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Research Paper

Characterization of the effect of pollution induced by service stations on pollution bioindicators: epiphytic lichens

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Abstract

To limit the impacts of air pollution, it is important to estimate its deposition and effects on ecosystems and human health. We have chosen the lichen bioindication qualitative approach to evaluate the effects of the pollutants generated by gas stations on bioindicators. For this purpose, we conducted an inventory of epiphytic lichens in our study area and transplanted non-polluted samples to the same stations to assess the stress caused by this type of pollution. This inventory enabled us to identify species that are mainly toxi-tolerant. Then, in the laboratory, through histological sections and the determination of chlorophyll level carried out on the transplanted lichens, we noticed physiological and morphological disturbances due to stress gas station pollution.

Keywords: Epiphytic lichens, pollution, service stations, transplantation, stress.

Introduction

Air pollution, in particular, urban pollution has constantly increased over time, due to the evolution of human activity resulting in alteration and reckless degradation of the environment. This pollution affects natural ecosystems in the long term, and living organisms are more or less sensitive to these pollutants[1]. The harmful effects generated by automobile pollution and especially on air quality have prompted researchers around the world to develop innovative solutions for preventing and controlling this type of pollution. Noting that it has a prominent role in the alteration of the environment, its toxicity for the ecosystem is obvious and is even increasingly aggravated through the trophic chain to become dramatic when reaching humans[2].

Numerous studies now prove unambiguously that this air contamination emitted by the automotive sector has an impact on human health, mainly with the emission of some elements having proven toxicity, such as gasoline compounds[3,4]. Toxicological studies indicate that the organic volatile compounds with a light chain (benzene, toluene, ethylbenzene, and xylene (BTEX)) are the most toxic components to humans emitted by service stations[5]. These hydrocarbons are easily absorbed and can cause a wide range of harmful health effects. These effects may vary depending on several factors, including differences in intensity and duration of exposure[6], chemical and molecular properties of the gasoline, and inter-individual differences due to the variation in the pharmacodynamics of individuals[7].

Lichens are considered as sensitive organisms, due to their biological characteristics. In this regard, lichens are effective biomonitors of atmospheric deposition and bioindicators of air pollutants[8-10]. Indeed, the use of living organisms for monitoring environmental quality should be a privileged method. However, for the assessment of air quality, the bioindication of lichen remains a practical tool because these organisms are most commonly used in lower plants. They are ubiquitous and being more or less sensitive to pollutants allows us to use them for assessing the qualitative and quantitative evolution of air quality of many environments. In our work, we have been interested in pollution generated by service stations and their effects on lichen diversity. For this, we carried out an inventory of species bordering the stations to study the state of biodiversity and the level of pollution. Besides, we also brought lichens from an unpolluted area and they were transplanted in the studied stations to assess the stress caused by the stations.

Materials and methods

Presentation of the study area

The province of Annaba is located in the East of Algeria, between latitudes 36°53′59″ North and longitudes 7°46′00″ East. It is bordered to the North by the Mediterranean Sea, to the East by the province of El Tarf, to the South by the province of Guelma and to the West by the province of Skikda. It covers an area of 1411, 98 km².

The province of El Tarf is located at the extreme North-East of Algeria and is bounded to the East by Tunisia, to the North by the Mediterranean Sea, to the South by the province of Souk-Ahras and to the West by the province of Annaba and covers an area of 3339 km². The GPS coordinates of El Tarf are: 36° 46' 1.2" North 8° 19' 1.2" East.
Climatology

The climate in our study region is warm and temperate. In winter, the rains are much more important than they are in summer and the average temperature is 17.2 °C. The rainfall here averages 767 mm, the precipitation varies 130 mm between the driest and wettest months. There is a difference of 14.3 °C between the lowest and highest temperature throughout the year. The hottest month of the year is August with an average of 24.8 °C and the coldest month of the year is January with an average temperature of 10.5 °C.

Sampling Methodology

Our approach is based on taking into account only the epiphytic lichens, following a simple technique of random sampling, which consists of taking with a knife the lichens present on the trees such as the pine, the olive trees and the elm that we found in the chosen service stations. The identification was made using the flora of Ozenda and Clauzade11 as well as the lichen guide of France by Chantal Van Haluwyn et al 201312

The lichen identification was determined by using a binocular microscope. All the morphological and structural characteristics of the sample were taken into account. Thus, we used the reagents usually used in lichenology: potassium hydroxide (10% solution), bleach, Paraphenylenediamine in 2% alcoholic solution.

Transplanted species

The samples of lichens that were collected for our study belong to the genus Parmelia, notably *Flavoparmelia caperata*. Species mainly epiphytic, growing on more or less acidic and fairly well lit bark, especially on deciduous trees, rarely on conifers, it can also be found on fence posts, siliceous rocks, tiles, etc...*Flavoparmelia caperata*, is quite sensitive to SO_2, tolerant to NOx and very sensitive to ammonia resulting from industry or released by the agricultural activity.

For the collection of the samples, a healthy site was chosen because it was not under any effect of anthropic pollution and it was the “Bougous forest” included in the El Kala National Park. For more assurance, we have taken the samples from trees also sheltering the species *Lobaria pulmonaria* whose it’s presence reflects a total absence of pollution[13,14].

Transplanting method

The "lichen transplant" technique developed by Brodo, 196115 involves taking samples from an uncontaminated reference station and transplanting them the study site. It is important that the lichens

must be less disturbed as possible during sampling and for this reason; the lichens are usually collected with their support (bark fragment or branch).
For our case, we have chosen to transplant branches covered with thallus that we fixed with a string at a height of 1.5 meter from the ground on various supports. The transplanting took place on 01/01/2019 in nine sites and for 03 months. We have chosen the sites where we placed the transplants, in the eight chosen service stations located near the national roads in the regions of El Tarf and Annaba (Table 1).
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Table 1: Description of transplant stations

Station	Storage capacity of tanks / Liter	Refilling frequency	The position in the gasoline station
Control station : Bougous forest	/	/	/
Site 1: "Ain Assel", a commune of the province of El Tarf,	85000	once a day	in the trees of the gasoline stations fences
Site 2: "Ain Charchar", is a commune located between the provinces of Annaba and Skikda, 21km from Fetzara Lake,	150000	once every three days	in the trees of the gasoline stations fences
Site 3: "Bouhamra" is a locality located in the commune of El Bouni in the north of the province of Annaba,	120000	once a day	in the trees of the gasoline stations fences
Site 4: "Berrahal" is located 30 km west of the province of Annaba,	38000	once a day	in the trees of the gasoline stations fences
Site 5: "Boutella Abdallah" is a locality in the commune of Ain Assel located in the North-East of the province of El Tarf.	66000	once every three days	in the trees of the gasoline stations fences
Site 6: "Sidi Kassi" is a commune of El-Tarf province, located 40 km from the city of El Tarf,	43000	once every two days	in the trees of the gasoline stations fences
Site 7: "Sidi Nacer" is a locality located between the provinces of Skikda and Annaba.	95000	once a day	in the trees of the gasoline stations fences
Site 8: "Lac des Oiseaux" is a commune of the province of El Tarf.	75000	once every three days	in the trees of the gasoline stations fences

Binocular and histological observations

The morphological study according to the method of Ozenda and Clauzade 1970 [13] was carried out by observing the different parts of the thallus under the binocular microscope (binocular Leica), and then the histological sections of the thallus were observed under the microscope at magnification (20×10) and (40×10) (ZEISS inverted microscope).

Chlorophyll dosage technique

We used the method established by Arnon, 1949[16] for the extraction of chlorophyll. One gram of the thallus sample was finely cut and gently mixed with a mortar. A quantity of 20ml of 80% acetone and 0,5mg $MgCO_3$ powder was added to this homogenized thallus and the materials were further grind gently. The sample was centrifuged at 500 rpm for 5 minutes and the supernatant was transferred to 100ml volumetric flask. The final volume was made up to 100ml with addition of 80% acetone. The absorbance of the solution was estimated by a spectrophotometer using 645nm and 663 nm wavelength. The measurements of the chlorophyll pigments (*a, b* and *a+b*) were calculated with the formula proposed by Lichtenthaler and Welburn, 1985[17].

Statistical analysis
The results of chlorophyll contents were expressed as mean ± standard deviation, three repetitions were performed (n = 3). Multigroup comparisons of the means were carried out by the analysis one way of variance (ANOVA) test followed by Student's t-test and Dunnett's test which were used to compare between the means of each transplant groups and control group. The statistical significance for all tests was set at $p<0.05$.

Results and discussion

1- Inventory of species bordering the stations
We conducted an inventory of lichens in the respective service stations, and only epiphytic lichens were taken into consideration. After sampling, we were able to identify 07 different species of lichens within all stations (Figure 01), which are:

a. Xanthoria parietina
Cosmopolitan foliose lichen present on all acidic substrates, bark, wood, tiles, rocks...etc. Very frequent species and more or less tolerant to nitrogen pollution. According to Bargagli 1989[18], *Xanthoria parietina* is a xerophytic lichen and can stand the driest climate of urban areas.

b. Lecanora conizaeoides
It is a ubiquitous crustose species of the Lecanoraceae family, its thallus is thick granular-soredate, greyish green, the small buff-brown or green-brown apothecia with sorediate margins. It has a greyish green or yellowish green colour if wet and greyish a little greenish if dry. Apothecia dispersed all over the thallus with single spores very largely elliptic.
It is also a nitrophilous species, very tolerant to the acidity of the support, which used to be abundant in cities on all kinds of acidic substrates due to the abundance of sulphur dioxide [19]. The presence of this species in service stations is due to sulphur dioxide emissions, which are produced by the combustion of fossil fuels such as diesel.

c. *Parmelia sulcata*
A ubiquitous species of the family Parmeliaceae, rosette-forming, apices truncated and incised, upper surface conspicuously embossed by an incomplete network of whitish pseudocyphella, grey-bluish, grey-greenish, pale grey when dry, lobe apices sometimes brownish, greenish, green-bluish when moist, lower surface blackish with rhizines, simple or forked. Often sterile or apothecia scarce and scattered, markedly sessile. little or no nitrophilous and relatively toxi-tolerant. It is often used for its accumulative properties of metals, radioactive isotopes and organic pollutants[20].

d. *Pertusaria amara*
A ubiquitous species of the family Pertusariaceae, it has a thick thallus that is generally well delimited but not zoned by a hypothallus, can cover large areas on the trunks and is very visible from a far distance. Surface with usually abundant, rounded or coalescing, white soralia with a bitter taste. It is acidophilus and nitrophilous, quite toxi-tolerant and present in more or less humid atmospheres.

e. Lecidella elaeochroma
A ubiquitous crustose species of the Lecanoraceae family, it has a thallus Inlaid in the tree trunk, bordered by a narrow blackish hypothalline line. Its apothecia are disc-shaped, visible and all black of about 1mm long. It is relatively resistant to pollution, and it is found in an environment where air pollution is quite high.

f. Phaeophyscia orbicularis
Cosmopolitan foliose species belonging to the Physciaceae family, it is characterized by a thallus with a small rosette shape, light to dark or brownish grey, green when wet, with very rare apothecia. Initially urn-shaped and later sessile. It is a mainly Mediterranean species, it has marginal soralia and is ubiquitous nitrophilous and not very toxi-tolerant[21].

g. *Physcia adscendens*

Ubiquitous species of the Physciaceae family, greyish-greenish, usually has a thallus with straightened ends, the lobes are inflated and hood-shaped, containing cream to yellow pale soredia, its lobes are bordered by long marginal lashes and have rare apothecia. It is an epiphitic lichen, sometimes present on artificial substrates (concrete, tombstones...) and always present in nitrophilous communities.

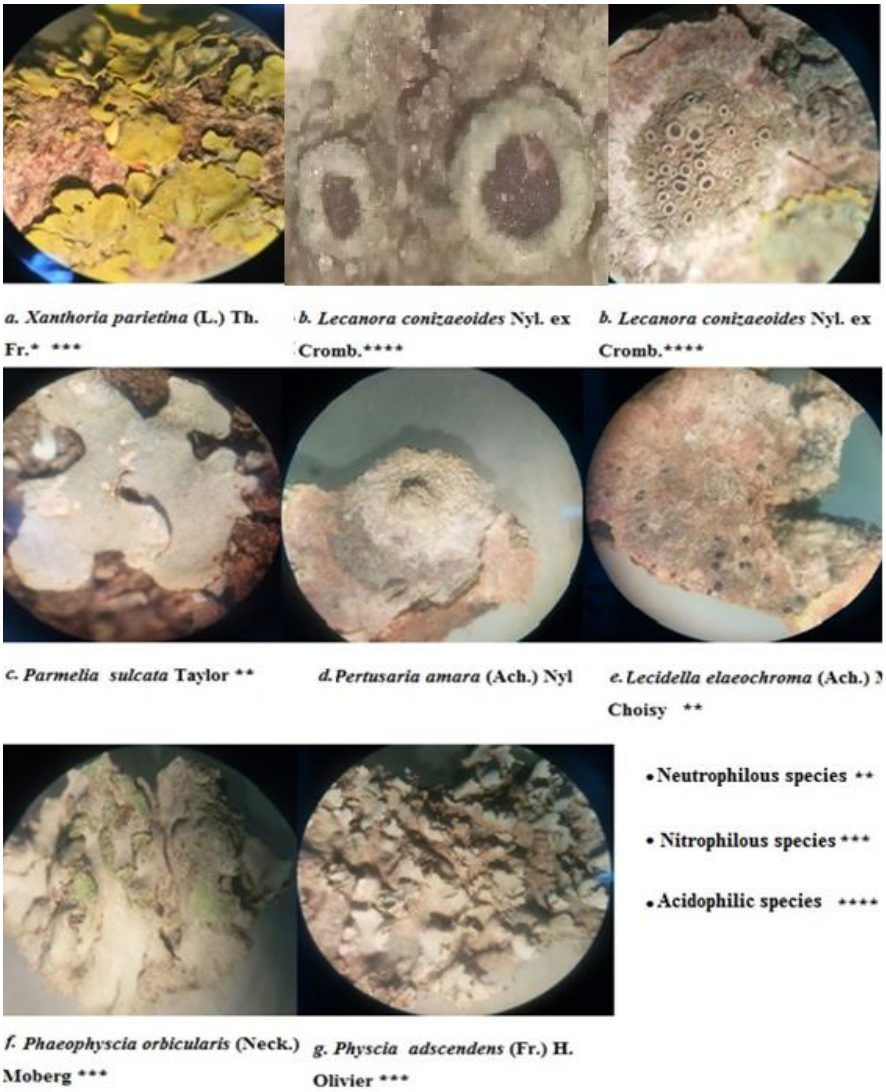


Figure 1: Lichens inventoried from different service stations

Results of the histological sections:

1. Control station: Bougous forest

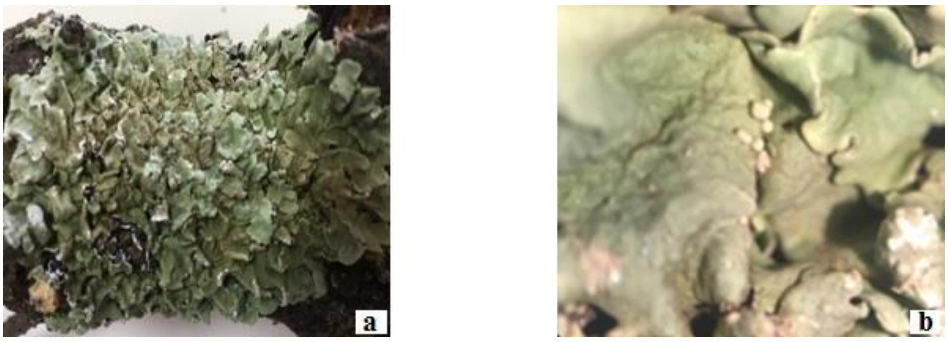


Figure 2: Bougous forest transplant, (a): direct observation of the transplant; (b): Observation with magnification x20)

Foliose thallus is generally large, yellow-green when wet, easy to detach from its support and its centre tends to detach naturally over time. The upper surface is more or less irregularly wrinkled. The underside is black except at the edges of the lobes where it is light brown over a few millimetres (Figure 02).

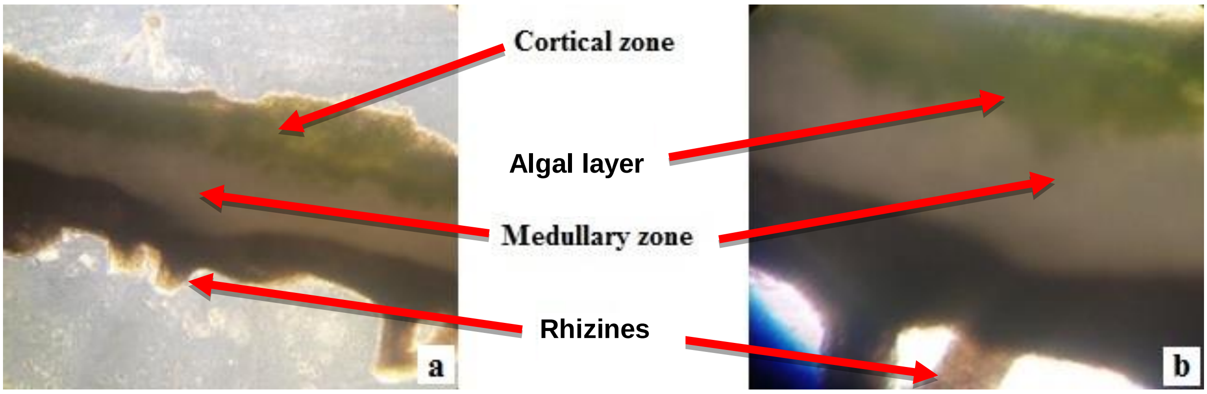


Figure 3: Microscopic observation of the lichen control; (a): histological section with magnification x200; (b): histological section with magnificationx400

By observing the histological sections of the control lichens (Figure 03), we can distinguish colourless hyphae of the fungus at the periphery forming a dense network of intertwined filaments constituting a protective layer called “the cortical zone”.

Towards the interior, we still see hyphae not tangled, but surrounding the greenish cells of the algae. Below this layer is the medullary zone formed exclusively of hyphae tissue. Underneath is a cortical zone that, together with filaments from the medullary zone forms false roots called rhizines positioned in the centre of the thallus.

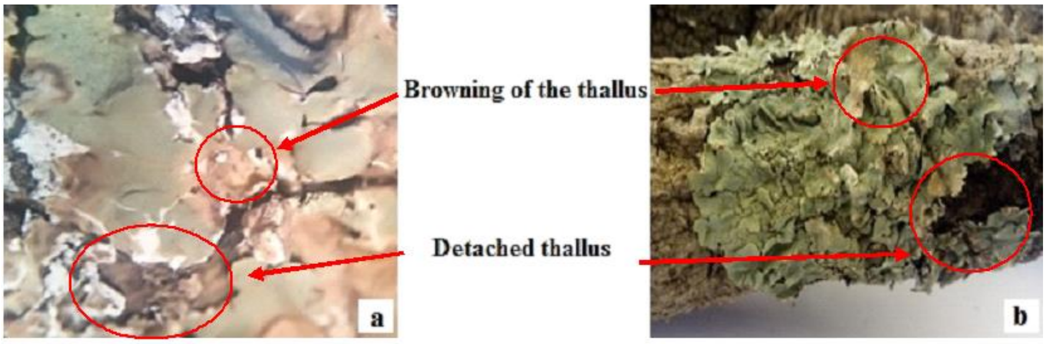


Figure 4: Ain Assal transplant (a) Observation with magnification x 20 (b) direct observation

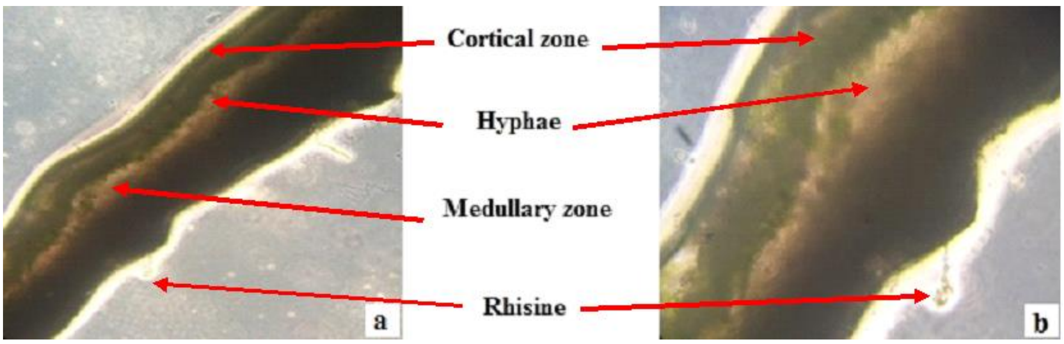


Figure 5: Microscopic observation of the Ain Assal transplant (a) Histological section with magnification x 200 (b) Histological section with magnification x 400

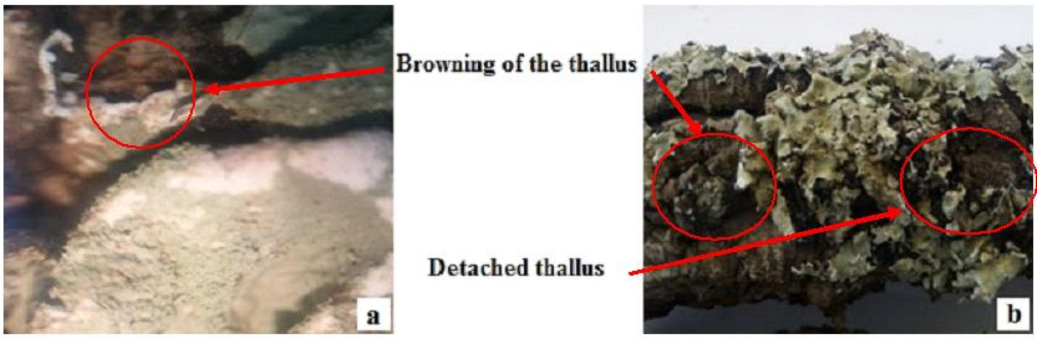


Figure 6: Ain Charchar station transplants (a) Observation with x 20 (b) direct observation

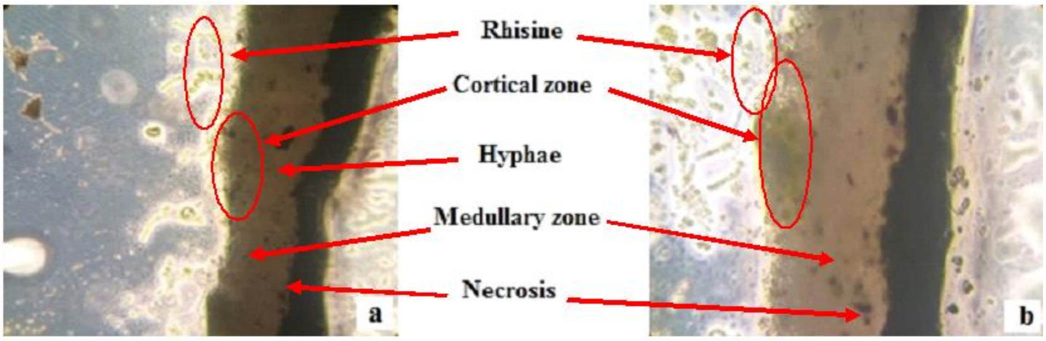


Figure 7: Microscopic observation of Ain Charchar station transplant (a) Histological section with magnification x 200 (b) Histological section with magnification x 400

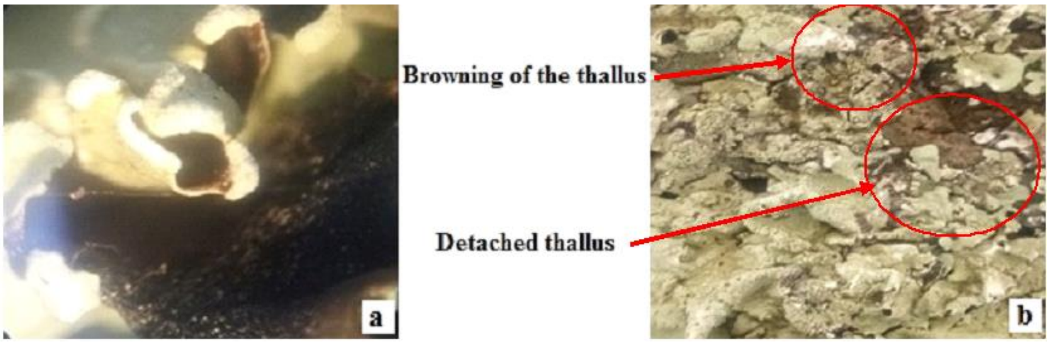


Figure 8: Berrahal station transplants (a) Observation with magnification x 20 (b) direct observation

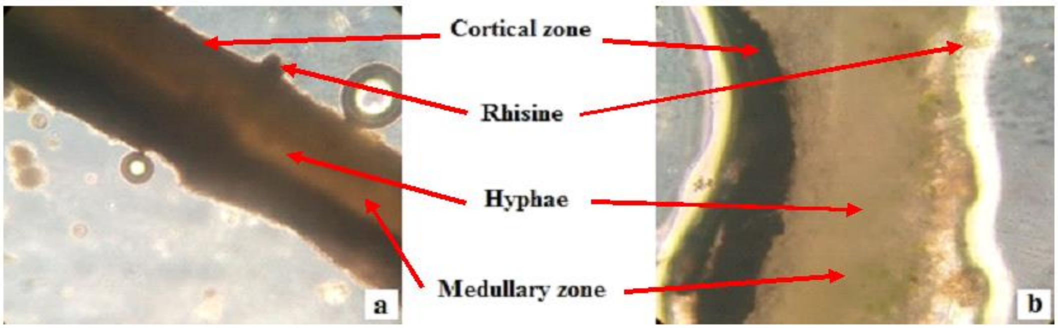


Figure 9: Microscopic observation of Berrahal station transplant (a) histological section with magnification x200 (b) histologocal section with magnification x400

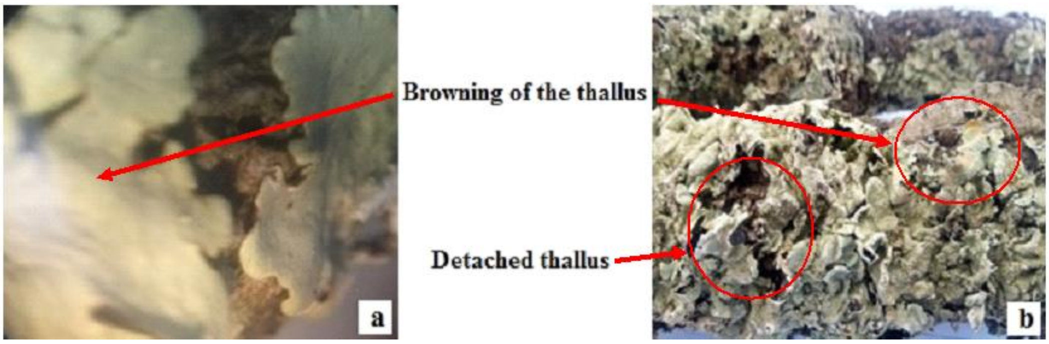


Figure 10: Bouhamra station transplant (a) Observation with magnification x 20 (b) direct observation

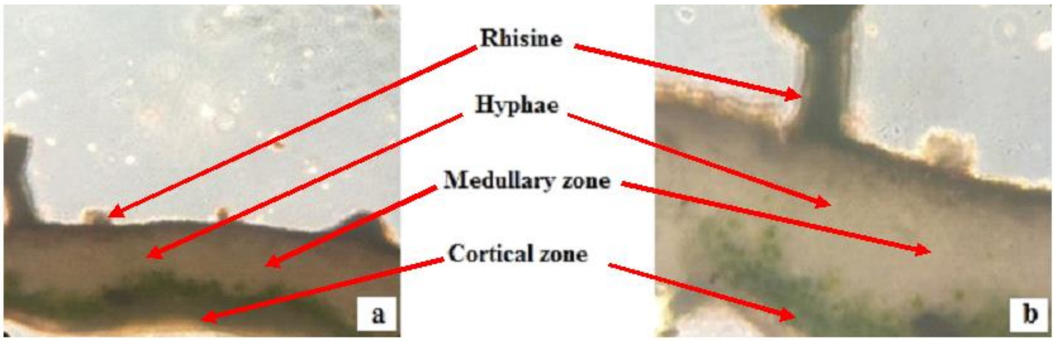


Figure 11: Microscopic observation of Bouhamra station transplant (a) histological section with magnification x200 (b) histological section with magnification x400

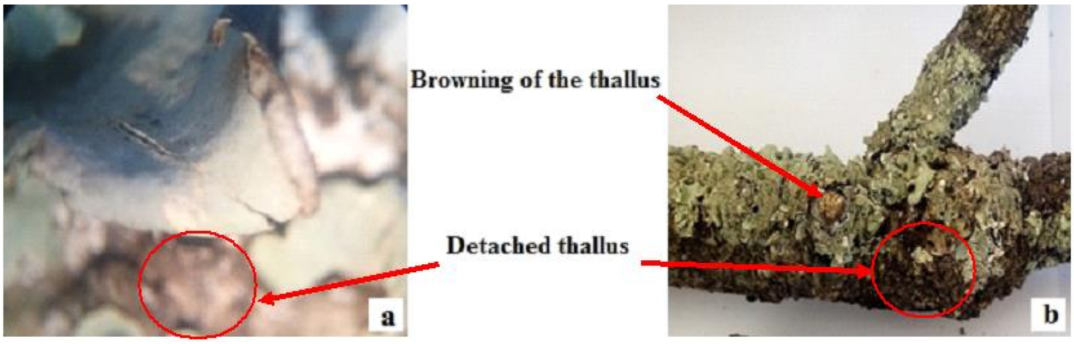


Figure 12: Boutella Abdallah station transplant (a) Observation with magnification x20 (b) direct observation

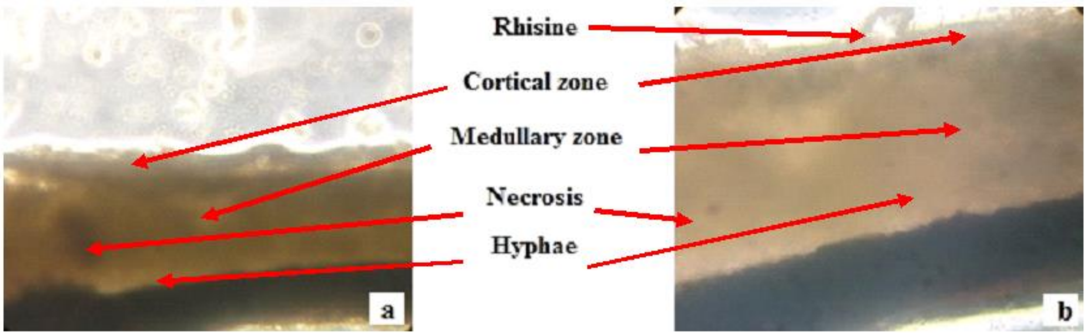


Figure 13: Microscopic observation of Boutella Ablallah station transplant (a) histological section with magnification x200 (b) histological section with magnification x400

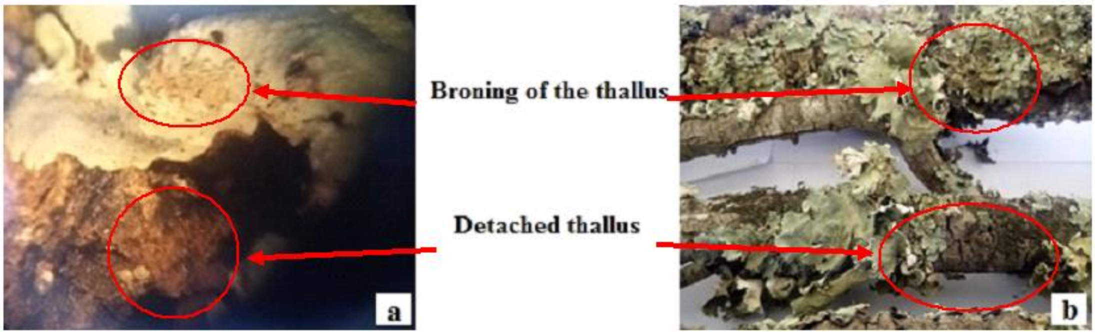


Figure 14: Lac des oiseaux transplant (a) Observation with magnification x20 (b) direct observation

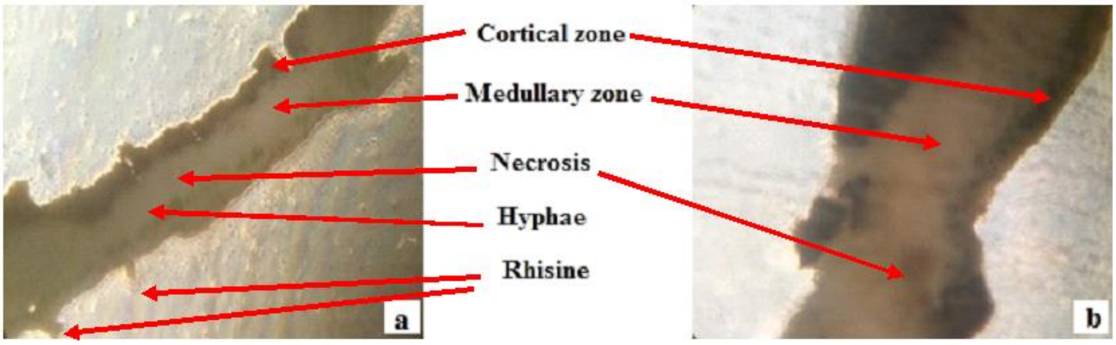


Figure 15: Microscopic observation of Lac des Oiseaux station transplant (a)histological section with magnification x200 (b) histological section with magnification x400

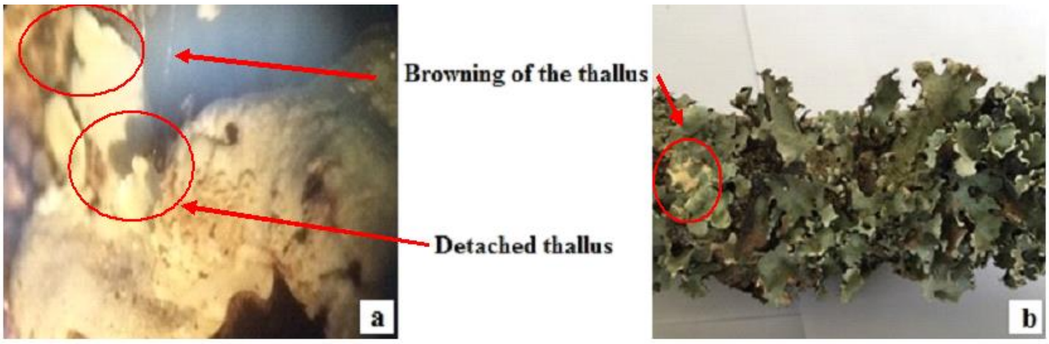


Figure 16: Sidi Kassi station transplant (a) Observation with magnification x 20 (b) direct observation

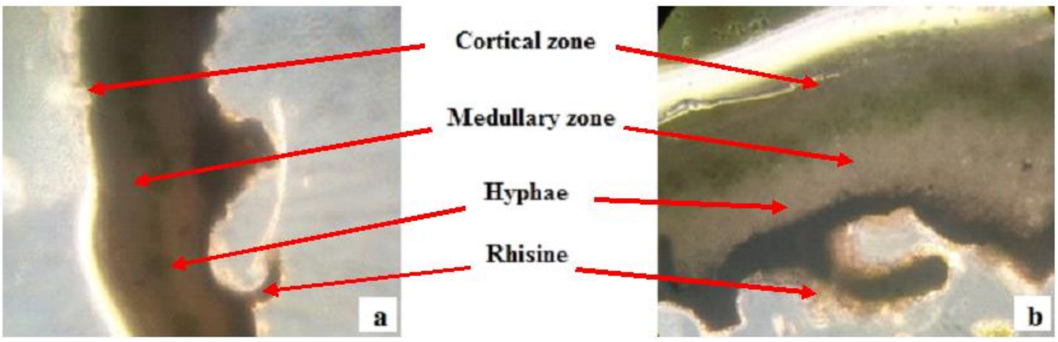


Figure 17: Microscopic observation of Sidi Kassi station transplant (a) histological section with magnification x200 (b) histological section with magnification x400

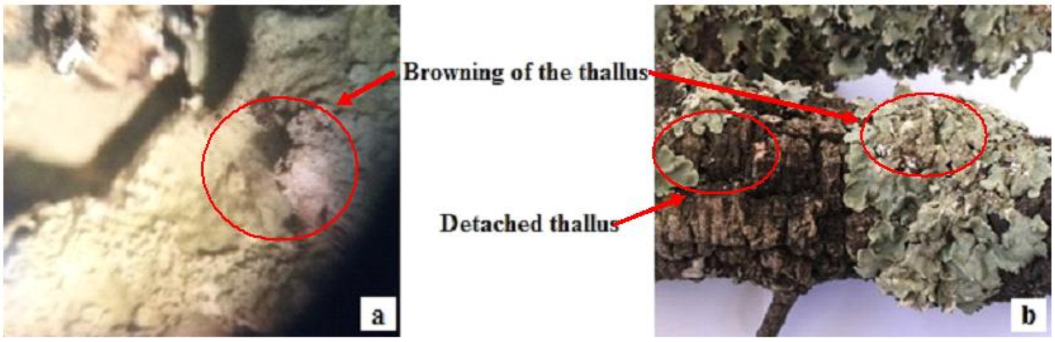


Figure 18: Sidi Nacer station transplant (a) Observation with magnifivation x20 (b) direct observation

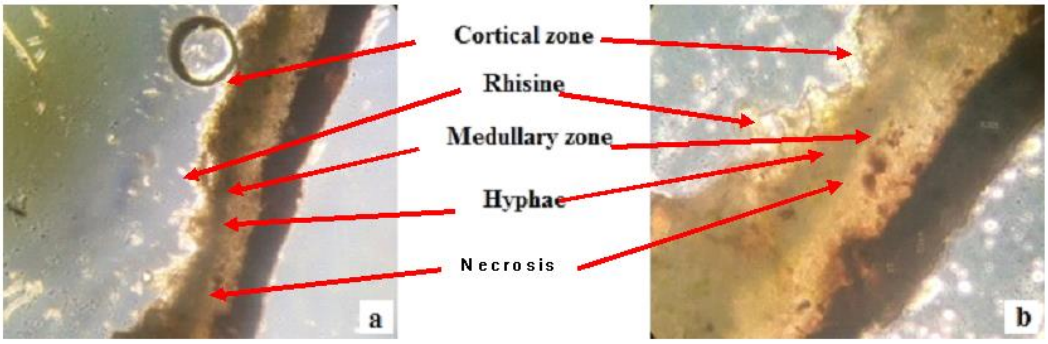


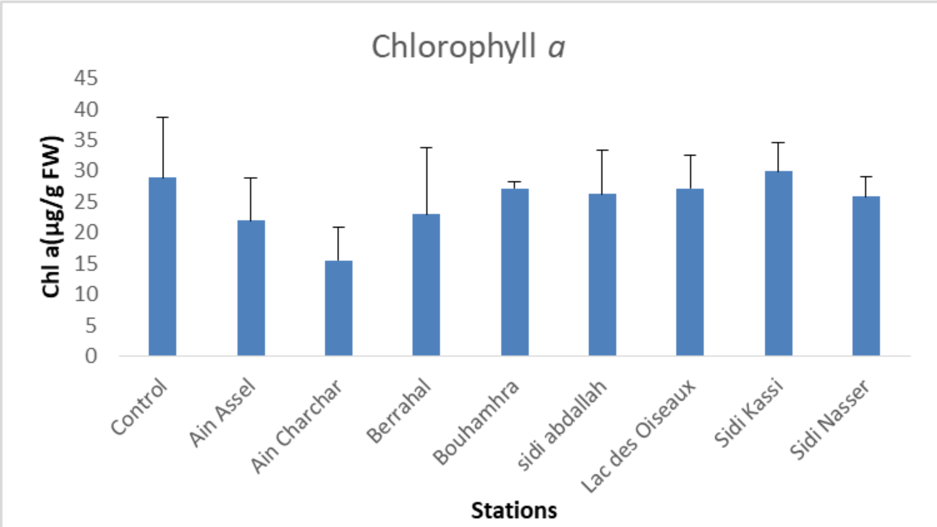
Figure 19: Microscopic observation of Sidi Nacer station transplant (a) histological section with magnification x200 (b) histological section with magnification x400

Table 2: Morphological observations and histological section from transplants different stations

Stations	Morphological observations	Histological observations
Ain Assel Figures 4 & 5	Detachment of the thallus and chlorosis leading to the browning of the thallus	Absence of rhizines in the centre of the thallus, slackening of hyphae and browning of the gonidia.
Ain Charchar Figures 7 & 6	Browning, drying out and detachment of the thallus	Necrosis of a number of gonidia, making it almost impossible to visualize the difference between the medullary and cortical areas
Berrahal Figures 8 & 9	Detachment and browning of the thallus.	The rhizines are rare in the centre of the thallus, the medullary and cortical areas are almost inseparable
Bouhamra Figures 10 & 11	Detachment of the thallus and chlorosis leading to the loss of the greenish colour.	The rhizines are rare in the centre of the thallus, the medullary and cortical zones are inseparables
Boutella Abdallah Figures 12 & 13	The transplants from Boutella Abdallah station showed the same disturbances that can be observed with a microscope and with the naked eye also as in the previous stations,	A rareness of the rhizines is observed, as well as the necrosis of the gonidia, making it impossible to visualize the difference between the medullary and cortical areas.
Lac des Oiseaux Figures 14 & 15	The transplants from the "Lac des oiseaux" station showed the same disturbances, which are observables with a microscope or the naked eye as the previous stations (those of Ain Charchar Ain Assel ...).	There is a loss of rhizines, a loss of green colour and the destruction of hyphae; the cortical and medullary areas are practically inseparables.
Sidi Kassi Figures 16 & 17	Detachment of the thallus and its browning.	The rhizines are less numerous than those compared to the control and few necrosis have been observed, moreover we can differentiate between the cortical and spinal zones.

Sidi Nacer Figures 18 & 19	The transplants from Sidi Nacer station show the same disturbances observable with a magnifying glass and the direct observation as in the Ain Charchar or Boutela Abdallah station with an accentuation of the detachment and browning of the thallus.	A rarity of the rhizines is observed in the centre of the thallus, as well as the necrosis of the gonidias, making it impossible to visualize the difference between the medullary and cortical areas.

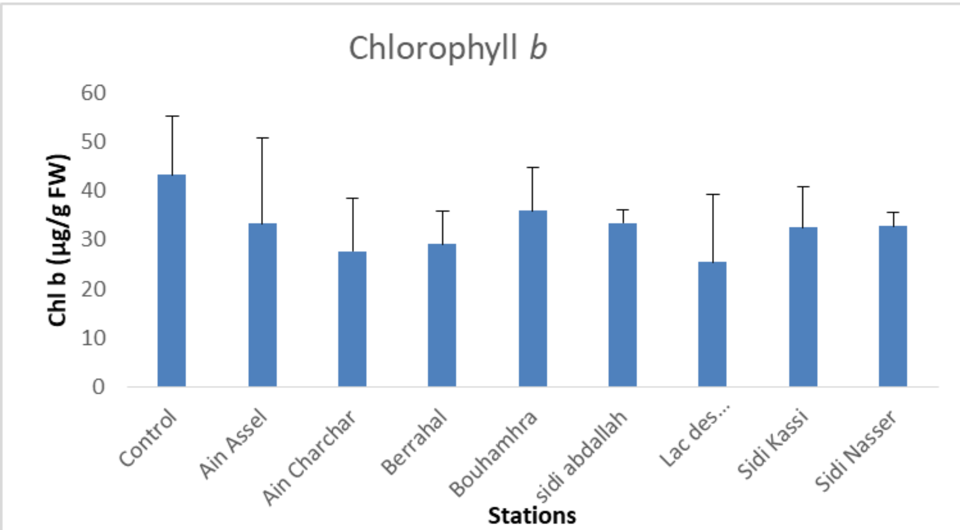
Results of chlorophyll dosage



+ Represents significant difference from the control group (Dunnett's, $p < 0.05$).
* Represents significant difference from the control group (Student's, $p < 0.05$).

Figure 20: Spatial variations in average levels of chlorophyll (*a*) µg / g FW of *Flavoparmelia caperata*

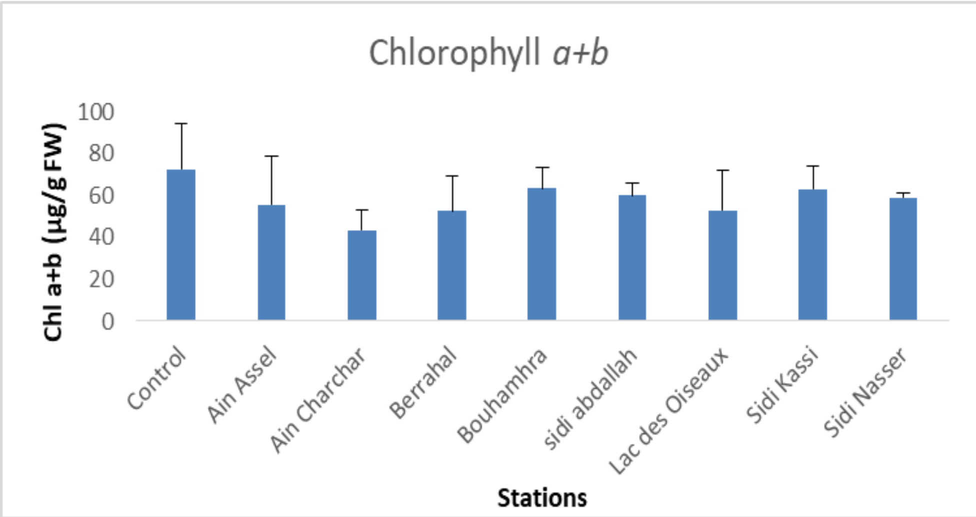
According to Figure 20, we record a decrease in Chlorophyll *a* levels in station 3 (Ain Charchar) with an average value of (15.5350 µg / g FW) compared to the control (28.8917 µg / g FW). On the other hand, we observe in the other stations a fluctuation in chlorophyll values. Statistical analysis reveals no significance in chlorophyll *a* levels at any transplant station ($p \geq 0,05$).



+ Represents significant difference from the control group (Dunnett's, $p < 0.05$).
* Represents significant difference from the control group (Student's, $p < 0.05$).

Figure 21: Spatial variations in average levels of chlorophyll (*b*) µg / g FW of *Flavoparmelia caperata*

According to Figure 21, it appears that Chlorophyll *b* values follow the same trend as Chlorophyll *a* and only with lower values compared to the control. However, in the station Ain charchar, the content of chlorophyll b decreased significantly (p <0.05) comparing with the control group (Student's test).



+ Represents significant difference from the control group (Dunnett's, $p < 0.05$).
* Represents significant difference from the control group (Student's, $p < 0.05$).

Figure 22: Spatial variations in average levels of chlorophyll (*a*+*b*) µg / g FW of *Flavoparmelia caperata*

Figure 22 shows that chlorophyll (*a*+*b*) follows the same trend as chlorophyll *a* and *b*. We note a decrease in chlorophyll levels in transplants for all stations compared to the control (72.7363 µg/g FW). Statistical analysis indicates no significance in chlorophyll *a*+*b* levels at all transplant stations compared to the control station (p≥0,05).

Service stations are generally indisputable sources of air pollutants due to the permanent presence of hydrocarbons and greenhouse gas emissions caused by the passage of vehicles that come to refuel. Pollutant emissions are influenced by not only on the characteristics of the vehicles on the road but also on the type of fuel they use. For example, in the case of gasoline-powered vehicles, if combustion were complete, all of the fuel would be transformed into CO_2 and H_2O. But in reality it is incomplete and the vehicle also emits sulphur and nitrogen monoxide[22]. Atmosphere particulate matter emissions are abnormal in the case of properly regulated spark-ignition engines since they only occur in the case of excessively rich fuel mixtures[21].

Among the different types of automobiles, diesel vehicles are the ones that are gaining ground primarily for their good fuel energical performance. However, diesel also has disadvantages in terms of the emission of particulates (soot, unburnt hydrocarbons) and nitrogen oxides NOx[23]. In our inventory of lichens bordering the stations, *Xanthoria parietina* was the most abundant species that we encountered at each station. The nitrophilous and toxi-tolerant properties of this specie justify the presence of nitrogen pollution in all stations[24]. According to Mc Mullin *et al.,* 2017[25] the absence of pollution-sensitive lichens in an area indicates a high pollution level. Other authors[26] have examined functional groups and used their presence or absence (depending on the traits) as indicators of air quality[27].

Parmelia sulcata is a toxi-tolerant species with metal-accumulating properties[20,28,25], its presence in Berrahal, Sidi Kassi and Lac des oiseaux stations could indicate the presence of trace metal elements in these different stations. The presence of the species *Lecanora conizaeoides* in the stations of Sidi Kassi and Lac des Oiseaux could be explained by the presence of sulphur pollution in these two stations, this agrees with the results of Laundon 2003[29]. Indeed, it has been shown that the decrease of SO_2 pollution in cities has led to recolonization by many SO_2-sensitive lichen species[30,31], and the increase of nitrogen pollution favours the increase of nitrophilous species[32-34,24]. The presence of toxi-tolerant species allows us to deduce that there is air pollution in general within these service stations. One of the consequences of the exposure of lichens to these various pollutions is the increase of stress. According to the measurement of the synthesis of photosynthetic pigments of our transplanted samples in these same service stations, we can highlight this stress at the algal level leading to a fluctuation in the synthesis of chlorophyll.

For chlorophyll *a*, the assay revealed a fluctuation in the synthesis of chlorophyll in the majority of the stations compared to the control, with the exception of Ain Charchar where a decrease in the chlorophyll *a* contents was noted. However, the comparison of the results of the control with those of the Sidi Kassi station, indicates that the synthesis in chlorophyll *a* was not disturbed. Which is probably because of the mycobiont that can draw part of its carbonaceous diet from an atmosphere loaded with CO_2. Furthermore, Holopainen 1983[35], attributes the increase of chlorophyll in *Hypogymnia physodes* transplants in polluted areas to a fertilizing effect due to nitrogen-rich dust deposition, as well as Vonarb and Brunold 1990[36], noted that *Parmelia sulcata* near Biel, Switzerland and in northern Switzerland contains high levels of chlorophyll in urban centres; they deduced that the transplanted lichens were not affected by NOx. This explains the observed disturbances of chlorophyll *a* contents in the transplanted lichens. Another more recent study carried out in Algeria in the province of Annaba indicates that the absence of pollution-sensitive lichens in polluted sites is in sharp contrast to the apparent improvement in the physiological state of pollution-tolerant lichens[37].

For chlorophyll *b* and *a+b* it can be seen that compared to the control, there is a slight decrease in chlorophyll at each station due to abiotic stress caused by air pollution at the service stations. Lichens undergo modifications and disturbances in their living conditions related to changes in their environment. These biotic and abiotic stresses can induce cellular dysfunction up to the death of algal cells[38]. There are different types of stress: physical stress (desiccation, temperature, pH, light); chemical stress (salinity, heavy metals, NOx, SOx) and physiological stress, which can act on the photosynthetic activity of the algal component of lichens[39].

According to morphological observations, discolouration of the transplants is observed with a restriction of their size, partial detachment and drying of the thallus. Indeed, a myriad of effects of pollution on lichens has been described. At the level of the whole thallus, researchers have described a decrease in thallus size and fertility[40-42], discolouration of the thallus[42,28]. Finally, in order to assess the disturbances at the tissue level, we made histological sections on our samples, observed under an inverted microscope.

According to the histological sections, we note on the one hand a less important quantity of rhizines in the centre of the thallus in samples of all different stations compared to the control, which gives a logical explanation for the detachment of the thallus, given that the rhizines are the organs that allow the fixation of the thallus on its support[38]. On the other hand, we observed a destruction of the hyphae, which have the power to capture algae in order to give a new lichen thallus. The fungus or mycobiont plays a preponderant role in the lichen symbiosis because it protects the algal cells against dehydration and light excess thanks to its hyphae that are in direct relation with the atmosphere and the substrate capturing water and mineral salts that are transferred to the photobiont[43].

In addition, the gonidia of all samples are less numerous except those of the control. Observations also showed that samples from Boutella Abdallah, Ain Charchar and Lac des Oiseaux stations show necrotic gonidia. It has been shown that an absence of gonidia and hyphae leads to a disturbance in the photosynthetic activity of the lichens[44]. The browning observed in the thallus is probably due to a disturbance in the synthesis of chlorophyll pigments caused by the necrosis of a large part of the algal cells.

Conclusion

The inventory of species bordering service stations reveals the presence of toxi-tolerant and nitrophilous species characteristic of a polluted environment. Indeed, the observations of the transplants showed physiological disturbances of the lichens exposed to the pollutants present in the service stations, thus our results highlighted fluctuations in the chlorophyll content (*a*,*b* and (*a* + *b*)). The disruption of the synthesis of photosynthetic pigments has caused on the morphological and physiological aspect necrosis leading to a discoloration of the gonidias. As for the detachment of the thallus of the transplants, the destruction of the hyphae is probably the cause. All these modifications observed within the thallus have sometimes led to its drying.

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