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ORIGINAL ARTICLE

Effect of metal content on chlorophyll fluorescence and chlorophyll degradation in lichen *Pyxine cocoes* (Sw.) Nyl.: a case study from Uttar Pradesh, India

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Abstract The major aim of the present study is to identify the relationship of physiological parameters of the photosynthetic system with the elemental content of the naturally growing lichen *Pyxine cocoes*. The epiphytic foliose lichen *P. cocoes* was used as biomonitoring indicator and effect of atmospheric pollutants on physiological integrity was examined. Potential quantum yield of Photosystem II (fluorescence ratio Fv/Fm), chlorophyll degradation ratio and quantitative estimation of Al, As, Cd, Cr, Cu, Pb, Fe and Zn contained in the lichen thallus were ascertained. Statistical analysis revealed significantly positive correlations between Fv/Fm and element contents (Al and Cr). The chlorophyll degradation as well as alteration in the pigment content was found to be the most sensitive parameters to assess the vitality of lichen thallus against polluted environment. The species accumulated higher amounts of elements (Al, As, Cu, Fe and Zn) in the polluted sites as compared to the non polluted sites. It was also evident from this study that vehicular emission played a significant role in the release of elements as pollutants in the surrounding environment. The effectiveness of this lichen could be further investigated by comparing this species with other biomonitors.

Keywords Chlorophyll fluorescence · Lichens · Elements · Photosystem II

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Introduction

Automated, continuous monitoring system is a useful tool for determining levels of atmospheric contamination, but it has certain limitations too. The need for implementing such automated monitoring systems in large number of sampling sites results in high cost of the project. Alternatively, many recent studies on atmospheric pollution have used biological samples such as lichen and mosses as biomonitors (Loppi et al. 2000). Biological monitoring is based on the assumption that any changes taking place in the microclimate, significantly affects the biota can be effectively used as an early warning system, to detect environmental changes (Garty 2001). The use of biomonitors allows monitoring of several sites at low cost and in addition can integrate pollutants over long period to unlike conventionally used instruments (Adriano 2001).

Lichens are one of the most valuable biomonitors of atmospheric pollution. They can be used as sensitive indicators to estimate the biological effects of pollutants by measuring changes at community or population level and as accumulative monitors of persistent pollutants (Loppi et al. 2000). The epiphytic lichens are widely recognized as organisms sensitive to the deleterious effects of atmospheric pollution and especially to the effects of acid rain (Nimis et al. 2002). Atmospheric pollution causes a decrease in photosynthetic levels (Gries et al. 1995), and a reduction in photosynthetic pigments in lichens (Garty 2001; Bajpai et al. 2012). Lichens are known to be sensitive to many types of pollution and are suitable for assessing damage caused by air pollution. They can be used to monitor pollution in three ways by identifying and mapping all lichen species in an area, transplanting healthy lichens into a polluted area and measuring thallus deterioration and sampling of an individual species and measuring

contaminants accumulated within the thallus. Due to their differential sensitivity to air pollution, lichens are potentially useful for air monitoring purposes (Richardson 1992). However, progress in this direction can only be achieved if sensitive and reliable methodologies are developed to assess pollution-induced stress-effects on these organisms.

An application of the pulse amplitude methodology (PAM) to measure chlorophyll fluorescence is a method to monitor the status of photosystem II in lichens exposed to air pollution. An alternative method to estimate the air-pollution impact on lichens is by determination of the integrity of the photobiont Chlorophyll (Ronen and Galun 1984). In the present work, we investigated the assumption that the ratio chlorophyll *a* to phaeophytin *a* in algal cells, indicative of the status of the photobiont chlorophyll, correlates with the Fv/Fm ratio in thallus exposed to pollutants under field conditions. The accumulation of certain air pollutants in the lichen thallus is assumed to coincide with the low Fv/Fm ratios and a low degradation ratio. These three parameters are exclusively related to the photobiont. The major objective of the present study is to identify the relationship of the physiological parameters of the photosynthetic system with the elemental content of the lichen *Pyxine cocoes.*

Materials and methods

Study area and sample collection

The study was undertaken in Sitapur district of Uttar Pradesh state of India with an area of 5,743 km^2. Lichen samples were collected from six sampling sites of Sitapur district (Fig. 1). The sites were selected on the bases of anthropogenic activities detected in the area (Table 1). In each site, the samples were taken from *Magnifera indica* tree bark and fulfill the standard criteria of (a) trunk more than 35 cm in diameter, (b) trunk inclination <75° (15° deviation from vertical), (c) apparently healthy and (d) height, 1.5–2 m above the ground. Ten samples of *P. cocoes* (average size of thallus is 3.0 ± 0.5 cm diameter) were collected from each site and cleaned with a brush to remove dust, leaf debris, fungus and degraded material deposited on the surface. The samples are then carefully removed from the tree bark and

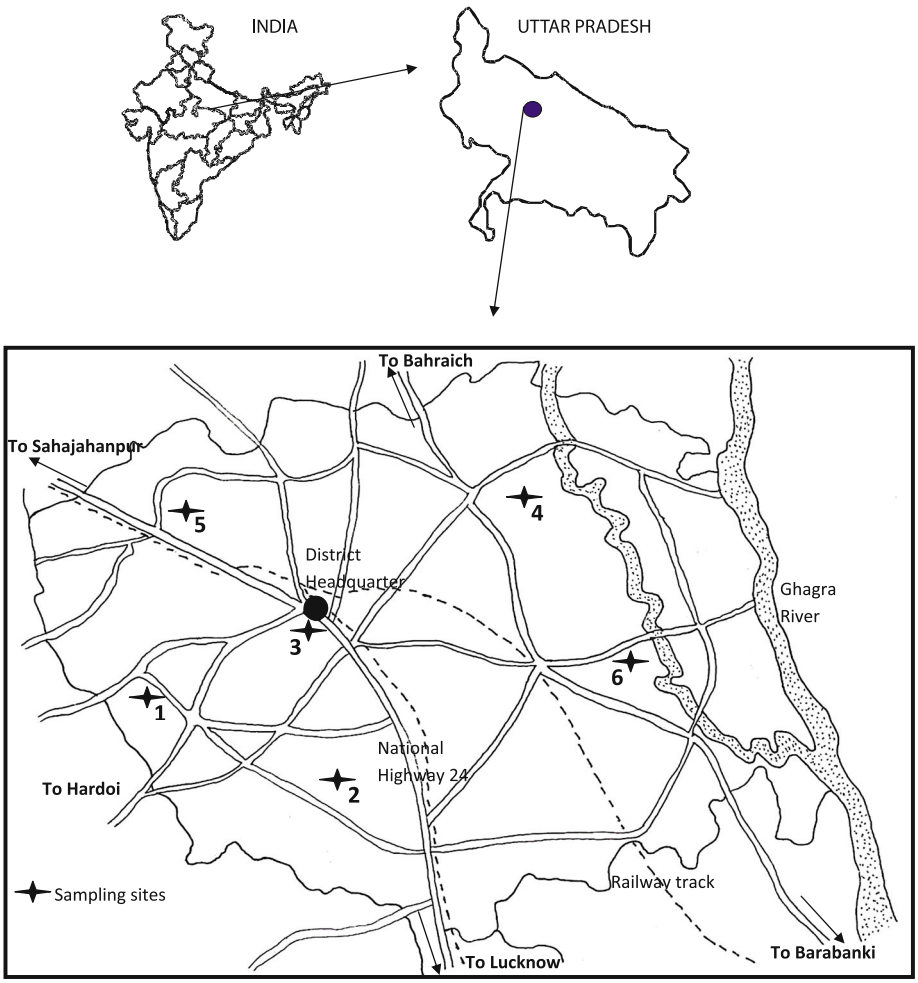

Fig. 1 Map of the study area with location of sampling sites

Table 1 Description of the sites selected for the collection of lichen

Sites	Site name	Longitude latitude	Altitude (mt.)	Remark
Site 1	Misrikh	N°27°35′53.10″ E°80°37′59.62″	139	Rice mill and sugar mill
Site 2	Naimisharanya	N°27°33′05.26″ E°80°40′40.88″	148	Traffic and other anthropogenic activities
Site 3	Ramkot	N°27°37′56.51″ E°80°40′49.11″	141	Traffic, road crossings, Sugar mills around 4–5 km
Site 4	Devicharan garden	N°27°35′26.56″ E°80°40′49.81″	140	Road side only vehicular activities
Site 5	Mohali	N°27°34′52.19″ E°80°38′57.44″	136	Traffic, railway track and other anthropogenic activities.
Site 6	Deviphoolmati Temple	N°27°35′38.45″ E°80°41′37.79″	138	No direct source of pollution

mixed together (single site) to make composite samples from which triplicate ($n = 3$) were drawn for pigments, fluorescence and elemental analysis separately.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured in the lichen *P. cocoes* with a pulse-modulated fluorometer (PAM 2500 Walz, Germany). Samples were moistened with a spray of distilled water and were dark adapted for 30 min prior to measurement. The thallus was exposed to a saturating flash of 0.6 s to measure variable to maximal fluorescence ratio (Fv/Fm). Each value was obtained as an average of ten individual measurements. The maximum quantum efficiency of the photosystem II (Fv/Fm) was calculated as described by Maxwell and Johnson (2000). The Fv/Fm ratio values obtained were used as an indicator to study effects of "stress" on the photosynthetic apparatus of *P. cocoes* (Schieleit and Ott 1994).

Chlorophyll Content

Triplicate air-dried samples from each of the sites were ground to produce sub samples of 0.5 g. Total chlorophyll ($a + b$) was determined according to Moran (1982) using 15.0 ml of dimethyl sulfoxide (DMSO; Merck, analytical grade) as an extraction solvent (Ronen and Galun 1984).

Chlorophyll degradation

Chlorophyll degradation, expressed as the phaeophytinization quotient, which reflects the ratio of chlorophyll *a* to phaeophytin *a* (Garty 2001) was assessed following method of Ronen and Galun (1984). The chlorophyll was extracted overnight in the dark in 5 ml dimethyl sulfoxide (DMSO Merck, AR grade). The ratio of chlorophyll *a* to phaeophytin was determined using Genesys 10 UV scanning spectrophotometer.

Elements estimation

Quantitative estimation of eight elements (Al, As, Cd, Cr, Cu, Pb, Fe and Zn) were undertaken. The dried lichen samples (triplicates) were ground to powder (weight $\approx$1.0 g each) and digested in mixture of concentrated HNO_3 and $HClO_4$ (v/v 9:1) for 1 h. Residues were filtered through Whatman Filter paper No. 42 and diluted up to 25 ml with double distilled water. Analysis was done with ICP-MS (Perkin Elmer SCIEX ELAN DRCe), the stock standards were used from Merck, India.

Statistical analysis

The results of the chemical and physiological analysis were evaluated by a one-way analysis of variance (ANOVA). Linear regression analysis was applied to test correlation between atmospheric pollutants (elements) and photosynthetic pigments. All statistical analysis was performed by Sigma plot Systat Software, Inc., Richmond, CA, USA.

Results and discussion

Changes in the photosynthetic activity of the lichen

The values of the chlorophyll content and potential quantum yield PSII are presented in Table 2. The total chlorophyll ($a + b$) was reported maximum (2.92 ± 0.36 μg^{-1} FW) in lichen thallus collected from site 6, i.e., unpolluted area while minimum chlorophyll content (0.92 ± 0.18 μg^{-1} FW) was found at most polluted areas (site 1 and 2). The minimum chlorophyll content at site 1 and 2 may be attributed to the high traffic density because of its location on railway crossing as well as city center and sugar mill area. The chlorophyll degradation values ranged from 1.48 ± 0.35 to 0.71 ± 0.04 in lichen collected in the surveyed area. The maximum chlorophyll degradation was observed at higher traffic area followed by industrial and lower traffic area (Table 2).

According to Garty (2001), chlorophyll degradation is one of the most frequently used parameters in lichen stress physiology expressed as the phaeophytization quotient, which reflects the ratio of chlorophyll *a* to phaeophytin *a*. In healthy lichens, the degradation ratio is about 1.4 while in polluted sites with high level of vehicular traffic activities, its values decrease. In the present study, the

Table 2 Pigment (Total chlorophyll, Chlorophyll degradation) content and Fv/Fm ratio analyzed in lichen thallus *P. cocoes* collected around Sitapur district

Pigments	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Total chlorophyll	$0.92 \pm 0.18^{a,d,i}$	$1.56 \pm 0.07^{a,d,i}$	$2.88 \pm 0.32^{a,d,i}$	$2.85 \pm 0.30^{a,d,i}$	$2.45 \pm 0.76^{a,d,i}$	$2.92 \pm 0.36^{a,d,i}$
Chlorophyll degradation	$0.71 \pm 0.04^{a,d}$	$1.15 \pm 0.41^{a,b}$	$0.95 \pm 0.04^{a,b,d}$	$0.90 \pm 0.06^{a,b,d}$	$1.48 \pm 0.35^{a,b,d}$	$0.92 \pm 0.02^{a,b,d}$
Chlorophyll *a/b*	1.29 ± 0.16^{d}	1.35 ± 0.10^{d}	$1.48 \pm 0.16^{d,i}$	$1.20 \pm 0.58^{d,i}$	$1.63 \pm 0.13^{d,i}$	$1.59 \pm 0.07^{d,i}$
Fv/Fm	$0.60 \pm 0.01^{a,b,c}$	$0.69 \pm 0.04^{a,b,c}$	$0.57 \pm 0.04^{a,b,c}$	$0.61 \pm 0.03^{a,b,c}$	$0.58 \pm 0.02^{a,b,c}$	$0.66 \pm 0.02^{a,b,c}$
One-way ANOVA						
F value	1,256.7**	987.43**	744.5**	15,667**	2,456.1**	456.1**
LSD	0.01	0.02	0.04	0.07	0.09	0.03

Values in each vertical column followed by the same alphabetic letter show there is no significant difference between them at $p < 0.05$ % level by least significant difference (LSD) analysis

Table 3 Heavy metal analysis in thallus of *Pyxine cocoes* around Sitapur district

Elements	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
Al	$1,277.33 \pm 4.04^{a}$	983.00 ± 10.14^{a}	889.33 ± 9.29^{a}	811.66 ± 10.40^{a}	772.66 ± 11.06^{a}	649.00 ± 7.93^{a}
As	1.91 ± 0.03^{b}	1.06 ± 0.07^{b}	0.92 ± 0.06^{b}	0.28 ± 0.05^{b}	0.23 ± 0.05^{b}	0.27 ± 0.04^{b}
Cd	$0.68 \pm 0.18^{b,c}$	$0.77 \pm 0.05^{b,c}$	$0.29 \pm 0.04^{b,c}$	$0.32 \pm 0.04^{b,c}$	$0.65 \pm 0.02^{b,c}$	$0.22 \pm 0.05^{b,c}$
Cr	2.9 ± 0.50^{d}	2.12 ± 0.08^{d}	1.16 ± 0.03^{d}	1.92 ± 0.12^{d}	1.09 ± 1.00^{d}	1.24 ± 0.04^{d}
Cu	8.00 ± 0.45^{e}	6.73 ± 0.33^{e}	5.48 ± 0.49^{e}	3.42 ± 0.66^{e}	2.73 ± 0.77^{e}	2.52 ± 0.32^{e}
Pb	13.36 ± 1.12^{f}	15.6 ± 0.75^{f}	10.83 ± 1.10^{f}	14.67 ± 0.48^{f}	5.8 ± 0.90^{f}	6.26 ± 0.60^{f}
Fe	663.96 ± 9.49^{g}	595.83 ± 10.55^{g}	484.84 ± 11.39^{g}	371.36 ± 8.66^{g}	319.6 ± 4.80^{g}	291.49 ± 4.75^{g}
Zn	27.16 ± 1.9^{h}	25.23 ± 0.42^{h}	21.8 ± 1.44^{h}	17.23 ± 1.7^{h}	16.63 ± 3.08^{h}	18.38 ± 1.84^{h}
One Way ANOVA						
F value	1,704.01**	443.89**	23.35**	72.05**	927.28**	54.24**
LSD	18.20	0.60	0.90	0.30	0.10	23.40

Values in each vertical column followed by the same alphabetic letter shows there is no significant difference between them at $p < 0.05$ % level by least significant difference (LSD) analysis

Mean $\pm$ SD, $n = 3$ in $\mu g g^{-1}$ Fresh weight

** Significance at the level of 0.05 %

chlorophyll degradation ratio increased with increased anthropogenic activities in the area and it significantly varied between site to site. Bajpai et al. (2010) also observed chlorophyll degradation around a coal-based thermal power plant area and found that maximum phaeophytization was observed at the vicinity of 5 km followed by 10 and 15 km in all the directions. Garty et al. (2000) also found the similar result with the lichen *Ramalina lacera*.

Distribution of elements

Accumulation of eight elements Aluminum (Al), Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Iron (Fe) and Zinc (Zn) in the thalli of *P. cocoes* were estimated (Table 3). Among eight elements, Al was accumulated in highest amount followed by Fe with the sequence of Fe > Zn > Pb > Cu > Cr > As > Cd. Maximum Al and Fe was found in the lichen collected around sugar mill and higher traffic areas (site 1 and 2). The significant variation was observed between the metals in this site. Among all the elements, Cd, Cr and As were reported in lowest concentration in all the sites. The probable reason of higher levels of metals at the site may be due to the presence of sugar and rice mills in the area. A significantly large volume of waste is generated during the manufacture of sugar and contains a high amount of pollution load particularly in terms of suspended solids, organic matter, and press mud, bagasse and air pollutants. The rice mills uses husk as fuel, which releases smoke and fly ash in the air it may be a reason for maximum Al, Fe content in this area. The above result also shows the similar findings of Kim et al. (2010) and Fang and Huang (2012). Accumulation of Al, Fe, Zn in the thalli of *P. cocoes* for all sides are significantly different from each site based on LSD analysis. Loppi et al. (1998) stated that Al and Fe two principal elements in the earth's crust are strongly correlated in lichens and environmental contaminants. In this

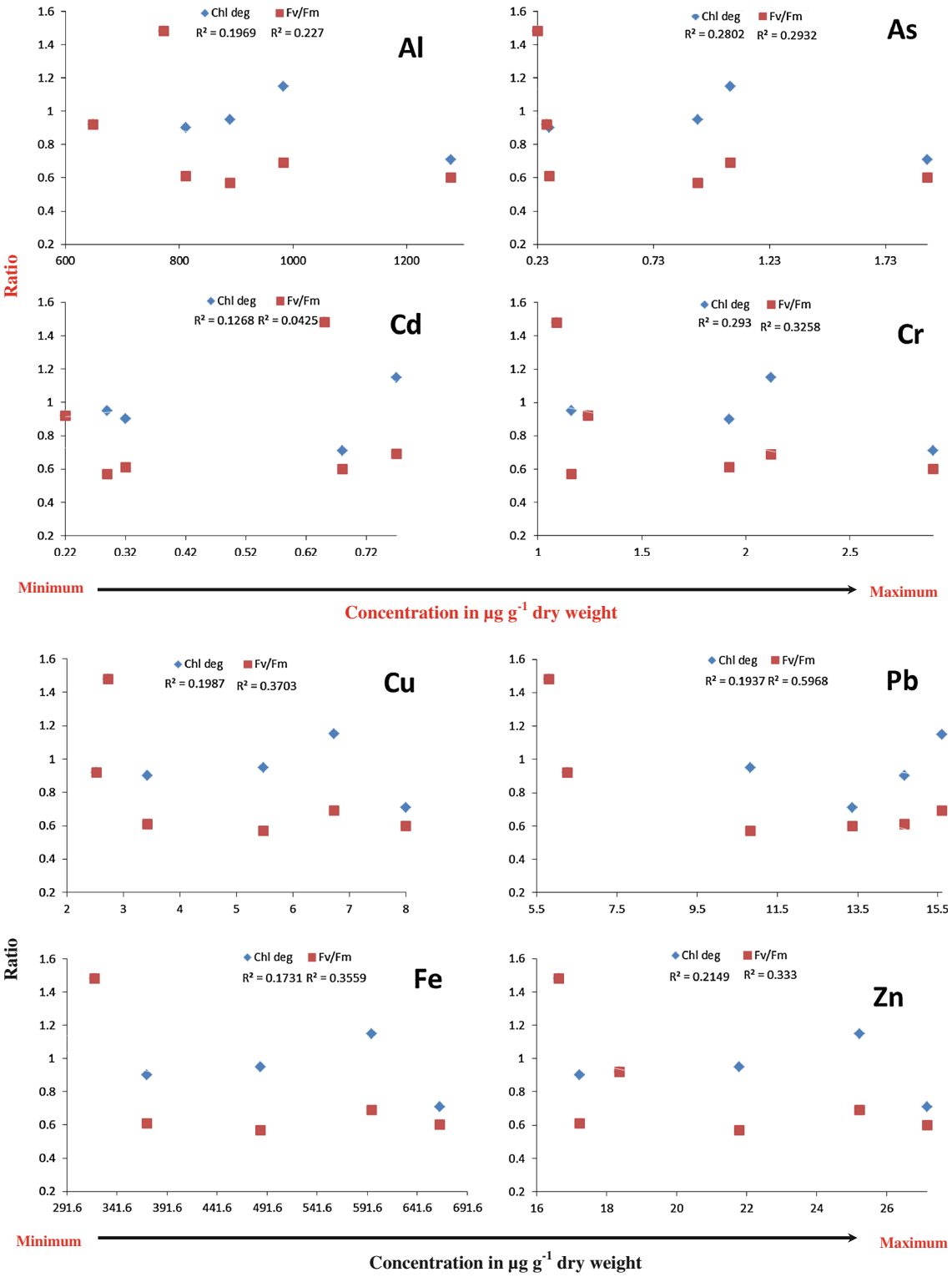

Fig. 2 Comparison between chlorophyll degradation, chlorophyll fluorescence and total elemental content found in the study area

study, Al and Fe was maximum reported at the vicinity of mill areas. A part from engine emission, Pb, Zn, Fe and Cr enter the surrounding environment due to abrasion of metallic vehicles parts. Pb indicates its origin from automobile exhaust, whereas Zn may be emitted by automobile tires and brake pads (Ward and Sampson 1989).

In the present study, arsenic also indicated its presence in the surrounding environment but detected in small

quantities as it ranges between 0.23 ± 0.05 and 1.91 ± 0.03 μg g^{-1} DW. Most of As was found around sugar mill areas followed by traffic areas. The past mining in an area, burning of fossil fuel as well as biomass and soil dust are the major source for huge deposition of As in the environment (Shukla and Upreti 2012; Wang 2013). In this study, the maximum As content in lichen collected nearby areas of mills may be due to incineration of coal/waste. According to Mrak et al. (2006), most of the Arsenic in air is present as inorganic arsenic in particulate form and transported through air.

Physiological status of the lichen relative to the elemental content of the thallus

Figure 2 shows fluorescence, chlorophyll degradation and correlation between the elemental content at different sites. The Fv/Fm positively correlated with all metals but the maximum positive correlation with Pb followed by Cu, Fe and Cr as R^2 values represented as 0.596, 0.370, 0.355 and 0.325, respectively. Fv/Fm is generally considered as an indicator of health of a photosynthetic organism and can be utilized to prepare their vitality index. In healthy, unstressed vascular plants, the ratio of Fv/Fm falls to 0.832. Values lower than 0.832 of Fv/Fm will be seen when exposed to stress, due to photoinhibition (Maxwell and Johnson 2000).

The reliably dead plant material, exhibits values to be <0.1. In normal lichens without stress the values range from 0.6 to 0.76 and sometimes healthy crustose and cyanolichens have lower values such as 0.5–0.6 (Jensen and Kricke 2002). In the present study, the Fv/Fm values ranges between 0.58 to 0.69 indicate the health of lichen thalli in the area whereas the same thallus contain the huge amount of elements in their thallus. Garty et al. (2000) also reported the similar result with the transplanted *R. lacera* lichen at 20 different sites having different degrees of pollution level and found that Fv/Fm ratios were correlated with Al, Cd, Cr and Cu elements. The effect of pollution on chlorophyll fluorescence revealed that there is a rapid change in induction kinetics of chlorophyll fluorescence (Gries et al. 1995).

Conclusion

The present study indicates that Pb, Cu, Fe and Cr significantly affect the fluorescence ratio of lichen whereas chlorophyll degradation was slightly affected. The present work reveals a link between chemical contamination and the lost vitality response of the photobiont in the lichen *P. cocoes*. An examination of the accumulated elements which exhibited a significant correlation with each sites and shows that maximum accumulation of elements were found around industrial area (sugar mill) followed by higher traffic as well as road side areas. The Fv/Fm ratio, chlorophyll degradation ratio and elemental contents in *P. cocoes* indicate the lichen under stress and its functions observed as environmental sensor. This study provides baseline data on metal concentration at different sites which will be helpful for carrying out future biomonitoring studies in the area.

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