

Ecophysiological Changes of *Pseudevernia furfuracea* Transplants: An Effective Tool for Monitoring Anthropogenic Impacts

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Abstract A five-year biomonitoring program (2019-2024) was conducted around a 15 MW Biomass Power Plant (BPP) by means of a network of transplants of the epiphytic lichen *Pseudevernia furfuracea*. It consisted of an easily replicable experimental design, especially by state environmental agencies, using only physiological state parameters as tracers of environmental change. Detection of the facility contribution to the deterioration of air quality in the study area was performed using: a) geographic sectors, b) kilometer bands, c) absolute station distance, and d) the Potential Number of Times the Wind passing through the point source zone Reaches each Stations (PNTWRS). It is noteworthy that the most compelling evidence for the impact-enhancing role of BPP was observed during a pandemic. This finding suggests a significant confounding effect of pollution sources at the local scale. All factors contributed to changes in transplant physiology, except geographic sectors. However, kilometer bands and PNTWRS showed superior performance. The parameters most frequently and intensely affected by exposure in the selected area were membrane damage (EC%), and peroxidation levels (TBAr), which showed a five-year mean increase of 230% and 155%, respectively, vs. Lichen Origin Area (LOA), although with considerable annual variation, while photobiont was significantly impaired (-36% vs. LOA) only in 2024. We consider such an outcome to be a valuable result, supporting the hypothesis that monitoring lichen ecophysiology may

facilitate the detection of both interannual changes in air quality and the contribution of point source development.

Keywords Biomonitoring, Ecophysiology, *Pseudevernia furfuracea*, Biomass Power Plant, Wind Quantitative Analysis

1. Introduction

The use of biomonitors to facilitate the tracking of a specific potential anthropogenic pollutant source and to identify environmental impacts has long been considered as an effective methodology to complement chemical-physical procedures [1,2]. The method is based on a three-level approach: population, community and functional levels. While all three levels are used in practice, the first two are particularly prevalent, especially in the context of environmental monitoring programs. As regards the evaluation of atmospheric conditions, mosses and lichens have often been preferred [3-5], although other plant species have been successfully used for similar purposes [6,7]. Basic and applied research can experiment and test new methodologies and organisms to improve the precocity and accuracy of the diagnosis of ecological alterations. On the contrary, state environmental protection agencies need to handle agile and repeatable procedures

that can lead to timely evaluation of environmental criticalities. This means that, especially in the case of lichens, the preferred approach has been that at the community level, i.e. the biodiversity analysis of their biocenosis [8], based on the diverse sensitivity of species, especially to gaseous pollutants [9]. Recently, the Italian Institute for Environmental Protection and Research (ISPRA) has proposed the first guidelines for the use of lichens as bioaccumulators of inorganic contaminants [10], using both native populations and transplanted organisms. In spite of the usefulness of mapping the spatial variation of trace element levels, which makes it possible to discriminate areas suffering high levels of contamination vs. those less affected, many environmental agencies still prefer to use active sampler monitoring techniques. Indeed, bioaccumulation data cannot be converted into air concentrations, which are the values expressed by the safety thresholds devoted to the environment and man protection. Unfortunately, the population approach is scarcely based on the use of biomarkers, although several protocols have been developed for their evaluation [11,12], possibly as a consequence of the high costs required by some of the analyses and the associated laboratory equipment. On the other hand, the evaluation of ecophysiological parameters of mycobiont and photobiont is substantially affordable and at the same time adequate to estimate how environmental conditions fit lichen requests [13]. In view of the above considerations, a biomonitoring program was planned based on *Pseudevernia furfuracea* transplants around a Biomass Power Plant (BPP). The biomonitoring program was conducted from the beginning of March to the beginning of May, for a duration of 5 years (except 2021 due to pandemic). The objective of this study was to assess the ecophysiological response of thalli following exposure to the study area and to determine the spatial and temporal consistency of potential impacts on air

quality ascribable to the facility.

2. Materials and Methods

2.1. Study Area

The selected experimental area (12.56 km²) (Fig. 1) includes the campus of the University of Calabria, attended by about 30,000 students, and two urban districts, Montalto Uffugo and Quattromiglia, with 2,000 and 22,000 inhabitants, respectively. The study area is crossed by a relevant road network (the A2 motorway, the SP241 and SS107 highways, and the provincial road 234), with an average traffic rate ranging from 350 to 520 vehicles h⁻¹. The main plant located within the industrial zone, is a 15 MW Biomass Power Plant (BPP) fueled by exhausted pomace and wood chips. In addition, there are several fields of cereals and arboriculture scattered throughout the area. The annual average climatic parameters (provided by the regional meteorological service) are the following: temperature 16.4 °C, humidity 65% (RH), and rainfall 1237 mm.

2.2. Design of Experiment

The objective of the study was to assess the efficacy of a straightforward experimental design, easily repeatable, that could be applied by a regional environmental protection agency for the routinely monitoring of air quality within an urban-industrial zone. This approach was selected for the purpose of assessing the impact of an anthropogenic point source, identified based on its primary potential role in atmospheric contamination, on the surrounding environment.

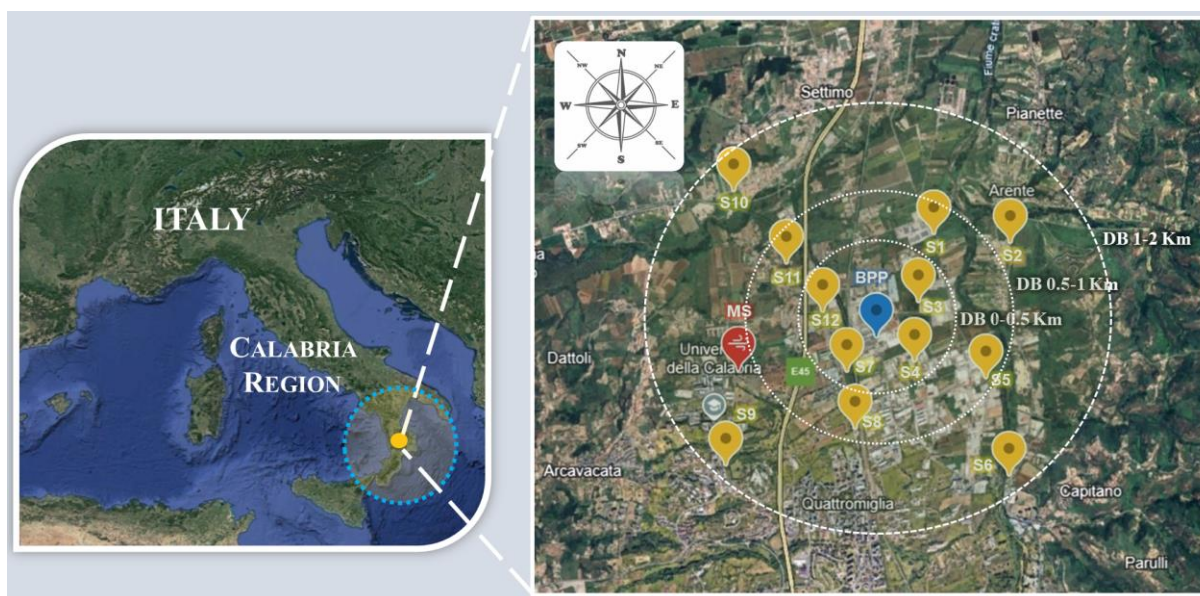


Figure 1. Study Area (figures taken from Google Earth and edited by the authors). MS = meteorological station (in red), BPP = Biomass Power Plant (in blue), in yellow = S1-S12 monitoring sites

The monitoring program was conducted by carrying out a transplant network of the epiphytic lichen *Pseudevernia furfuracea*, a widely used organism in studies on air pollution processes [14-16]. Thalli were attached (in triplicate) to wooden poles or tree branches at a height of 2.5-3 m above the ground. A total of 12 stations were selected to achieve a final average density of 0.95 stations per km², which is adequate for effective biomonitoring purposes [17-19]. The air emission point source (BPP) was located in the middle of the study area, and 4 Geographical Sides (GS) were defined around it: northeast (NE), southeast (SE), southwest (SW), and northwest (NW). Additionally, 3 kilometers bands (KB) were delineated, comprising distances of 0-0.5 km, 0.5-1 km, and 1-2 km.

We planned to expose the transplants in the period from the beginning of March to the beginning of May for five consecutive years (from 2019 to 2024). The first lichen network setting was in March 2019, but due to the pandemic outbreak, the 2021 campaign was cancelled, and the last one was in 2024. Lichen thalli were collected inside the Sila National Park, in a semi-pristine location (locality "La Fossia" designated as Lichen Origin Area (LOA)). The average annual climatic parameters, are as follows: temperature 10 °C, humidity 76.8% (RH), rainfall 1644 mm. Due to the practice of thinning branches from the lower part of the trunk to prevent fires, the thalli were collected from different sites each year. Based on such an experimental design, the variation of Ecophysiological Parameters (EPs) of lichen transplants was evaluated according to the following criteria: temporal, a) comparison between pre-exposure (LOA) and post-exposure (SA) in each of the five years, b) comparison between the five years (where EPs were expressed as % variation vs. pre-exposure values), spatial a) LOA vs. GS, b) LOA vs. Kilometer Bands (KB) progressively distanced from the BPP. The EP values measured at each monitoring site were also related to the Absolute Station Distance (ASD) from the facility and to the parameter PNTWRS (Potential Number of Times Wind Reaches Stations). The latter has already been successfully used by the authors to detect the contribution of point and diffuse sources to the spatial variation of inorganic and organic lichen bioaccumulation [20-21]. It represents the frequency with which the wind passing through the area where the potential contamination source is located, travels the absolute distance between the stations and the source. This parameter is expressed as the number of times that the wind, potentially carrying the emitted contaminants, reaches the stations located at each of the geographical sites during the lichen exposure period. The data needed for its calculation are: the direction, frequency and speed of the wind in the study area on each of the 4 selected Geographical Sides, the absolute distance of the monitoring sites from a point source and the lichen exposure time. In practice, it is the ratio between the time the wind flows in each of the GS and the time the wind takes to reach the stations (located in each GS) travelling the distance from the BPP to them.

2.3. Weather Monitoring and Data Elaboration

In order to monitor local-scale wind trends, humidity, and temperature - all of which have been demonstrated to affect lichen physiology [22], and to protect the instrumentation from potential vandalism, a meteorological station (Vantage Vue, Davis Instruments) was situated in close proximity to the BPP within the perimeter of CREA-OLI (Research Centre for Olive and Oil Industry).

The recording interval for the environmental parameters was set at once every 30 minutes, resulting in a final dataset of 2880 observations for each parameter. All the series were reversed on a PC and subsequently elaborated using an Excel spreadsheet. In the case of wind parameters, the default compass rose with 16 geographical sides was reduced to 4 sides, and the PNTWRS of each station was computed using the values referring to the wind flowing from the side opposite to that of the station selected for the PNTWRS calculation. Furthermore, in order to obtain a more accurate estimation of the suitability of humidity for the ecological requests of the lichen thalli, we combined the procedures suggested by Jonsson et al. [23] and Reiter et al. [24] to estimate the percentage of Net Photosynthesis vs. the Potential Maximum of the measured values of percentage of Relative Humidity (% RH) in the study area.

2.4. Ecophysiological Parameters

2.4.1. Dehydrogenase Activity

The assay measures mitochondrial dehydrogenase activity in accordance with Bačkor & Fahlset [25]. The protocol briefly states that 15 mg of thallus were added to 2 mL of 0.6% of 2,3,5-Triphenyl Tetrazolium Chloride (TTC) dissolved in 0.05 M phosphate buffer (pH 6.8), containing 0.005% Triton X-100 and incubated at 25 °C for 20 h. The Triphenyl Formazan (TPF) resulting from the reduction of TTC was then extracted first by dimethyl-sulfoxide and then by n-hexane. The hexane phase was isolated by centrifugation at 400 g for 15 minutes and the levels of TPF were quantified as Units of Absorbance at 492 nm by means of a Spectrophotometer (Perkin Elmer lambda 40).

2.4.2. Membrane Damage

The alteration of membrane integrity is a crucial parameter as it may indicate both an impairment of exchanges between the intra- and extra-cellular environments, as well as modifications in the cell ionic strength. The degree of membrane damage can be evaluated by a conductometric assay [26]. 100 mg of thalli were carefully rinsed, and subsequently immersed in 50 mL of deionized water, whose conductivity was measured before and after thalli introduction (1 h shaking). Finally, the thalli were boiled for ten minutes. The conductivity was measured again, considering it as the Maximum Possible Level (MPL) of membranes rupture. The value registered after 1 h shaking is expressed as percentage of the MPL of

membrane rupture.

2.4.3. Peroxidation Levels

Many atmospheric contaminants may affect organisms by promoting the formation of ROS, whose oxidation activity results in peroxidation, especially of molecules with unsaturated bonds. The estimation of this type of damage is frequently performed by Thiobarbituric Acid Test [27]. The thalli, 50 mg, were rinsed with distilled water and then homogenated into 2.5 mL of 0.1% Trichloroacetic Acid (TCA). The suspension, 1.5 mL, was centrifuged for 20 minutes at 12,000 g and 0.5 mL of supernatant added to 1.5 mL of 0.6% of Thiobarbituric Acid (TBA) and 10% TCA. The mixture was transferred to an oven set at 95 °C for 20 minutes, then centrifuged at 12,000 g for 10 minutes. The absorbance of the supernatant was measured at 532 nm (corrected for non-specific absorbance at 600 nm) with a Spectrophotometer (Perkin Elmer lambda 40). The main reaction product, resulting from the degradation of peroxides during the assay, was Malondialdehyde (MDA), which was quantified using the extinction molar coefficient of the adduct TBA-MDA ($155 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.4.4. Photosynthetic Pigments

The preliminary operation consisted in the repeated washing of lichens with CaCO_3 to remove lichenic substances that could denature pigments. The thalli, 60 mg, were extracted twice, under green light, with two different volumes of dimethyl sulfoxide. The initial homogenization of the samples occurs in 3 mL of the chemical solution, to which additional 4 mL were added. The samples were then incubated for 18 h. Subsequently, centrifugation at 400 rpm for 10 minutes followed, after which 3 mL of dimethyl sulfoxide were added to the precipitate. The suspension was incubated for another 6 hours. Finally, the two volumes were combined, and the concentrations of Chlorophyll a, Chlorophyll b and the fraction Xanthophylls+Carotenoids were calculated by measuring the absorbance at 480, 649 and 665 nm, and applying the Wellburn equations to the spectrophotometer readings [28].

2.4.5. Photosynthetic Efficiency

This parameter is a predictor of the conversion efficiency of radiative energy into chemical energy at the level of Photosystem II (Maximum Quantum Yield). It was measured based on the following procedure. The lichen thalli were hydrated at room temperature for 1 h. Thereafter, two fluorescence measurements were conducted using a portable fluorimeter (Handy Pea, Hansatech Instruments). The first measurement was taken after a 10-minute period in the dark, and the second was taken following exposure to a saturating flash of light ($2400 \mu\text{mol s}^{-1} \text{ cm}^{-1}$). The two fluorescence values were indicated as F0 and Fm, and their difference as Fv. The photosynthetic efficiency was expressed as Fv/Fm [29].

2.4.6. Statistical Analyses

The appreciable variation of interannual EPs values of LOA, due to lichen collection in different sites of the same zone as well as climatic differences, makes the use of a general 5 years mean ineffective, resulting in a loss of statistical significance. Therefore, a t-test was performed to conduct yearly comparisons between LOA and SA Ecophysiological Parameters values, whereas the % change of variation vs LOA (% CL) was calculated for interannual comparisons. The effect of geographical sides and kilometer bands, located around the facility, on the variation of EPs was investigated by means of 1-Way Parametric ANOVA with a Tukey post hoc test. The association between the spatial variation of EPs and both the absolute station distance from BPP and PNTWRS was determined through the calculation of Pearson correlation coefficient. All statistical analyses were performed using the Minitab 20 software.

3. Results and Discussion

3.1. Weather Monitoring

Table 1 showed the climatic parameters recorded by a meteorological station situated within the study area for each of the five bimesters during which the biomonitoring activities were carried out. As regard temperature, the central tendency values were quite comparable, with a minimum of 14.04 °C and a maximum of 15 °C, a range that appeared favorable to the physiology of *Pseudevernia furfuracea*, a meso-xerophytic organism [30] with tolerance temperature limits ranging, on average, from -5 °C to 20 °C [31]. Prior experimental findings indicated that this lichen exhibits temperature-dependent bioaccumulation of contaminants, with a notable decline in such capabilities above 20 °C [32]. This implies that when the ambient temperature overcomes the aforementioned threshold, biological stress no longer responds linearly to contaminant exposure, thereby compromising its role as a biomonitor. During the course of our investigation, the average values of temperature were about 5 °C below the upper tolerance limit of *P. furfuracea*, with 87% of all recorded values falling below this threshold. The interannual standard deviation was 4.45, and the coefficient of variation was 5%.

Such an outcome suggests that the limited variation in temperature between the five years is unlikely to have affected pollutants uptake as much as the physiological response of the thalli. Likewise, the Relative Humidity data seem to match the ecological requests of *P. furfuracea*. Indeed, several studies indicated that the coniferous trunk cover of this lichen is quite satisfying at humidity values between 60% and 80% [33,34], a range within which the yearly calculated means fall (Table 1).

Table 1. Mean values and related dispersion of the five years' data of Temperature and Relative Humidity recorded during the transplant exposure bimester (beginning of March – beginning of May) through 2019-2024 period and calculated Percentage of Net Photosynthesis (% NP) vs. the Potential Maximum Level

Years	2019	2020	2022	2023	2024
Temperature (°C)					
Mean	15	14.86	14.69	14.04	14.74
Standard Deviation	3.41	2.06	3.38	3.24	2.98
Coefficient of Variation	21.71	13.89	22.99	23.10	20.23
Humidity (% RH)					
Mean	70.38	67.78	64.88	69.26	66.46
Standard Deviation	6.37	4.81	10	11.06	7.75
Coefficient of Variation	9.06	7.09	15.41	15.97	11.67
% NP					
Mean	89.31	87.96	87.26	88.4	87.87
Standard Deviation	1.88	1.48	3.1	3.32	1.99
Coefficient of Variation	2.10	1.68	3.55	3.75	2.26

However, to test better how much the whole RH% record series influences lichen metabolism, we initially calculated the thallus hydration corresponding to the measured humidity [23], then we determined the relative % Net Photosynthesis vs. maximum attainable, on average, at 70% of water content [24,35]. Table 1 showed that the photobiont, in each year, demonstrated noteworthy potential photosynthetic performance. Indeed, only 10.3% of the five-year data set fell below the 85% of potential maximum values. Based on these outcomes, it can be reasonably concluded that climatic parameter variation does not result in a bias versus an accurate evaluation of physiological status change induced by the atmospheric contaminants to which lichens were exposed subsequent to their transplantation from LOA to SA. With regard to the climatic data series, in cases where they were unfavorable in comparison to biomonitor ecological requests, the detection of an “absolute” physiological delta due to local atmospheric pollution was less accurate.

However, a “relative” delta (i.e. spatial variation within the study area) could be considered reliable. To prevent unexpected interannual climatic variation, which has the potential to reduce the effectiveness of the methodology, a possible solution may consist in performing transplantation, beside in the SA, in a site within a zone: a) located in the opposite direction of most frequent winds, b) “relatively” far from the point source to be investigated, c) free of anthropogenic pressures (natural/seminatural zones), and d) with climatic conditions overlapping with those in the study area (Blank Site = BS). In order to pursue this experimental strategy, BS will be used in place of LOA.

3.2. Ecophysiological Parameters

3.2.1. Air Quality Evaluation: Temporal Criteria

Tables S1-S5 (Supplementary material) show the whole

data series in relation to the five-year monitoring program. A statistically significant difference between the mean of each ecophysiological parameter measured before exposure in the SA and the mean after exposure was considered an “actual change”. However, the ecological significance of this change would depend on the dimension of the delta (i.e. a real impairment to air quality). As detailed in the Design of Experiment section, the collection of thalli was not performed each year in the same zone due to the systematic denudation of the lower part of the trunks to prevent fires. In light of the interannual climatic variations, a notable discrepancy was observed in the LOA values associated with each transplantation event. As a consequence, a statistical comparison was performed between the LOA and SA ecophysiological parameters mean year by year, rather than a whole five-year mean. Tables 2 and 3 show the results of the related t-tests. In addition, to make possible comparisons between the five years, the absolute values of EPs were expressed as % change vs. LOA (% CL) in order to compare the five-year monitoring data. Based on these conversions, it was also possible to calculate the five-year mean for each EPs of the % CL. The latter were illustrated in Figures 2-3, while the former in Figures 4-5. TBARs (+155%), and EC% (+230%) showed the highest five-year mean variations, followed by Chl a (-18%), suggesting a clear deterioration in thalli physiology after two months of exposure to SA. As regards A492 and Fv/Fm, both showed a quite modest change, with values of +7% ($t = -2.04$, $p = 0.045$), and -6% ($t = 2.81$, $p = 0.007$) respectively. However, the values of photosynthetic efficiency are typical of lichens in a good condition [36], whereas the increase in dehydrogenase activity may be due to a slight activation of metabolism which is a common response to exposure to unfavorable environmental conditions. Since station density, about 1 per km², was such as to realize an affordable estimation of

spatial variation of physiological status of lichens, based on the combination of all the statistical significant cases in relation to both kilometer bands and geographical sectors, we calculated the area with a negatively affected air. The highest amount of impacted area was detected in 2024 (100%), followed by 2022 and 2023 (75%), and 2019 (68%). In contrast, 2020 showed the lowest level of involvement of the study area (16%). This is an interesting outcome. Indeed, 2020 corresponded to the onset of the pandemic, i.e. a period during which there was a significant reduction in both automotive traffic and industrial manufacturing/power production, leading to an improvement in environmental conditions [37-40]. This made it possible to detect how strong and systematic were the anthropic pressures. A comprehensive analysis of the results clearly indicates that the mycobiont seems to be much more susceptible to the effect of exposure in the SA than the photobiont. Both the five-year mean and each annual mean of oxidative stress and membrane damage showed a remarkable and statistically significant difference vs. LOA.

Nevertheless, there was a discrepancy in the transplant dehydrogenase activity levels in comparison to the pre-exposure levels, particularly in the five-year mean and the 2022 and 2023 comparisons. With regard to photobiont parameters, the percentage changes were lower than those observed for the mycobiont, and statistically significant differences were evident in a reduced number of cases (five-year mean: Chlorophyll a and Fv/Fm; yearly mean:

Chlorophyll a: 2023, all pigments: 2024). This result is contrary to the prevailing theoretical expectations. Indeed, the existing literature on lichen physiology impairment following transplantation in urban-industrial areas suggests that both partners are affected by exposure to atmospheric pollutants [41-43]. However, it should be noted that most of lichen biomass is made up of fungus partner with the photobiont located inside the thalli. This suggests that the mycelial component is directly exposed to airborne contaminants, and that the physical nature of stressors is likely to influence the degree of exposure of the partners to them. Indeed, the available literature data indicates that gaseous pollutants predominantly interact with algae, due to their rapid permeation, resulting in a detrimental effect [44-47] as well as a eutrophication-promoting effect [48,49]. When chemical agents are associated with particulate of different sizes, they are not immediately uptaken and remain trapped at the external surface of mycelial biomass or within the intercellular space. The mobilization of these compounds occurs as a result of the release of complexing molecules or leaching due to rainfall, with the majority of absorption occurring at the fungal cellular level [50-52]. Accordingly, it may be hypothesized that there will be an increase in both the prevalence and toxicity of gaseous/volatile atmospheric components in 2024. This was based on the observation that, on average, the concentration of pigments showed a statistically significant reduction of two- to three-fold compared to the LOA, in comparison to previous years.

Table 2. T-test relative to comparisons of yearly means of mycobiont EPs between pre-exposure (LOA) and post-exposure values (SA)

	A492		TBArS		EC%	
	t	p	t	p	t	p
2019	0.37	0.422	8.62	<0.00005	8.37	<0.00005
2020	2.17	0.073	-5.14	0.001	-3.8	0.008
2022	-2.6	0.023	-6.92	<0.00005	-12.61	<0.00005
2023	-8.9	<0.00005	-6.06	0.001	-8.38	<0.00005
2024	-0.54	0.598	-7.75	0.001	-11.31	<0.00005

Table 3. T-test relative to comparisons of yearly means of photobiont EPs between pre-exposure (LOA) and post-exposure values (SA)

	Chl a		Chl b		Xan+Car		Fv/Fm	
	t	p	t	p	t	p	t	p
2019	2.1	0.107	1.26	0.277	1.17	0.29	2.25	0.133
2020	1.63	0.138	1.04	0.338	1.08	0.315	0.34	0.067
2022	0.44	0.398	0.52	0.685	0.97	0.592	89.7	<0.00005
2023	3.2	0.011	1.9	0.153	2.24	0.075	0.47	0.657
2024	7.17	<0.00005	5.94	0.002	6.1	<0.00005	2.22	0.045

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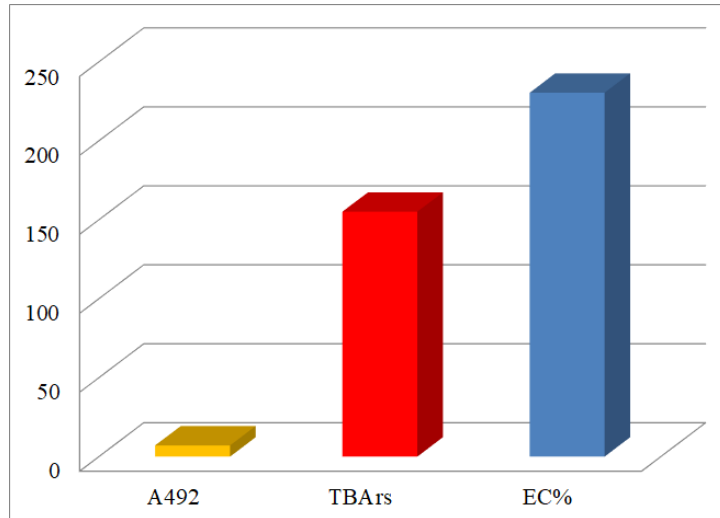


Figure 2. 2019-2024 means of percentage change of mycobiont EPs vs. LOA. * = statistically significant difference between LOA and SA means

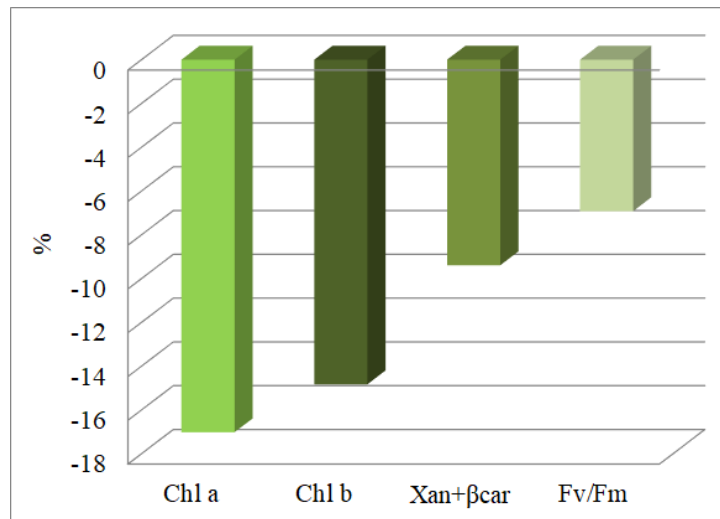


Figure 3. 2019-2024 means of percentage change of photobiont EPs vs. LOA. * = statistically significant difference between LOA and SA means

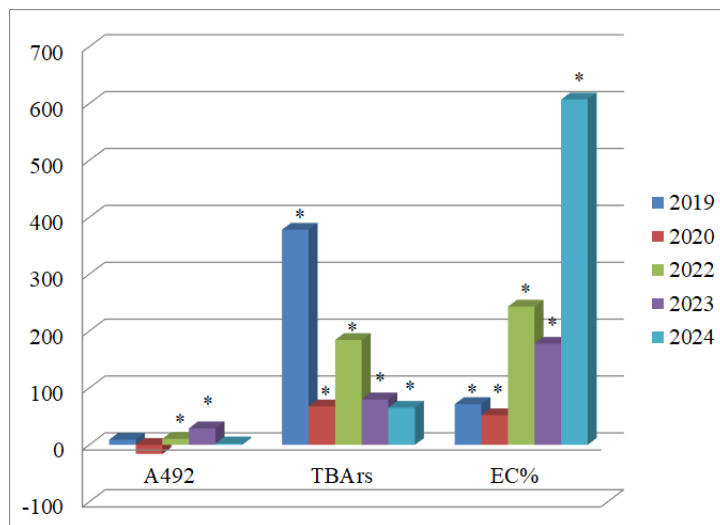


Figure 4. Yearly means of percentage change of mycobiont EPs vs. LOA. * = statistically significant difference between LOA and SA means

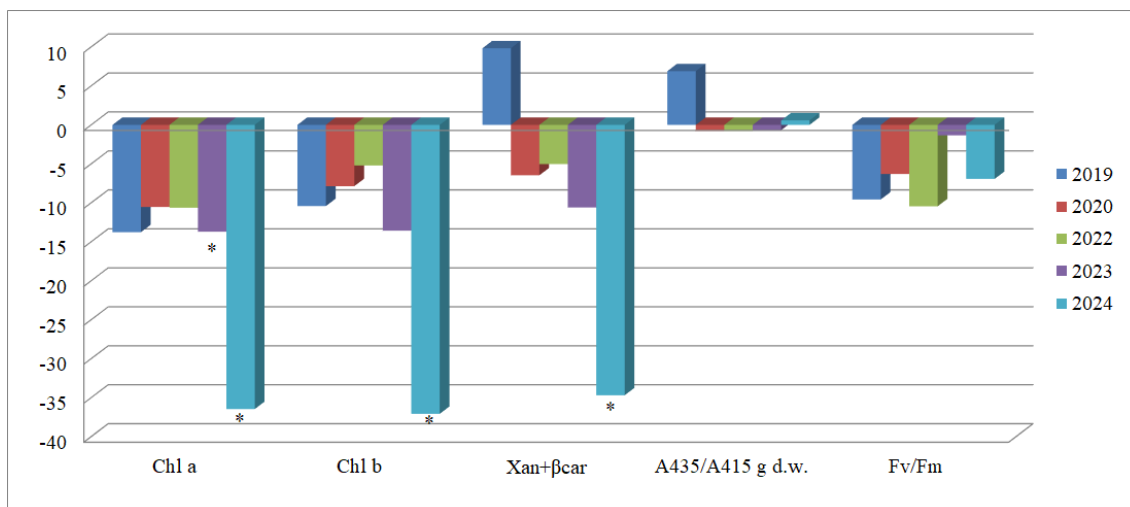


Figure 5. Yearly means of percentage change of photobiont EPs vs. LOA. * = statistically significant difference between LOA and SA means

3.2.2. Air Quality Evaluation: Spatial Criteria

Table 4 depicts the frequency and velocity of wind events. During the five-year monitoring period winds blew predominantly originated from the South (mean frequency = 60%). However, the mean higher velocity is associated with North winds, with the NW sector showing the highest value (2.78 m s⁻¹). The wind speed is typically below the threshold for the dispersion of pollutants [53], suggesting that local emissions are rarely effectively diluted, which may result in potentially hazardous exposure. A comparison of the total number of statistically significant differences in relation to the comparisons between means of geographical sectors and that of LOA revealed that 69% are located in the south (SE and SW) and 31% in the north. These data apparently appear to conflict with the wind direction, suggesting that local point sources may mask the general spatial trend of pollution due to BPP emissions. However, when the potential frequency of the station being reached by wind (passing through Point Source site) was calculated (using the PNTWRS model), rather than considering the simple wind frequency, it could be seen that the totals (over five years) of the North and South sides were virtually the same (104556 vs 102368). Furthermore, the variation of PNTWRS associated with the Three Top South Stations (TTSS) represented 63% of that associated with all remaining stations, whereas the Three Top North Stations (TTNS) represented 53%. Moreover, the percentage change versus the LOA of TBARs and EC% associated with TTSS accounted for 48% and 28%, respectively, of the total change over the entire monitoring period. This suggests that this group represents, especially in the case of oxidative stress, a criticality with regard to air quality in the SA. Such a result indicates that when the effect of a potentially Point Source (PS) is masked by the emissions of local sources, as is the case in urban-industrial areas, only relating geographical sectors (selected based on the location of the PS) to the wind direction may be an inadequate approach to evaluating the extent to which a PS

negatively affects air quality.

Table 4. Mean values of wind frequency (%) and velocity (m s⁻¹) calculated on the basis of the entire two months' data series

	Wind	2019	2020	2022	2023	2024
NE	Frequency	16.22	20.64	15.29	11.12	15.46
	Velocity	2.24	1.46	2.51	2.49	2.27
SE	Frequency	30.05	23.35	21.35	25.63	30.95
	Velocity	2.14	1.53	2.56	2.41	2.42
SW	Frequency	33.14	30.32	32.84	36.13	38.02
	Velocity	1.63	2.22	2.97	2.87	3.1
NW	Frequency	20.56	25.67	30.5	27.11	15.55
	Velocity	2.02	2.63	3.26	3.37	2.64

A better methodology would be to “calculate/quantify the potential contribution” of wind to the diffusion of pollutants in the geographical sides. The analysis of the results of the association between increasing “kilometer bands”, each containing 4 stations, from the BPP and the related values of means of Eps showed that 12.5% of statistically significant differences from LOA supported an effect of facility on the variation of the physiological status of the thalli (2020 and 2023). Conversely, only one of the calculated correlation coefficients between absolute station distance and EPs was found to be statistically significant (2020). The study cases are particularly complex and challenging to detect the biological stressing effect of a point source due to the lack of true replication. As a consequence, a frequent solution is the application of a recovery gradient, i.e. evaluating the potential improvement of physiological/biochemical parameters of biomonitor organisms following an increase in distance from the contaminants source.

This approach is based on the assumption that distance is a surrogate for the atmospheric dilution of emitted substances. Such a method suffers at least two potential

sources of bias. Firstly, the confounding effect of other factors (in our case, local-scale sources) that may affect temporal and spatial variation of selected biological parameters is not adequately considered [54-56]. Secondly, distances may be an inadequate estimator of contaminant deposition trajectories, given that the latter are also affected by the frequency and velocity of the wind along the distance separating the source and monitoring stations [57-59]. As previously stated, the confounding effect of local polluting sources was strongly (although not completely) reduced following the outbreak of the pandemic in 2020. This resulted in a condition favorable to detecting the dimension of environmental impact ascribable to BPP. In such a case, kilometer bands (statistical significant differences between 0-0.5 and 0.5-1 km bands and LOA) explained a higher amount ($F = 10.86$, $p = 0.001$, $r^2 = 66\%$) of the variation of TBARs than that explained by absolute station distance ($r = -0.68$, $p = 0.013$, $r^2 = 46\%$). It is possible that the association between KB and EPs is less affected by confounding due to residual emissions from local sources than the correlation between each “absolute distance-EPs” pair. This is because mean calculated values that are more affected by local sources are compensated for by values that are more affected by facility emissions (the trend is not hidden), whereas this does not occur in the case of the sum of the products of the (x-X) and (y-Y) differences due to the relatively low number of (x-y) pairs.

In 2020, the highest amount of variation ($r^2 = 75\%$) in TBARs was explained by the variation in PNTWRS, with a correlation coefficient (r) of 0.87 ($p < 0.00005$). This association was considered “strong” [60], suggesting that best evidence of the polluting effect of the facility (stress on lichen physiology) was showed by “quantifying” the potential role of wind as a contaminant vector. When confounding factor was more active, the association between EPs (EC%) and selected “factors” weakened, although it remained detectable when using kilometer bands (a statistically significant difference was observed between the 0.5 km band and LOA, $F = 4.48$, $p = 0.025$, $r^2 = 0.41$, in 2023) and PNTWRS ($r = 0.65$, $p = 0.023$, $r^2 = 0.41$, in 2024). Considering the totality of the results, it is recommended that the application of KB and PNTWRS be aligned with the objective of identifying potential impacts at both local scale (hundreds of squared meters from PS) and whole study area (squared kilometers). This approach will facilitate the formulation of an environmental diagnosis that encompasses the full spectrum of spatial biological stress dimensions.

4. Conclusions

The experimental activity carried out between 2019 and 2024 demonstrated that the sole spatial-temporal variation of the ecophysiological status of lichen transplants within a selected urban-industrial area can detect alterations in air quality following exposure to local anthropic activities, as

well as due to a potential main point source. The monitoring of interannual climatic parameters at high resolution made possible to verify that changes in the ecophysiology of lichen thalli could not be attributed to unfavorable values of temperature and humidity, which are paramount variables in lichen functionality. The results of temporal monitoring suggest that *P. furfuracea* transplants were, in each of the five years, strongly impacted by the local atmospheric physical and chemical characteristics. The fungus is the partner most systematically affected, while the alga showed an appreciable impairment only in the last monitoring year. We hypothesize that such a result may be attributable to the predominant association of contaminants with particulate emissions. The rapid permeation of gas/volatile pollutants across the lichen organism, and their subsequent damage to the photobiont, located inside the lichen thallus, increase their contribution to the contamination process exclusively in the year 2024. Spatial variation monitoring clearly demonstrated that the contribution to EPs alteration by BPP was difficult to detect due to the contemporary contributions of other local scale pressures. Best evidences of the impact of BPP were detected when “background disturbances” were reduced, as during a pandemic outbreak. The absolute distance of stations from BPP is the less effective parameter for associating the spatial impairment of EPs with the facility. The best performance was showed by PNTWRS, i.e. when was “quantified” the potential role of wind in transporting contaminants emitted from BPP to transplantation sites, i.e. taking into consideration, at the same time, wind frequency, direction and velocity that affected the dimension of pollutants diffusion trajectories. We consider our experience useful in providing suggestions for the development of guidelines that can be adopted by state environmental agencies for the use of lichen EPs to monitor atmospheric impacts within a study area, as well as the contribution of a main point source to these impacts. The aforementioned considerations are as follows: a) select the appropriate biomonitor and relative physiological status parameters, b) select a study area based on the point source whose biological effect you want to detect, c) perform a high-resolution monitoring of climatic variation during the transplantation period and, if necessary, add a Blank Site, d) choose an adequate station density (1 km²), e) plan to repeat the spatial monitoring design for some years, given that pressures intensity can modify over time, f) calculate, each year, the % change of variation of EPs following exposure vs the Transplant Origin Area (TOA), in order to avoid bias due to spatial and temporal variation of TOA organism physiology, g) the use of “kilometer bands” as a factor to detect the development of a spatial recovery gradient, i.e. an impact at the local scale (hundreds of squared meters) h) quantify the potential role of wind, passing through the zone where the point source is located, in the transport of contaminants to “each of the stations” for a more accurate detection of pollution contribution by

the selected facility (impact at the level of the entire study area).

Xan = Xanthophylls

List of Abbreviations

- ASD = Absolute Station Distance
- BPP = Biomass Power Plant
- Car = Carotenoids
- Chl a = Chlorophyll a
- Chl b = Chlorophyll b
- EPs = Ecophysiological Parameters
- GS = Geographical Sides
- KB = Kilometers Bands
- LOA = Lichen Origin Area
- MDA = Malonaldehyde
- MPL = Maximum Possible Level
- NP = Net Photosynthesis
- PNTWRS = Potential Number of Times the Wind passing through the point source zone Reaches each Stations
- PS = Point Source
- RH = Relative Humidity
- ROS = Reactive Oxygen Species
- SA = Study Area
- TBA = Thiobarbituric Acid
- TBARs = Thiobarbituric Reactive Species
- TCA = Trichloroacetic Acid
- TOA = Transplant Origin Area
- TPF = Triphenyl Formazan
- TTC = 2,3,5-Triphenyl Tetrazolium Chloride
- TTNS = Three Top North Stations
- TTSS = Three Top South Stations

Supplementary Material

Table S1. Ecophysiological parameters values related to thalli pre (LOA) and post (S1-S12) transplantation in Study Area. Monitoring year 2019. TBARs = $\mu\text{Mol g}^{-1} \text{d.w.}$; pigments = $\text{mg g}^{-1} \text{d.w}$

2019	A492	TBARs	EC%	Chl a	Chl b	Xan+ β car	Fv/Fm
S1	1.33	19	39	689	218	360	0.68
S2	0.99	64	36	779	246	405	0.421
S3	1.4	19	33	805	230	400	0.381
S4	1.66	33	27	854	260	434	0.395
S5	1.37	42	26	972	277	459	0.589
S6	1.39	49	30	752	230	378	0.628
S7	0.83	36	20	649	199	356	0.717
S8	1.04	44	31	1048	304	492	0.679
S9	1.27	12	20	845	257	437	0.683
S10	1.2	21	28	780	235	397	0.563
S11	1.45	16	23	923	263	449	0.655
S12	0.46	48	27	676	220	357	0.684
LOA	1.13	7	13	1255	357	383	0.642
LOA	1.39	8	17	854	239	383	0.651
LOA	1.02	7	17	925	268	413	0.612
LOA	0.975	7	18	844	251	344	0.677

Table S2. Ecophysiological parameters values related to thalli pre (LOA) and post (S1-S12) transplantation in Study Area. Monitoring year 2020. TBARs = $\mu\text{Mol g}^{-1} \text{d.w.}$; pigments = $\text{mg g}^{-1} \text{d.w}$

2020	A492	TBARs	EC%	Chl a	Chl b	Xan+ β car	Fv/Fm
S1	1.39	17	9	2027	561	825	0.777
S2	1.46	13	9	1543	433	670	0.778
S3	1.13	20	14	1731	504	757	0.731
S4	0.91	26	11	1876	591	817	0.757
S5	1.06	20	19	1715	484	724	0.535
S6	0.90	18	8	1984	557	833	0.799
S7	1.07	22	8	1090	321	518	0.744
S8	0.84	19	15	1535	441	685	0.674
S9	1.11	18	12	1590	453	684	0.772
S10	1.16	15	9	1939	559	871	0.713
S11	1.13	17	7	1926	546	821	0.714
S12	1.41	17	12	1280	368	637	0.757
LOA	1.24	13	7	1638	432	681	0.784
LOA	1.58	13	8	2053	561	843	0.738
LOA	1.26	10	9	1928	555	804	0.799
LOA	1.30	8	6	1920	556	824	0.792

Table S3. Ecophysiological parameters values related to thalli pre- (LOA) and post (S1-S12) transplantation in Study Area. Monitoring year 2022. TBARs = $\mu\text{Mol g}^{-1} \text{ d.w.}$; pigments = $\text{mg g}^{-1} \text{ d.w}$

2022	A492	TBARs	EC%	Chl a	Chl b	Xan+ β car	Fv/Fm
S1	1.34	19	11	1321	360	595	0.71
S2	1.32	20	12	1506	428	657	0.569
S3	1.25	16	17	1156	321	526	0.715
S4	1.24	19	13	955	261	464	0.735
S5	1.46	39	22	831	251	432	0.659
S6	1.55	37	20	796	219	394	0.677
S7	1.32	31	22	999	362	529	0.687
S8	1.41	23	27	734	231	389	0.637
S9	1.16	29	17	1164	332	526	0.744
S10	1.69	24	10	1134	333	556	0.616
S11	1.15	17	23	840	247	418	0.545
S12	1.58	23	19	978	292	483	0.712
LOA	1.25	9	5	1433	403	617	0.747
LOA	1.25	11	5	1106	297	513	0.745
LOA	1.26	7	5	1213	335	538	0.736
LOA	1.20	8	5	878	244	426	0.752

Table S4. Ecophysiological parameters values related to thalli pre- (LOA) and post (S1-S12) transplantation in Study Area. Monitoring year 2023. TBARs = $\mu\text{Mol g}^{-1} \text{ d.w.}$; pigments = $\text{mg g}^{-1} \text{ d.w}$

2023	A492	TBARs	EC%	Chl a	Chl b	Xan+ β car	Fv/Fm
S1	1.53	11	17	1702	603	803	0.755
S2	1.46	14	17	1844	647	862	0.753
S3	1.59	8	17	1561	558	744	0.757
S4	1.70	13	30	1679	606	784	0.712
S5	1.59	24	18	1414	520	688	0.67
S6	1.74	12	16	1763	643	849	0.802
S7	1.55	15	16	1208	486	625	0.811
S8	1.78	22	17	1405	544	696	0.721
S9	1.70	11	17	1353	529	693	0.787
S10	1.52	11	18	1662	599	812	0.765
S11	1.92	10	18	1616	602	768	0.778
S12	1.86	13	15	1458	565	725	0.796
LOA	1.28	8	11	1853	662	856	0.747
LOA	1.30	7	10	1902	781	909	0.729
LOA	1.28	6	10	1805	659	861	0.801
LOA	1.29	8	12	1650	560	748	0.8

Table S5. Ecophysiological parameters values related to thalli pre- (LOA) and post (S1-S12) transplantation in Study Area. Monitoring year 2024. TBARs = $\mu\text{Mol g}^{-1} \text{ d.w.}$; pigments = $\text{mg g}^{-1} \text{ d.w}$

2024	A492	TBARs	EC%	Chl a	Chl b	Xan+ β car	Fv/Fm
S1	1.05	17	20	870	289	448	0.786
S2	1.30	15	25	1274	377	594	0.718
S3	1.32	24	36	1671	470	707	0.757
S4	1.30	29	16	1176	353	524	0.62
S5	1.01	27	6	798	251	378	0.735
S6	1.28	35	12	985	317	462	0.552
S7	1.52	30	7	966	322	510	0.758
S8	1.44	17	14	946	308	470	0.687
S9	1.77	15	17	1173	346	521	0.794
S10	1.28	22	9	1191	366	535	0.741
S11	1.16	26	9	1600	472	719	0.666
S12	1.40	19	6	1394	411	604	0.732
LOA	1.28	13	2	1877	616	896	0.776
LOA	1.28	14	3	1855	537	798	0.771
LOA	1.30	14	2	1931	618	874	0.785
LOA	1.27	15	2	1705	499	736	0.729

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