences the synthesis of Chl *a*.

3.3. Choice of lichen species for biomonitoring

The ascending hierarchical classification of lichen species, according to their presence/absence in different sites, allowed us to gather 3 groups of species according to the Euclidean distance (see Fig. 3). This distance is 1.25 between groups 1 and 2, 1.21 between groups 1 and 3 and 1.19 between groups 2 and 3. the first group contains the species P. le, C.fir, C.fo.en, R.ca, C.gr, Am.pu, Sp.cr, Tr.gle and R.la, the second group contains E.pr, A.cu, C.ch, Xpa, R.po, P.ad, C.st and a third group contains C.ran, C.ce, C.fim, C.ram, C.ca, C.ci and C.fol.

The presence of the species X.pa, P.ad and C.st in all sites and their high bioaccumulations of metals compared to the other species (Fig. 1 additional) justify the choice of them for the remainder of the study. C.st shows the highest bioaccumulation potential for Ni, Pb and Zn while X. pa and P.ad show comparable spectra, slightly lower than that of C.st. For Cd, the 3 species have almost identical spectra and weakly accumulate this metal (Fig. 4). Tukey's HSD test reveals non-significant differences between these three species for the levels of the 4 bioaccumulated MTEs.

The P.ad species, which shows the weakest reaction against oxidative stress (H_2O_2), appears to be the most resistant compared to the other two species. This finding explains, at least in part, the lower damage to biological membranes materialized by a relatively lower level of MDA (Fig. 5). However, there are not significant differences between these three species with respect to these two reactions ($p \gg 0.5$). It is therefore concluded that a similar bioaccumulation of MTEs detected between these three species triggers similar physiological responses.

Regarding the synthesis of chlorophyll pigments (Fig. 6) there are highly significant differences between the three species ($p = 0.00018$) for chl *a* and chl *b*.

4. Discussion

Several studies have shown that lichen richness and diversity are influenced by several factors. The higher specific richness recorded in the sites located to the north of the C26 road (Nd1, Nd2 and Nd3) than that of the sites located to the south (Sd1, Sd2 and Sd3) could be related to the influence of the wind. Indeed, according to the wind rose of the region studied, the prevailing wind throughout the year is from the WNW to NW sector, especially during the spring (Abazar, 2015). Wind direction and proximity to the sea are factors that influence the distribution of lichen species (Brahimi et al., 2007). Other factors can influence this distribution, namely, tree essence, circumference, recovery and orientation of the sampling site (Paoli et al., 2006). Indeed, the four lichen groups (crustaceans, leaves, fruticose and scaly) show a disparity according to the studied sites which can be the result of all these factors. This lichen diversity can, on its own, constitute a method for evaluating (the air quality of a given ecosystem. The most used method is that of the Haluwyn and Lerond scale (Van Haluwyn and Lerond, 1986) which is based on lichenic association, and which makes it possible to distinguish 7 atmospheric quality zones ranging from A to G. In the current study, the presence of lichen association of *Evernia prunastri*, *Xanthoria parietina*, *Physcia adscendens* and *Cladonia stellaris*, allows to classify the air quality of the forest of Dar-Chichou in zone E representing the average pollution level (Van Haluwyn and Lerond, 1986).

We always base ourselves on the specific richness, we can advance that the sites of the south (Sd1, Sd2 and Sd3) are more polluted than the sites of north (Nd1, Nd2 and Nd3). Indeed, and according to several works (Paoli et al., 2006; Larsen et al., 2007; Calvelo et al., 2009), the diversity of lichens constantly decreases with the increase in stress linked to atmospheric pollution (Sujetoviene and Sliumpaite, 2013). Our results confirm those of the file as for the crustacean species known for their weak exchanges compared to the foliaceous and fruticose species. The crustacean species such as P.le, Am.pu, sp.cr, A.cu accumulate less air pollutants that the leafy and fruticose species X.pa, P.ad, C.st (Fig. S2).

In the case of rapid thallus growth, the MTE contents in the lichens may express temporary contamination and in the case of slow growth such as the case of X.pa, they may indicate chronic contamination (Daillant, 2003). Indeed, according to the scale of Nimis and Bargagli

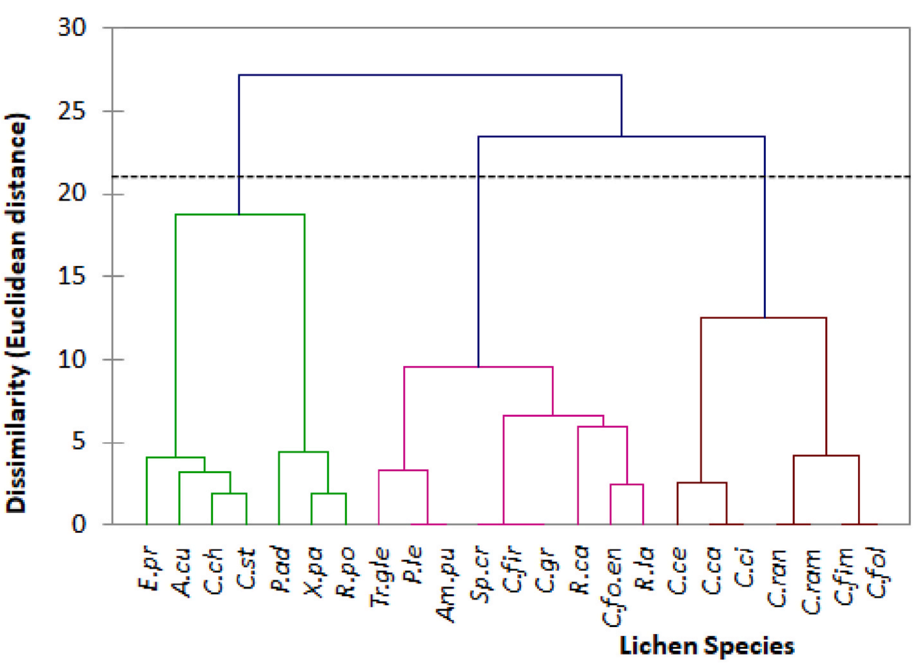

Fig. 3. Dendrogram showing groupings of lichen species according to Euclidean distance clustering. (X. pa) *Xanthoria parietina*, (P.le) *Pertusaria leioplaca*, (C. gr) *Cladonia grayi,* (Am.pu) *Amandinea punctata,* (C. gr) *Cladonia grayi* (C.fol) *Cladonia foliacea,* (Sp.cr), *Sporodophoron cretaceum*, (P.ad) *Physcia adscendens*, (C.st) *Cladonia stellaris*, (R.la) *Ramalina lacera* (A.cu) *Alyxoria culmigena*, (C.fim) *Cladonia firma*, (C.ch) *Cladonia chlorophaea*, (R.po) *Ramalina pollinaria*, (R. ca) *Ramalina canariensis*, (C.ran) *Cladonia rangiformis*, (C.ce) *Cladonia cervicornis*, (C.fir) *Cladonia fimbriata*, (C.fo.en) *Cladonia foliacea endiviifolia*, (C.ram) *Cladonia ramulosa,* (C.ca) *Cladonia carneola*, (E.pr) *Evernia prunastri* and (C.ci) *Cladonia ciliata*.

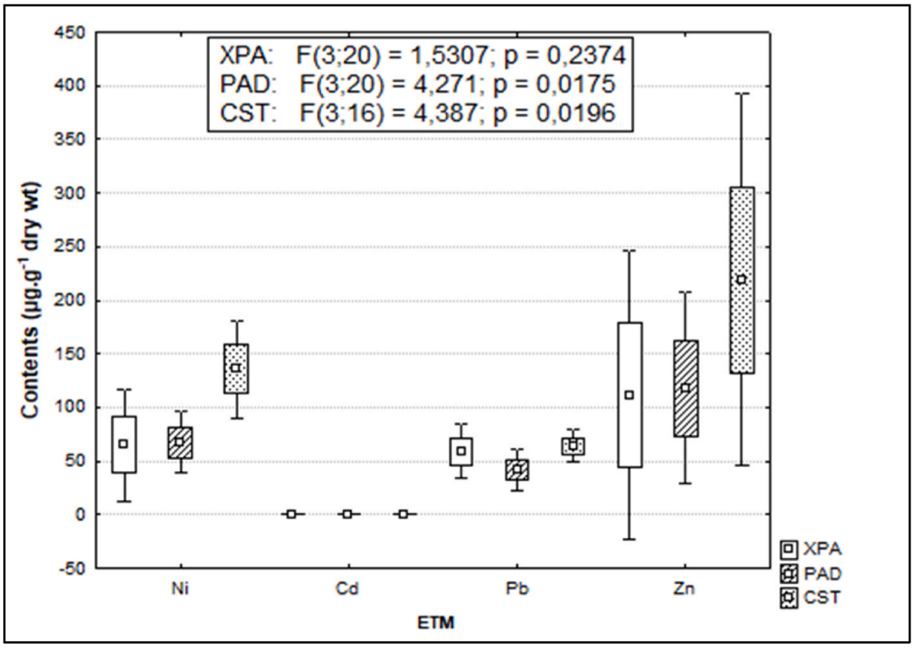

Fig. 4. Boxplots of Ni, Cd, Pb and Zn concentrations (μg.g^{-1} DW) in thallus of *Xanthoria parietina,* (XPA), *Physcia adscendens,* (PAD) and *Cladonia stellaris* (CST). Values are given as means ± 2SE.

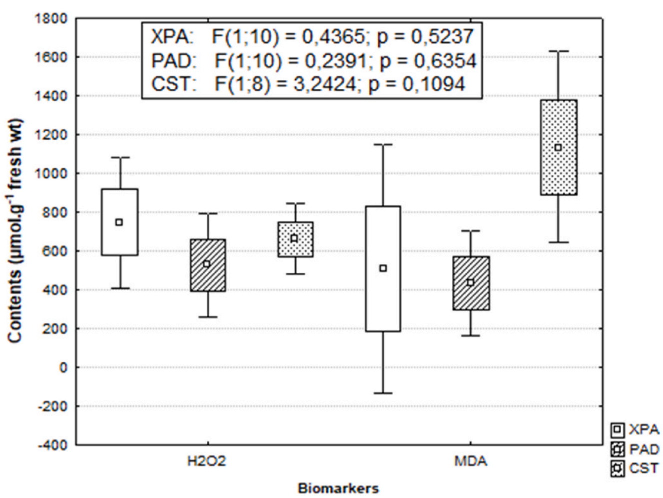

Fig. 5. Boxplots of Hydrogen Peroxide (H_2O_2) and malondialdehyde (MDA) content (μmol.g^{-1} FW) in thallus of *Xanthoria parietina,* (XPA), *Physcia adscendens,* (PAD) and *Cladonia stellaris* (CST).. Values are given as means ± 2SE.

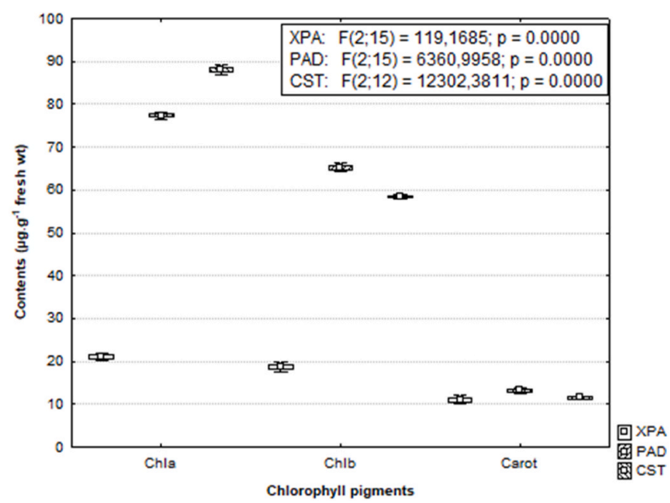

Fig. 6. Boxplots of Chl *a*, Chl *b* and Carotenoid (Carot) content (μmol.g^{-1} FW) in thallus of *Xanthoria parietina* (XPA), *Physcia adscendens* (PAD) and *Cladonia stellaris* (CST). Values are given as means ± 2SE.

(Draper and Hadley, 1990), certain values of the accumulation of Zn far exceed the threshold of alteration.

The impairment of certain physiological functions in lichens in connection with the accumulation of certain pollutants makes them good bioindicators (Nimis et al., 1999; Bouziane, 2006). Indeed, many studies establish a direct link between metallic trace elements and oxidative stress in higher plants (Cuny et al., 2002). They have shown a deleterious effect on chlorophyll pigments but also on membranes by increasing the concentration of malondialdehyde (MDA) (Nimis et al., 1999). Metal trace elements have many toxic effects. They affect the various metabolic pathways such as photosynthesis, respiration, and others (Cuny et al., 2002; Garty et al., 1985; Chettri et al., 1998). According to Brown and Beckett (Brown et al., 1985), membrane damage by trace elements is the primary cause of their toxicity.

The PCA study of the correlation between the MTE contents accumulated by the entire lichen biocenosis of Dar-Chichou and the various chemical and biochemical parameters studied (Table 2) shows differences in correlations according to the metal studied and according to the chemical and/or biochemical parameter considered.

Concerning Zn, Pb and Ni, significant positive correlations were recorded between each of these metals and the carotenoid contents on the one hand and the H_2O_2 and MDA contents on the other hand. It sems that neither the synthesis of chl *a*, nor that of chl *b* are disturbed by the contents of MTE studied exception between chl *a* and Ni. According to some authors, pollutants cause damage to photosynthesis, decrease the integrity of cell membranes (Zambrano and Nash, 2000; Piccotto et al., 2011; Paoli et al., 2011), and/or induce oxidative stress (Carreras et al., 2009; Oztetik and Cicek, 2011b). Contrary to all expectations, the levels of chlorophyll compounds are very high in some lichenic species of polluted sites compared to control lichens (Cuny et al., 2002). Von Arb and Brunold (Von Arb and Brunold, 1990) and Carreras and Pignata

(Carreras et al., 1998) showed that the concentrations of chlorophylls *a and b* were high in samples exposed to downtown pollution. This has been attributed to a fertilizing influence of pollutants (Carreras et al., 2009). Air pollutants are probably responsible for the degradation and increase of pigments at the same time (Carreras et al., 2009; Reed et al., 1989). This could explain the positive correlation between the chl *a* and the high Ni contents in the samples of this study.

The content of a pollutant in a lichen species may also depend on the type of the photobiont partner in this symbiotic association. Generally, the photobiont is more sensitive to heavy metals than the mycobiont. This sensitivity determines that of the lichen (Reed et al., 1989; Vankataraman et al., 1992). Brown and Beckett (Brown and Beckett (1983) found that Zn, Cd and Cu inhibited photosynthesis in lichens containing cyanobacterial protobionts at significantly lower concentrations than in lichens containing green algae as photobionts. In this work, it was found that the Zn tolerance was proportional to the Zn content. This could be related to some molecules produced by lichens. Indeed, a greater production of usnic acid by some lichenic species or by the same species under different conditions, is at the origin of a greater accumulation of Zn (Cuny et al., 2002). This could be the case for the species X.pa in Nd1 (408.21 μg g^{-1} DW), C.st in Nd3 (561,675,408, μg.g^{-1} DW) and P.ad in Nd3 (276.726 μg g^{-1} DW). However, this production of usnic acid is itself dependent on other factors such as road traffic, the source of some pollutants, itself influenced by topographical and meteorological characteristics (Carreras et al., 2009).

In addition, the type of substrate carrying the lichen can also be the cause of the difference in the bioaccumulation of MTEs by some lichen species. In fact, the elements transported from the substrate to the thallus, by the particle trapping mechanism, depend above all on the physicochemical characteristics of the substrate. A study carried out by Weed and Norton (1991) on liquid extracts from a lichen colonizing sandstone rock, shows the presence of oxalate-iron structures. On basalts rocks, lichens accumulate magnesium and iron (Iskandar and Syers, 1972). The disintegration of granitic rocks, rich in aluminum, shows the transformation of biotite into hydro-y-aluminum vermiculite (Pietro et al., 1994). On limestone rocks colonized by *Cladonia callopisma, Diploschistes ocellatus, Squamarina oleosa,* and *Protoblastemia testacea,* calcite has decreased considerably due to the activity of oxalic acid (Ascaso et al., 1982).

Among the physiological responses of lichens to pollutants is the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2). Under normal growth conditions, cell production of ROS is low in the chloroplast (240 μM $s^{-1}O_2^{-}$ and 0.5 μM H_2O_2) (Polle, 2001). Several stress can disrupt cell homeostasis and stimulate the production of ROS (240–720 μM s^{-1} O_2^{-} and 5–15 μM H_2O_2) (Calvelo et al., 2009). This corroborates with our results for the whole of the lichen biocenosis collected. In fact, significant positive correlations between the Pb, Ni and Zn contents and the H_2O_2 content were recorded for all the lichenic species of Dar-Chichou Forest. These ROS are capable of oxidizing various cellular compounds and can lead to oxidative destruction of the cell. However, they also act as messengers for the activation of defense systems. Faced with the increase in ROS, the cell activates these detoxification mechanisms, and it would be interesting in future work to research the contents of certain antioxidants produced in the event of metal stress (Example: catalase, …). This would make it possible to test the differential competence of different lichen species to protect themselves against this type of stress. The significant positive correlation recorded between the content of carotenoids and the level of H_2O_2 in the lichenic species studied confirms the advanced conclusions by Adams et al. (1993), which confirm that carotenoids participate in photosynthetic activity in plants and that they contribute to the protection of chlorophylls by reducing their photo-oxidation.

The primary targets of ROS are lipids, especially those present in cell and subcellular membranes. Membranes rich in polyunsaturated fatty acids (PUFA) are very sensitive to oxidation due to their high degree of unsaturation (Pamplona et al., 2002; Hulbert, 2005). Among the products formed during lipid peroxidation, malonedialdehyde (MDA), studied as a marker of lipid peroxidation. MDA, a terminal product of lipid degradation and whose content is closely related to cell membrane degradation, is an early indicator of toxic aggression and can therefore be used as a biomarker of oxidative stress (Ladhar-Chaabouni et al., 2007). Indeed, significant positive correlations were recorded between the level of H_2O_2 and the content of MDA in higher plants such as wheat. In lichens, increased MDA concentration has been shown to be an indicator of oxidative damage (Canas et al., 1997; Gonzalez and Pignata, 1994). This early biomarker of oxidative stress is correlated with the contents of some metals (Sujetoviene and Sliumpaite, 2013). Other Work have shown a direct relationship between the levels of S, Al, Pb and the MDA content in the thalli of some lichens [76].

According to the PCA and the correlation study, X.pa, C.st and P.ad are the three species which best characterize the distribution and therefore constitute the essential link in any lichen group that can be used in bioindication of Dar-Chichou. The study of these three species, allowed us in the rest of the work to compare the variation of the spatial accumulation of the studied MTE, the variation of their biochemical responses to the different contents of MTE and to look for the impact of this metallic pollution on the synthesis of some photosynthetic pigments. The wide distribution, in all the study sites, of these three species could explain their similarity of accumulation of MTEs and which would be related to their types of thalli having a large accumulation surface compared to the covered surface. Indeed, the thallus is foliaceous for X. pa and P.ad while it is fruticose for C.st (Canas et al., 1997).

In addition, the comparison of the three species concerning on the one hand the contents of Chl *a* and chl *b*, and their reactions to oxidative stress (H_2O_2) and lipoperoxidation (MDA) on the other hand allows to deduce that the three species have not the same photosynthetic physiology, nor the same biochemical reactions against contamination by certain MTEs. P.ad seems to be the most sensitive, C.st the most resistant species and X.pa takes an intermediate place between the two. C.st, having the greatest potential for bioaccumulation of MTE, seems that its resistance offers it stronger protection against the degradation of chlorophyll parameters. This is confirmed by a stronger reaction against lipoperoxidation (MDA content almost double that of the other two species), which offers it a strong protection against the degradation of the chlorophyll parameters. According to Brown (Canas et al., 1997), the multicellular and reticular form of many lichens makes it possible to trap insoluble particles containing metals, which will contribute to the analysis of total metals but will not have an immediate impact on their metabolism.

5. Conclusions

This global study combining chemical and biological parameters to assess the effect of road traffic on air quality, provided the first database in Tunisia referring to contamination by MTE and their probable biological effects. In this work, we studied the bio-uptake of 4 MTE (Ni, Cd, Pb, Zn) in the thalli of some species of lichens near a road crossing the forest of Dar-Chichou (NE-Tunisia). In this study, 23 species of lichens with different thalli were detected and the amounts of MTE in the thalli of several species were determined.

The spatial distribution of the specific accumulation of MTEs (Pb, Ni, Cd and Zn) showed that the species X.pa, P.ad and C.st are the most accumulating. However, *Cladonia firma* (C.fim), *Cladonia ramulosa* (C. ram) and *Evernia prunastri* (E.pr) are the poorest in these ETMs. These species can thus be used to identify the naturalness threshold of the Dar-Chichou ecosystem according to the scale of Nimis and Bargagli (Nimis et al., 1999). Indeed, according to this scale, all Cd values are below the naturalness threshold. For Pb, the values are between class 3 (average naturalness) and class 6 (high weathering). Concerning Zn, the median value is lower than the threshold of naturalness. Finally, for Ni, all values indicate very strong weathering.

Principal component analysis (PCA), dealing with the sampling sites and the parameters studied, makes it possible to identify on the PC1 axis a first group formed by the three bioaccumulated metals (Ni, Pb, Zn) and the stress markers oxidative (H2O2) and lipid peroxidation (MDA). On the side of the positive pole of the PC2 axis, an increasing gradient of chlorophyll parameters (Chl a and Chl b) is individualized. As for the lichen species, this PCA also made it possible to identify the characteristic species of the different sites in relation to their bioaccumulative properties and their biochemical reactions against oxidative stress and lipid peroxidation.

The ascending hierarchical classification of lichen species, according to their presence/absence in the different sites, allowed us to distinguish 3 groups of species, including the one which contains the species X.pa, P. ad and C.st noted in all the sampling sites and having more or less similar accumulation profiles of MTEs. This distinction justifies our choice to use these species as bioindicators of possible metallic air pollution from road traffic.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgment

The authors wish to thank Karim ben Hamed and Aderrezek Smaoui (LPE Borj-cédria) for their help in identifying lichen species and carrying out certain analyzes, and the anonymous reviewers for their constructive comments and the editor for his review of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.indic.2022.100211.

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