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Physiochemical Response of the Lichen genus *Everniastrum* as Bioindicator of Ambient Air Nitrogen Deposition along with an Elevation Gradient in a Temperate-alpine Habitat of Western Himalaya

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ABSTRACT

The high elevation habitats of the Himalayas in the past few decades are experiencing intense land use changes due to tourism-related anthropogenic activities. These habitats receive moderate to higher deposition of Nitrogen through vehicular, agricultural practices, and other urban exhausts. Nitrophobic lichens of the family Parmeliaceae largely populate the high elevation habitats. The current study explores the capability of the Parmelioid genus *Everniastrum* as a bioindicator of nitrogen pollution in a temperate-alpine habitat. The study explored the correlation between the nitrogen accumulation in the thallus of two common *Everniastrum* species of the landscape to the photobiont chlorophyll, carotenoids, and chlorophyll degradation. The study concluded that one of the species *Everniastrum cirrhatum* can be an appropriate bioindicator of ambient air N deposition and can be used in long-term biomonitoring experiments.

INTRODUCTION

The Himalayas are among some of the youngest montane landscapes in the world. The Himalayan environments are characterized by steep topographical inclines resulting in large-scale variations in associated climatic parameters such as atmospheric pressures, temperature, precipitation, and edaphic parameters (i.e., soil pH, microbial activity, water availability, etc.). The heterogeneous changes in the Himalayan habitats have resulted in stratified eco-climatic zones, i.e., subtropical foothills ($\leq$2,000 m), temperate habitats ($\leq$3,000 m) at intermediate elevations, and alpine at higher elevations ($\geq$3,000 m) (Singh and Singh 1987). The zonation of ecosystems ultimately epitomized in vegetational stratification of the higher plant as well as symbionts such as lichens (Singh and Singh 1987, Upreti 1998). The natural vegetation in the Himalayas, due to the increased human inhabitation and related rapid change in land use, has undergone a drastic change in the community structure of forests and ecosystem services in the last few decades (Rao and Pant 2001; Tiwari 2008, Fayaz et al., 2020; Shrestha et al., 2022). Among the various anthropogenic perturbations excessive ambient air nitrogen (N) deposition has been recognized as one of the nine factors that have reached such a critical level that is threatening the very existence of humanity on the planet (Rockstrom et al. 2009).

Lichen, a symbiosis between a dominant fungus and algae and/or cyanobacteria, has frequently been used for biomonitoring various pollutants, e.g., heavy metals, Polycyclic Aromatic Hydrocarbons (PAHs) etc. (Garty 2001; Lucadamo 2021). In the recent two decades, lichens have been extensively studied as bioindicators/ biomonitors for nitrogen pollution, predominantly in Europe (Rai and Gupta 2022). The lichen biomonitoring studies in Asia are limited to a few studies related to floristic analysis, heavy metal, and PAH accumulation in lichens (Rai and Gupta 2022). The current study was done in the temperate-alpine woodland and meadows of the western Himalayas to determine the ambient air nitrogen biomonitoring capabilities of a common parmelioid lichen genus *Everniastrum* along an elevation gradient and the increasing distance from the motor road, an apparent point source for N pollution.

MATERIALS AND METHODS

Study Area

The study was conducted in the Khaliya landscape, a temperate-alpine woodland, and meadow near the town of Munsiyari, Pithoragarh district in the western Himalayan state of Uttarakhand (Fig. 1). The Khaliya landscape is highly montane with a steep slope (i.e., 60°-90°) and

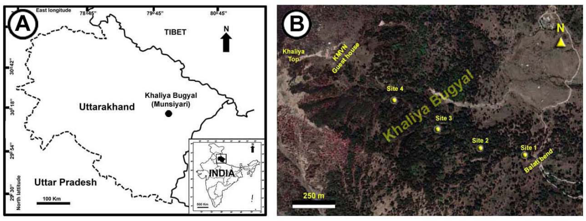

Fig. 1: Location map of sampling sites in Khaliya landscape, Munsiyari, Pithoragarh, western Himalaya.

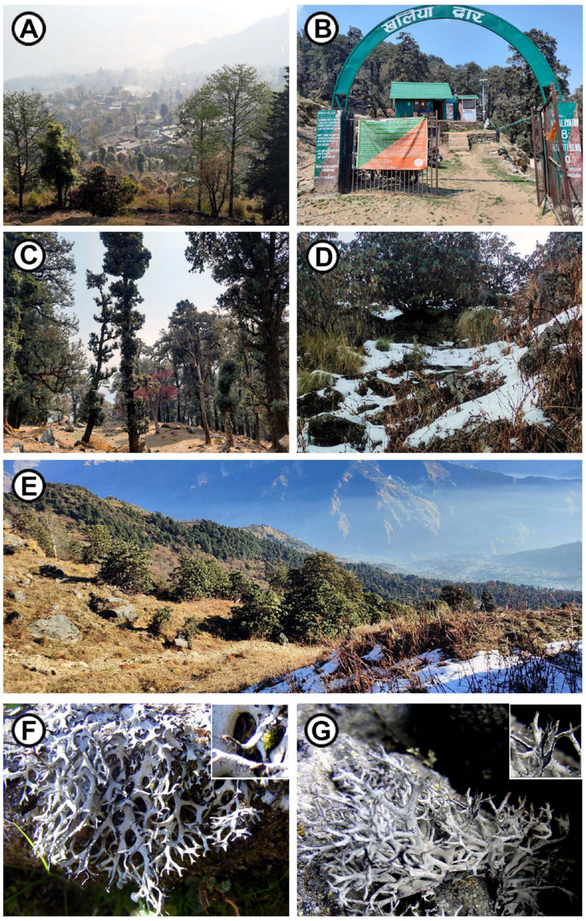

Fig. 2: Environs of Khaliya landscape, Munsiyari, Pithoragarh, western Himalaya. A. The Munsiyari township, B. The official approach gate to the Khaliya landscape forests at Balati bend, C. The open canopy Quercus-Rhododendron mix woodland at mid-elevations, D. Alpine scrubland at higher elevations (2700-3000 m), *E. Scrubland* fading into alpine grasslands (≥3100 m) near the highest point of the Khaliya landscape the Khaliya top (about 3500 m), *F. Habitus of Everniastrum nepalense, G. Habitus of Everniastrum cirrhatum.* The insat images in F and G highlight the exposed ventral portion of respective thallus.

elevation ranging from 2653–3437 m. The vegetation of the area is dominated by *Quercus-Rhododendron* mixed forest up to 2800 m (Fig. 2C), which fades in initially into alpine scrubland dominated by stunted *Rhododendron* coppices followed by alpine grassland above 3000 m elevation. The alpine grassland at higher elevations is dominated by grasses and frequently traversed by patches of Rhododendron coppices (Fig. 2E). The woodland is a predominately open canopy forest. Throughout the Khaliya landscape area, large open temperate grasslands are maintained through permit-controlled grazing by traditional Gujar herders. Khaliya landscape, though has traditionally been conserved by the forest department as a medicinal plant conservation area, is a famous hiking destination in Kumaon Himalayas, now regulated through monetary fee levying. Land use by the local human population ranges from urban to semi-pastoral. As the town in the last two decades has developed in major hiking and other tourist activities, the urbanization has led to higher vehicular density and increased amount of ammonia/ nitrogen deposition through automobile and domestic exhaust. The study was conducted on the two species of *Everniastrum*, i.e., *Everniastrum cirrhatum* (Fr.) Hale (Fig. 2F) and *Everniastrum nepalense* (Taylor) Hale ex Sipman (Fig. 2G), collected from four sites in the Khaliya landscape.

Field Methods and Data Recording

The sampling of *Everniastrum* was done using a two-way random stratified sampling method where the distance from the road, the primary source of nitrogen pollution was the primary stratifying variable along the increasing elevation gradient of the study area (Greig-Smith 1983; Krebs 1989; Holt et al. 2007; Rai et al. 2012). A portion of collected samples was curated for taxonomic authentication in handmade standardized paper packet folds. About 3-6 grams of sample was pooled from each site and was transported to the laboratory in zip-locked airtight polyvinyl bags to avoid further contamination. The geotagging, elevation data, and distance from the road of sites were recorded using a hand-held GPS unit (Garmin GPSMAP® 76S™).

Lichen Sample Identification and Authentication

The lichen samples were identified up to the species level at the lichenology laboratory, CSIR-National Botanical Research Institute (CSIR-NBRI), Lucknow, Uttar Pradesh, India, using standard morpho-anatomical examination, chemical tests (i.e., spot tests, thin-layer chromatography, and relevant taxonomic literature (Awasthi 2007; Orange et al. 2001; Elix 2014). The samples were authenticated through permanent deposition as voucher specimens in the herbarium LWG of CSIR-NBRI.

Table 1: The lichen genus collected from the four sites of Khaliya landscape forests, and their geo-topological attributes

Sites	*Elevation (m)*	*Coordinates*	*Lichens*	*Canopy cover*	*Distance from road (m)*
Site 1	2683	N 30° 03' 34.3" E 80° 13' 11.6"	*E. cirrhatum*	Open	280
Site 2	2848	N 30° 03' 36.9" E 80° 12' 57.6"	*E. cirrhatum*	Moderately closed to open	780
			E. nepalense		
Site 3	2915	N 30° 03' 38.7" E 80° 12' 52.3"	*E. cirrhatum*	Moderately closed to open	1300
			E. nepalense		
Site 4	3092	N 30° 03' 46.9" E 80° 12' 40.8"	*E. nepalense*	Open	2500

Table 2: The percentage nitrogen, corresponding pigment concentration and chlorophyll (Chl.) degradation in lichen thallus of the lichens collected from the four sites of Khaliya landscape forests.

Sites	*Lichens*	*(%) Nitrogen*	*Chl. a*	*Chl. b*	*Total carotenoids*	*Chl. degradation ratio*
Site 1	*E. cirrhatum*	1.49 ± 0.23	0.19 ± 0.01	0.25 ± 0.01	0.70 ± 0.04	0.78 ± 0.01
Site 2	*E. cirrhatum*	0.68 ± 0.37	0.39 ± 0.03	0.33 ± 0.01	0.68 ± 0.01	1.01 ± 0.03
	E. nepalense	0.38 ± 0.10	0.53 ± 0.01	0.17 ± 0.02	0.68 ± 0.02	1.00 ± 0.01
Site 3	*E. cirrhatum*	0.26 ± 0.22	0.68 ± 0.01	0.38 ± 0.02	0.55 ± 0.02	1.11 ± 0.01
	E. nepalense	0.26 ± 0.35	0.55 ± 0.02	0.27 ± 0.01	0.44 ± 0.03	1.12 ± 0.03
Site 4	*E. nepalense*	0.30 ± 0.15	0.62 ± 0.02	0.48 ± 0.02	0.68 ± 0.01	1.38 ± 0.01

Analysis of Thallus Elemental Nitrogen (N) Content

The percentage thallus N concentration was used as a proxy for the estimation of ambient air N deposition. Total thallus N content was estimated using the flash combustion procedure with a CNS-O Elemental Analyzer Vario EL III. Air-dried lichen thallus in triplicates of 10 mg for each estimation was used. Sulphanilamide was used as the standard to ensure the accuracy and recovery of the elemental N.

Extraction and Estimation of Photobiont Chlorophyll (Chl.) and Chl. Degradation

Chl. a, Chl. b and total carotenoids were assayed using the spectrophotometric method (Boonpragob 2002) using DMSO as the sole extracting solvent (Karakoti et al. 2014; Králiková et al. 2016) employing Wellburn equations (Wellburn 1994; Králiková et al. 2016). About 20 mg of air-dried lichen thallus was kept in 15 ml of DMSO in a borosilicate test tube (sealed with parafilm), in dark at room temperature overnight for extraction of pigments. The aliquot was removed from the extraction mixture and was analyzed against a blank of 100% DMSO in a UV-Vis spectrophotometer at a specific wavelength according to Wellburn equations (Wellburn 1994). The pigment concentration is expressed in $\mu g\ ml^{-1}$. The Chl. degradation was estimated as the ratio of Chl. a to phaeophytin spectrophotometrically by measuring the A435 nm: A415 nm (Ronen and Galun 1984).

Data Analysis

The bivariate two-tailed correlation analysis was done (Pearson's correlation coefficients) to compare explanatory variables– elevation and, distance from road and response variable– lichen thallus % N concentration, Chl. a, b, carotenoids, and Chl. degradation using IBM® SPSS® Statistics ver. 20 (Rai et al. 2012; Gupta et al. 2017).

RESULTS

The study recorded two *Everniastrum* species in the four sites sampled along the increasing elevation gradient of the Khaliya landscape – *E. cirrhatum* and *E. nepalense* (Table 1). The geo-topographical attributes of collection sites are given in Table 1. The physiochemical analysis of the samples recorded decreasing % N thallus concentration, and total carotenoids, along the increasing elevations in both the *Everniastrum* species (Table 1). The study further recorded an increase in Chl. a, b and Chl. degradation ratio along the increasing elevation of the Khaliya landscape.

Table 3: Values of correlation coefficient between the thallus % nitrogen concentration, various chlorophyll (Chl.) parameters and site of collection distance from road in *Everniastrum* cirrhatum in the Khaliya landscape forests.

	(%) Nitrogen	Chl. a	Chl. b	Total carotenoids	Chl. degradation ratio
Chlorophyll a	-0.959				
Chlorophyll b	-0.999[a]	0.972			
Total carotenoids	0.836	-0.957	-0.861		
Chlorophyll degradation ratio	-0.999[a]	0.946	0.996	-0.811	
Distance from road (m)	-0.982	0.996	0.990	-0.925	0.973

[a]Correlation is significant at the 0.05 level (2-tailed).

Table 4: Values of correlation coefficient between distance of collection site from road, thallus % nitrogen concentration and, the various chlorophyll (Chl.) parameters in *Everniastrum nepalense* in the Khaliya landscape forests.

	Distance from road (m)	(%) Nitrogen	Chl. a	Chl. b	Total carotenoids
(%) Nitrogen	-0.470				
Chlorophyll a	0.960	-0.697			
Chlorophyll b	1.000[a]	-0.490	0.966		
Total carotenoids	0.223	0.756	-0.058	0.201	
Chl. degradation ratio	1.000[b]	-0.483	0.964	1.000[b]	0.208

[a]Correlation is significant at the 0.05 level (2-tailed); b Correlation is significant at the 0.01 level (2-tailed).

The correlation analysis concluded that the % N thallus concentration in the *E. cirrhatum* was negatively correlated to the Chl. a, Chl. b, Chl. degradation ratio -OD 435/OD 415 and distance from road (Table 3). The study also concluded that in the *E. cirrhatum* total carotenoids were positively correlated with %N thallus concentration (Table 3). Further, the correlation analysis also found that Chl. a and b were negatively correlated with total carotenoids (Table 3). Chl. degradation ratio and total carotenoids were negatively correlated with the increasing distance of the sampling sites from the road (Table 3). The bivariate correlation analysis in *E. nepalense* recorded a negative correlation between the increasing distance of the sampling sites from the road and % N thallus concentration, whereas Chl. a-b, total carotenoids, and Chl. degradation ratio was positively correlated (Table 4). Further, in *E. nepalense* Chl. a, Chl. b and, Chl. degradation ratio was negatively correlated with % N thallus concentration, whereas total carotenoids were positively correlated (Table 4).

DISCUSSIONS

Both the recorded lichen species showed higher concentrations of nitrogen in their thallus from the sites in proximity to the apparent source of nitrogen pollution i.e., vehicular exhaust from the road and domestic exhaust from the Munsiyari township (Frati et al. 2006; Tretiach et al. 2007; Gupta et al. 2013; Pinho et al. 2014; Rai and Gupta 2022). The higher N thallus content near the pollution source correlated with lower Chl. a, Chl. b and higher carotenoid content in both species (Riddell et al. 2008; Satya and Upreti 2009; Rai and Gupta 2022). In both the *Everniastrum* species the samples collected near the N pollution source recorded lower Chl. degradation ratio due to higher phaeophytinization of chlorophyll a, which was in accordance with Satya and Upreti (2009) and Frati et al. (2011). The increased ambient air N in the form of NOx and/ or NHx makes the atmosphere acidic which causes a decrease in photobiont chlorophyll and increase in the degradation of chlorophyll a to phaeophytin a, decreasing the chlorophyll degradation ratio (Garty et al. 2000). Among the two *Everniastrum* Species, *E. nepalense* was found inhabiting mid to higher elevation of the landscape, away from the primary source of pollution (i.e., vehicular exhaust), which was the reason that the chlorophyll physiology was less correlated/affected by N pollution (Malaspina et al. 2018; Rai and Gupta 2022).

CONCLUSION

The Khaliya landscape is influenced by intense anthropogenic land use ranging from extensive agriculture, animal husbandry, and urbanization which act as the source of extensive discharge of ammonia and nitrate/

nitrites. These N pollution sources negatively affect the photobiont physiology of the foliose Parmelioid lichens, which predominately grow in the region. The study hereby highlighted the N pollution indicator capability of *Everniastrum*. Among the two lichens species specifically, *E. cirrhatum* showed a high correlation with biochemical parameters of plastids and their degradation, which can act as appropriate biomonitoring lichen species for higher elevational habitats in the Himalayas.

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