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# Combining spectroscopic techniques and chemometrics for the interpretation of lichen biomonitoring of air pollution

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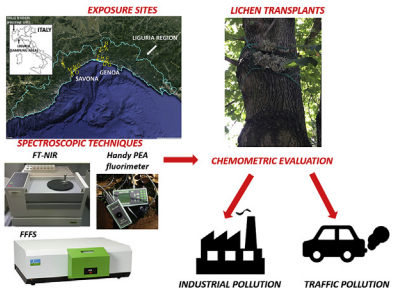
## HIGHLIGHTS

- Lichens, used as biomonitors of air pollution, were analyzed by spectroscopic techniques.
- Analytical data were elaborated by means of chemometrics tools.
- Results obtained with spectroscopic techniques were compared with the values of atmospheric pollutants.
- NIRS was able to differentiate between samples exposed in a polluted or not polluted area.
- FFFS was able to highlight different type of pollution, industrial vs. traffic.

## GRAPHICAL ABSTRACT

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## ABSTRACT

A screening evaluation of lichen thalli, based on spectroscopic techniques coupled with chemometrics, is proposed as fast, simple and "green" method for the biomonitoring of air pollution. For two consecutive years, lichen thalli of *Pseudevernia furfuracea* were exposed for three months in selected sites of Liguria (NW-Italy) according to different levels and types of air pollution. At the end of the exposure period, transplanted thalli were analyzed by a set of monitoring techniques, including Front-Face Fluorescence Spectroscopy (FFFS), Near Infrared Spectroscopy (NIRS) and Plant Efficiency Analyser (PEA). Data were compared with values of air pollutants recorded during the exposure period by the Regional Agency for Environmental Protection, in order to relate lichen physiological indicators with the effects of atmospheric concentrations.

A chemometric evaluation of the analytical signals, including principal component analysis (PCA) and quadratic discriminant analysis (QDA), was performed; the mean prediction rate of the discriminant models calculated on the FFFS emission spectra ranged from 70 to 75% on the external test sets. Front-face fluorescence spectroscopy proved to be a promising technique for the determination of level and type of pollutants in lichen thalli.

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## 1. Introduction

Lichens are symbiotic associations between a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, which can be either a green alga or a cyanobacterium (Nash

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III, 2006). Lacking organs for active water uptake, structures for regulating gas exchanges and permeability barrier for water, lichens are susceptible to absorb water, nutritive substances and gases directly from the atmosphere. Thus, they are extremely sensitive to the presence of substances that alter the atmospheric composition (e.g. $SO_2$ and $NO_x$) and are among the most widely used biomonitors of air pollution (Bargagli and Mikhailova, 2002). For biomonitoring studies, lichens may be used as bioaccumulators, to estimate the accumulation of trace elements within the lichen thalli over space and time (Bargagli and Mikhailova, 2002), or as bioindicators, to assess any alteration of the community diversity and composition (Giordani, 2007) and to estimate changes of physiological biomarkers in response to atmospheric pollutants (Jensen and Kricke, 2002; Mikhailova, 2002). From a physiological perspective, it has been widely demonstrated that the exposure of lichens to many gaseous pollutants (i.e. $SO_2$ and $NO_2$) may causes membrane injury, ultrastructural alterations, pigment degradation and/or impairment of photosynthetic function (Deltoro et al., 1999; Malaspina et al., 2015, 2018). Conventionally, these biomarkers may be evaluated by means of spectrophotometric or fluorimetric techniques. Recently, the assessment of the efficiency of the photosynthetic process in the algal population is one of the most common biomarkers used (Calatayud et al., 1996; Maxwell and Johnson, 2000; Malaspina et al., 2018). The use of direct light fluorimeter (Plant Efficiency Analyser, PEA) allows obtaining information on the efficiency of the photosynthetic processes on the tylacoid membranes of the algal chloroplasts, from the connectivity between PSII reaction centres to the electron flow to PSI. Particularly, PEA records the maximum quantum yield of primary photochemistry of the photobiont (measured by the ratio $F_V/F_M$) and other fluorescence parameters, which can be consider as highly sensitive and reliable tools for studying changes in photosynthetic apparatus and in its working efficiency caused by the negative effects of atmospheric pollution. Differently, when consider lichen as a bioaccumulator, we can obtain information on their trace elements content, thus on the atmospheric contaminants.

The main conventional analytical techniques used to determine element concentration consist of atomic absorption spectrophotometry techniques such as ICP-AES and ICP-MS (Bargagli and Nimis, 2002). Although these techniques are accurate and reliable in giving a quantitative result, they require long laboratory procedures and they are not able to establish unambiguously a relation between any change in the lichen physical and chemical properties and the individuals pollutants in the atmosphere (Nimis et al., 2002).

In this paper, we tested an alternative approach, which combining information from different analytical sources, could potentially provide a comprehensive evaluation of the complex chemical phenomena that occur in complex matrices. For this reason, spectroscopic techniques (e.g. visible (VIS), near infrared (NIR) and mid infrared (MIR) spectroscopy) were considered in order to integrate the assessment of atmospheric pollution by means of lichens. Spectroscopic analysis exploits the interaction of electromagnetic radiation with atoms and molecules to provide qualitative and quantitative chemical and physical (structural) information that is contained within the wavelength or frequency spectrum of energy that is either absorbed or emitted. Spectroscopy in the visible, near and mid-infrared ranges is an increasingly growing technique due to its cost, rapidity, simplicity, and safety, as well as its ability to measure multiple attributes simultaneously without monotonous sample preparation, making it suitable to be implemented on a routine basis. Near infrared spectroscopy (NIRS), Front-Face Fluorescence Spectroscopy (FFFS) and Plant Efficiency Analyser (PEA) are not expensive and 'green' because no reagents are required and thus no waste is produced.

By using the application of mathematical and statistical techniques, chemometrics allows to extract chemical and physical information from complex multidimensional data (Wold and Sjöström, 1972), which are currently observed in spectroscopy techniques. Chemometrics often relies on visualization to help the chemist to obtain the required information, and the most used method in this respect is principal component analysis (PCA). PCA extracts information from data tables by transforming them into plots (Massart and Vander Heyden, 2004).

In our previous work (Casale et al., 2015), we showed that NIR spectroscopy coupled with chemometrics was able to generate a lichen 'fingerprint' capable of discriminating between samples exposed in a polluted or non-polluted area. Differently, FFFS is usually applied on food samples for classification purposes (Ruoff et al., 2006; Sádecká et al., 2009), whereas, according to our knowledge, this technique was not investigated for lichen biomonitoring.

The present study aimed at testing the use of different analytical spectroscopic approaches, coupled with chemometrics, as rapid and simple tools for assessing effects of air pollutants on lichen thalli. For achieving this goal, thalli of the fruticose lichen *Pseudevernia furfuracea* (L.) Zopf v. *furfuracea*, collected from a pristine area, have been transplanted for three months to 15 sites in the Liguria region (NW-Italy), characterized by contrasting levels and type of atmospheric pollution, as measured by the regional Environmental Protection Agency (ARPAL). Lichen samples have been analyzed by FFFS, NIRS and PEA and data elaborated by multivariate data analysis (chemometrics), in order to compare the performances of these spectroscopic techniques and to highlight possible synergic or complementary information.

## 2. Materials and methods

### 2.1. Lichen sampling and sample preparation

The fruticose epiphytic lichen *Pseudevernia furfuracea* (L.) Zopf v. *furfuracea* was selected because it is widely used in biomonitoring studies with transplants (Blasco et al., 2011; Kodnik et al., 2015; Nascimbene et al., 2014; Sorbo et al., 2008; Tretiach et al., 2007a, 2011).

Lichen thalli were collected from northerly exposed barks of *Picea abies* (L.) H. Karst in a forest area of Valtournenche (Valle d'Aosta, Italy) at 1900 m a.s.l., far from local sources of air pollution (Malaspina et al., 2014). Collecting lichens from the north side of tree allows work with material adapted to homogeneous regime of diffuse light (Tretiach et al., 2007b). Samples were picked up, at 1.5–2.0 m above the ground, together with a piece of the supporting branch, using garden shears. The material was taken to the laboratory in paper bags and left to dry out at room temperature and low light overnight ($\approx 5\ \mu mol\ m^{-2}\ sec^{-1}$), to minimize a rise in the $F_V/F_M$ caused by recovery from natural photoinhibition (Gauslaa and Solhaug, 2004). Samples were divided into two groups: one, including samples that were never exposed in the experimental sites, were kept in freezer until the end of the experiments (control), whereas the second group included one hundred and fifty thalli which were randomly selected and prepared to be exposed in the 15 exposure sites. In the laboratory, lichen thalli were fixed by means of plastic bands on plastic nets (of ca. 25 × 15 cm) and put into paper bags.

### 2.2. Study area and sampling sites

Fifteen sites (A - Q) distributed in an area of ca. 200 $Km^2$ in Liguria region (NW Italy) (Fig. S1) were selected for exposure.

Particularly, lichen samples were exposed in the urban – industrial area of Genoa (D – H) and Savona (M – Q) and in their hinterland (A – C, and I – L, respectively) (Table 1). Site A and B, characterized by high levels of air pollution, were located in two hinterland districts of the province of Genoa nearby the highway and an oil refinery. Like the two previous ones, site C was located in the hinterland of Genoa but it differs from the previous one because it is mainly an urban area characterized by a lower level of air pollution. Site D and E were located in the city centre of Genoa near the principal traffic congested roads, whereas site F and H were located in the city of Genoa near the industrial harbours and close to the shipyards. Site G was in a little green area in the centre of Genoa surrounded by a small traffic road. Site I and L were in the hinterland of Savona (NW-Liguria), the first in a small village with low traffic and the other near a big industrial settlement. Finally, site L – Q were in the urbanized area of Savona subjected to different traffic density.

### 2.3. Sample exposure

In each experimental site, 10 lichen thalli (fixed on three plastic nets as described above) were attached on the trunk of adjacent three trees, at approximately 2.5 m above the ground, protected by the canopy from direct sunlight.

The sampling was performed for 2 consecutive years, 2015 and 2016, in order to take into account the temporal variability. Fig. S1 shows the map of the 15 exposure sites in the Liguria region (NW-Italy).

In 2015, the lichen deployment was carried out in two consecutive days in July. Lichen samples were transplanted to 15 sites, close to (<50 m) the monitoring stations of the Liguria Regional Environmental Protection Agency (ARPAL, http://www.arpal.gov.it), in the province of Genoa and Savona, according to expected contrasting levels of atmospheric pollution. Thirteen thalli were not exposed and thus considered as control samples. In the second year, the experimental effort was reduced on the basis of the information provided by the results obtained in the first year. Accordingly, in July 2016 only 5 of the 15 stations monitored during 2015 were selected as a representative set, in terms of level and type of atmospheric pollution and geographical location. Only 6 thalli were reserved for controls.

In both years, the sampling lasted three months. Hereafter, samples were retrieved packed in paper bags, protected from sunlight, and transported back to the laboratory, where they were detached from branches, carefully cleaned from debris and dead or senescent parts, and kept in dark conditions at ambient temperature until analysis. Unfortunately, in both sampling years not all thalli were found at the end of the exposure period of three months. Table 1 shows the list of the remaining samples that were analyzed. For some stations, the number of samples analyzed by FFFS, NIRS and PEA can be different.

### 2.4. Air monitoring pollution data

Data on concentrations of the main air pollutants (Benzene, $NO_2$, $SO_2$ and $PM_{10}$) were continuously (hourly) recorded in each experimental site and over the entire exposure periods (2015 and 2016 campaigns) by the devices of the Liguria Regional Environmental Protection Agency (ARPAL), located close to the transplanted thalli. In Table 1, for each site, we reported the hourly average concentrations of the main air pollutants (Benzene, $NO_2$, $SO_2$ and PM10 expressed in $\mu g/m^3$) recorded by the ARPAL during the 3 months of exposure. These data were used to categorize the sampling sites on the basis of their level and type of pollution.

### 2.5. Instrumental analysis

All the instrumental measurements described in this section were performed on control (i.e., not exposed) and on transplanted thalli, (i.e., at the end of exposure periods). For the FFFS and NIR analyses, each lichen sample was firstly pulverized with a ball mill, and then the powder was divided in two portions: 0.5 g were used for FFFS analysis and 1.0 g for NIR measurements. Differently, PEA analyses were performed directly on the top of the lacinia of lichen thalli.

#### 2.5.1. Front-face fluorescence spectroscopy (FFFS)

Emission spectra were recorded using a Perkin–Elmer LS55 (Perkin-Elmer Ltd., Beaconsfield, U.K.) luminescence spectrometer equipped with a Xenon lamp and a variable angle front-surface accessory. The incidence angle of the excitation radiation was set at 56° to ensure that reflected light and scattered radiation were minimized. Samples were placed in cuvettes with a circular surface of diameter 15 mm. Excitation and emission slits were both set at 10 nm. Emission spectra were recorded between 300 and 500 nm

**Table 1**
List of the experimental sites (locality) with the corresponding code (site code). For each site are reported summary of samples analyzed in 2015 and 2016 (N analyzed samples), of the average concentrations of the main air pollutants (Benzene, NO2, SO2 and PM10 expressed in μg/m3) recorded by the ARPAL in the 3 months of exposure (pollution data) and relative pollution category.

| Site code | Locality | N analyzed samples | | | | | | Pollution data | | | | | | | | Pollution category |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2015 | | | 2016 | | | 2015 | | | | 2016 | | | | |
| | | FFFS | NIR | PEA | FFFS | NIR | PEA | $NO_2$ | $SO_2$ | PM10 | Benzene | $NO_2$ | $SO_2$ | PM10 | Benzene | |
| CTR | Controls, not exposed | 13 | 13 | 13 | 6 | 6 | 6 | | | | | | | | | Control |
| A | Busalla-Piazza Garibaldi | 7 | 7 | 7 | | | | 36.2 | 9.2 | | 1.38 | 31.5 | 10.0 | 23.8 | 1.29 | Industrial |
| B | Busalla-Sarissola | 7 | 4 | 6 | 10 | 6 | 6 | 15.8 | 7.2 | | 0.80 | 26.7 | 8.3 | | 1.22 | Industrial |
| C | Campomorone | 6 | 4 | 6 | | | | | | | 1.59 | 22.2 | 6.0 | 12.9 | 0.37 | Low traffic |
| D | Genova-Corso Buenos Aires | 9 | 9 | 8 | | | | 40.2 | | 28.8 | 1.71 | 40.1 | | 31.7 | 1.80 | High traffic |
| E | Genova-Corso Europa | 7 | 3 | 7 | 9 | 9 | 5 | 44.4 | | 35.4 | 1.70 | 51.4 | | 29.6 | 2.19 | High traffic |
| F | Genova-Multedo | 4 | 4 | 4 | | | | | 9.4 | | 1.20 | | 9.1 | | 0.71 | Industrial |
| G | Genova-Quarto | 6 | 6 | 6 | | | | 28.1 | 3.9 | 16.0 | 0.66 | 11.2 | 4.7 | 14.0 | 0.66 | Low traffic |
| H | Genova-Via Buozzi | 6 | 6 | 6 | 9 | 6 | 5 | 48.9 | 12.3 | | 1.70 | 50.1 | 7.9 | | 1.49 | Industrial |
| I | Cairo Montenotte-Loc Bragno | 6 | 6 | 6 | | | | 10.0 | 5.7 | 17.5 | 0.90 | 10.3 | 5.4 | 17.9 | 1.01 | Low traffic |
| L | Cairo Montenotte-Loc Mazzucca | 8 | 6 | 8 | 4 | 4 | 2 | 14.6 | 8.1 | 19.5 | 1.62 | 15.4 | 9.7 | 19.3 | 2.07 | Industrial |
| M | Quiliano | 6 | 3 | 6 | | | | 14.6 | 3.0 | 21.9 | 0.79 | 15.3 | 4.0 | 20.9 | 0.35 | Low traffic |
| N | Savona-Corso Ricci | 7 | 7 | 6 | | | | 25.8 | | 12.6 | 1.28 | 29.6 | | 16.5 | 1.33 | Low traffic |
| O | Varaldo | 6 | 3 | 6 | | | | 11.0 | 5.0 | 17.6 | 0.37 | 13.4 | 3.9 | 17.6 | 0.20 | Low traffic |
| P | Vado Ligure | 3 | 3 | 3 | 10 | 6 | 6 | 37.5 | 5.1 | 24.8 | 3.32 | 25.4 | 4.8 | 20.7 | 1.83 | High traffic |
| Q | Albissola | 7 | 7 | 7 | | | | | | 15.2 | 1.44 | | | 15.4 | 1.32 | Low traffic |

(with 0.5 nm resolution) at excitation wavelength of 270 nm. Intensities were plotted as a function of the emission wavelength. For each sample, measurements were done in triplicate to minimize remaining scattering effects and the average signals were used in the multivariate data analysis. The BL Development software (PerkinElmer) was used to register the fluorescent signals.

#### 2.5.2. Near infrared spectroscopy (NIRS)

NIR measurements were carried out using an FT-Near-Infrared Spectrometer, based on a Polarization Interferometer (Buchi NIRFlex N-500), in the 4000–10,000 $cm^{-1}$ range with 4 $cm^{-1}$ resolution. NIR Operator software (Buchi) was used to register the NIR spectra.

For each sample, approximatively 1 g of powder was placed in an optical glass Petri dish and analyzed in reflectance mode. An average of 64 scans was taken for each spectrum. The optical glass dish was washed in warm water, accurately rinsed and dried before carrying out the three replicates of each sample. The average signals were used in the multivariate data analysis.

#### 2.5.3. Plant Efficiency Analyser (PEA)

Chlorophyll *a* fluorescence (Chl *a*) measurements were performed using Handy-PEA chlorophyll fluorometer (Plant Efficiency Analyser, Hansatech instruments Ltd, Norfolk, England). Prior to taking the measurements, samples were sprayed with deionised water until wet and adapted to darkness for 15 min. Three Chl *a* measurements were performed on each thallus. The Chl *a* fluorescence transients were induced by a red light (peak at 650 nm) provided by an array of three high-intensity LEDs. Data were recorded after a saturating light pulse (3500 μmol $m^{-2}$ $sec^{-1}$) of 1 s. The gain of the PEA was 0.8. The fluorescence transient rises from $F_0$ (when all PSII reaction centres are open, i.e. when the primary acceptor quinone is full oxidized) to $F_M$ (when all the PSII reaction centres are closed, i.e. the full reduction of the primary acceptor quinone). The potential quantum yield of primary photochemistry ($F_V/F_M$) was calculated as $(F_M–F_0)/F_M$. We also considered other parameters to describe the ability of the photobiont in transferring trapped photons along the tilacoid membrane from PSII to PSI (Strasser et al., 2000, 2004; Tsmilli-Michael et al., 2000). For a detailed description of the parameters and formulae, see Table S1.

### 2.6. Data analysis

#### 2.6.1. Data matrices organisation

Two data matrices were elaborated for each analytical techniques (FFFS, NIRS and PEA): F1, N1 and P1 containing the data relative to year 2015 and F2, N2 and P2 the data of year 2016, respectively.

Regarding FFFS, F1 had 108 rows (samples) and 397 columns (variables acquired between 300 and 500 nm, with 0.5 nm resolution). F2 had the same number of variables as F1 but only 48 rows (samples). F1 and F2 were pre-processed using standard normal variate (SNV) for correcting for shift.

As far as the NIR data are concerned, the part of the spectra from 8000 to 10,000 $cm^{-1}$ was removed since it was not informative, thus N1 had 91 rows (samples) and 1001 columns (variables acquired between 8000 and 4000 cm-1, with 4 $cm^{-1}$ resolution); N2 data matrix had 37 rows (samples) and 1001 columns (variables, between 8000 and 4000 $cm^{-1}$). NIR spectra were pre-processed using Standard Normal Variate (SNV) to eliminate the unwanted variation due to light scattering.

With regards to PEA, three replicates for each thallus were acquired, so that P1 had 315 rows (105 *3) and 21 columns (parameters of efficiency) and P2 had 90 rows (30 *3) and 21 columns.

#### 2.6.2. Chemometrics analysis

Principal Component Analysis (PCA) was applied as a data display method on the six spectroscopic data matrices (F1, N1, P1, F2, N2 and P2) and on the pollution data matrix. Quadratic Discriminant Analysis (QDA) was performed as a classification technique on the six spectroscopic data matrices.

PCA is the most used tool in exploratory data analysis and uses an orthogonal transformation to convert a set of correlated variables into a set of uncorrelated variables called principal components (Wold, 1987). QDA is a probabilistic parametric classification technique, which represents an evolution of Linear Discriminant Analysis (LDA) (Vandeginste et al., 1998) for nonlinear class separations. Also QDA, like LDA, is based on the hypothesis that the probability density distributions are multivariate normal but, in this case, the dispersion is not the same for all of the categories. It follows that the categories differ not only for the position of their centroid but also for the variance-covariance matrix (different location and dispersion).

For the year 2015, the QDA discrimination rules were validated using both a cross-validation procedure with five cancellation groups (5CV) and an external test set. The test set samples were selected randomly assigning 25% of the samples to the external test and 75% to the training set. For the year 2016, the QDA discrimination rules were validated only in cross validation (5CV) considering the low number of samples. QDA results were expressed as the total prediction rates, that is the ratio of correct predictions to the total number of predictions and it measures the predictive ability.

## 3. Results and discussion

### 3.1. Principal component analysis

#### 3.1.1. Air monitoring pollution data

Data collected by ARPAL in the 15 monitoring stations in the province of Genoa and Savona, in the period July–September 2015 are reported in Table 1. In the study area, the largest sources of $SO_2$ in the atmosphere include industrial processes, ships and other vehicles emissions and heavy equipment that burn fuel with a high sulfur content (Mazzei et al., 2008; Schembari et al., 2012). Benzene, particles less than 10 μm in diameter ($PM_{10}$) and nitrogen oxides ($NO_x$) are the main urban air pollutants due to traffic. Fig. 1 shows respectively the PCA loading (Fig. 1a) and score (Fig. 1b) plots of these pollution data. $SO_2$ was the only variable showing negative loadings on PC1; $NO_2$, Benzene and $PM_{10}$, had positive loadings on PC1. Therefore, PC1 was associated with the type of pollution, 'industrial pollution' at negative values of PC1 and 'pollution from traffic' at positive values. Stations A, B and C, located in the northern hinterland of Genoa, were characterized by a high content of $SO_2$ and this is potentially due to a soap factory (C) and an oil-refinery producing 700 t/year of $SO_2$ (A and B). Station H also showed a very high content of $SO_2$ and this can be explained because this station was in front of the Genoa harbor where many ships dock. Stations E and D are in the most traffic congested streets in the centre of Genoa and station P is a touristic area close to the sea and therefore very popular during summer.

Fig. S2 (a and b) shows the PCA loading (S2a) and score (S2b) plots of pollution data collected by ARPAL in the period July–September 2016. The information provided was very similar to that extracted from the 2015 data.

According to the information obtained from the pollution data, samples were divided into 4 classes, in terms of type and level of pollution, which characterized the sites of exposure:

1. not exposed: Control samples (CTR).

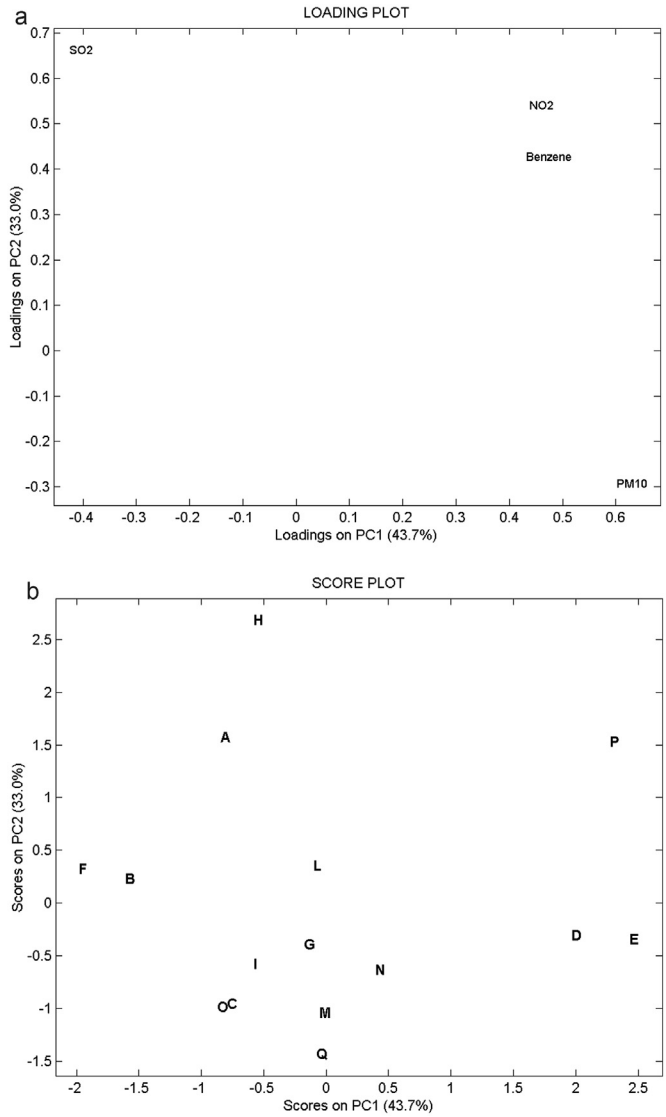


**Fig. 1.** Loadings (1a) and score plot (1b) of pollution data collected by ARPAL in the 15 monitoring stations in the province of Genoa and Savona, in the year 2015.

2. Exposed in stations characterized by industrial pollution: A, B, F, H, L
3. Exposed in stations characterized by high-congested traffic: D, E, P
4. Exposed in stations characterized by low traffic: C, G, I, M, N, O, Q.

Based on the results of the previous survey, for the year 2016 the category "low traffic" was excluded from the analysis.

### 3.1.2. FFFS

Fig. 2 shows the score plot of the data set F1 (year 2015), in the space of the 2 first PCs. The control samples were at higher positive scores on PC1 that explains the 48% of the total variance and they were all clustered, highlighting a good homogeneity of the starting samples. On the contrary, samples exposed in 4 industrial sites (A, B, F and L) were associated with negative scores of PC1. Samples from traffic sites (both high-congested and low traffic) showed a less uniform pattern with respect to samples exposed in industrial sites, however half of the samples from traffic sites (E, M, N, O and P) were associated with negative scores of PC2.

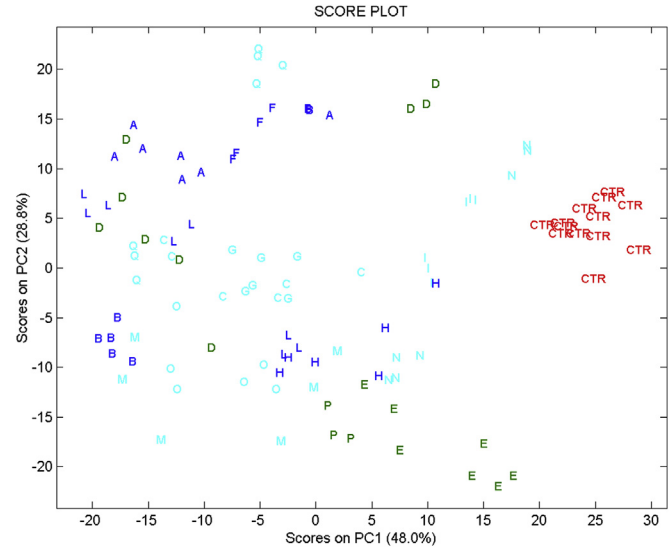


**Fig. 2.** Score plot of the FFFS emission spectra acquired on the lichens thalli exposed during 2015. Samples are indicated by their pollution classes: red control; blue industrial pollution; green high congested traffic; light blue low traffic. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. S3 shows the score plot of the data set F2 (year 2016), in the space of the 2 first PCs. Control samples were associated with negative scores of PC2 and form a defined cluster with respect to samples transplanted in the exposed sites. The separation of the pollution classes was evident along PC1: industrial sites were associated to negative scores whereas traffic sites with positive ones.

### 3.1.3. NIRS

Fig. 3 shows the score plot of the data set N1 (year 2015), in the space of PC1-PC4. Control samples formed a well-defined cluster,

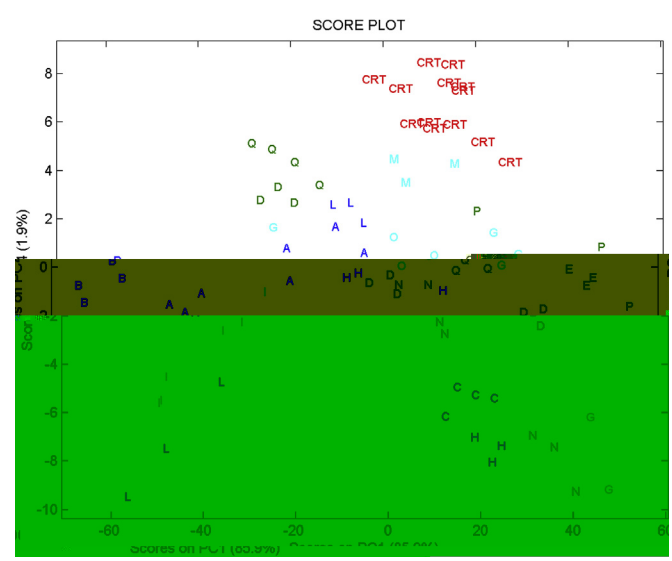


**Fig. 3.** Score plot of the NIR spectra acquired on the lichens thalli exposed in the year 2015. Samples are indicated by their pollution classes: red control; blue industrial pollution; green high congested traffic; light blue low traffic. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
QDA results on the FFFS (F1 for 2015 and F2 for 2016), FT-NIR (N1 for 2015 and F2 for 2016) and PEA (P1 for 2015 and P2 for 2016) data of the year 2015 and 2016. Results are the mean correct prediction rate expressed as percentages (%).

| Data set | Control | Industrial | High-congested traffic | Low traffic | Weighted mean |
|---|---|---|---|---|---|
| F1 | 100.0 | 80.0 | 100.0 | 25.0 | 70.0 |
| F2 | 100.0 | 75.0 | 71.4 | // | 75.0 |
| N1 | 90.0 | 64.7 | 66.7 | 25.0 | 51.3 |
| N2 | 83.3 | 62.5 | 66.6 | // | 67.6 |
| P1 | 100.0 | 26.1 | 33.3 | 45.4 | 40.0 |
| P2 | 100.0 | 66.7 | 80.0 | // | 77.8 |

by short-term environmental variations. In lichens, many organic compounds can produce NIR and FFFS absorptions, including e.g., polycyclic aromatic hydrocarbons and organic acids that come from environmental pollution or endogenous organic acids that may increase as a response to stresses such as pollution (Casale et al., 2015).

## 4. Conclusion

Lichen biomonitoring is widely used for detecting air pollution patterns and can be especially useful in remote areas where the use of instrumental recording is hindered by difficult access to sites and difficult management of mechanical and electrical devices.

In this study, the combined use of several rapid analytical approaches, coupled with chemometrics, as rapid and simply tools for assessing the effects of air pollutants on lichen thalli was investigated. Lichen samples were analyzed by FFFS, NIRS and PEA, in order to compare the performances of these analytical spectroscopic techniques, and to highlight possible synergic or complementary information.

Despite the fact that it seems hard to discriminate between similar levels of atmospheric pollution, the explored techniques and in particular FFFS were able to highlight different type of pollution (namely, industrial vs. traffic). Considering the biological variability of the lichen thalli, the classification performances achieved by QDA can be considered more than satisfactory.

This could pose the basis for promising development of spectroscopic techniques for exploring possible range of impact of different sources of emissions in a complex context.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.01.136.

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