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Contrasting physico-chemical responses in Himalayan lichens as indicators of nitrogen and heavy metals stress

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ABSTRACT

The shortage of data on nitrogen (N) air pollution in the Himalayas points to the opportunity to utilise lichens as bioindicators to understand ecological impacts. In this study, we examine the chemical variability of two widely occurring lichens (*Usnea* spp., *Hypotrachyna* spp.), considering physico-chemical properties and responses along two Nepalese forest transects representing N pollution gradients, along which we measured atmospheric ammonia (NH_3) concentrations for the very first time in the region. The measured atmospheric NH_3 concentrations ranged from 3.01–4.84 μg m^{-3} in the Kathmandu transect and 2.51–4.74 μg m^{-3} in the Annapurna Conservation Area (ACA) transect. We found higher thallus N, ammonium, and metal ion concentrations in both lichen species closer to local air pollution sources. The highest values were observed for lichens at the ACA transect, including for both physico-chemical parameters (electrical conductivity, chlorophyll content, chlorophyll fluorescence, phenolic content) and oxidative responses (radical scavenging, catalase activities), consistent with the higher levels of NH_3 air pollution. We conclude that the atmospheric NH_3 concentration has already exceeded the ecological threshold for effects on lichens in this region, highlighting a major risk of biodiversity loss. In the absence of large-scale air quality monitoring, the measured physico-chemical properties and oxidative responses can be used to inform the application of lichens as bioindicators of N and metal pollution. Further studies on other lichen species are recommended to better understand the functional biology explaining contrasting responses between lichen species of the Himalayan and (sub)tropical regions to strengthen their applicability for ecological monitoring.

1. Introduction

Lichens, a symbiosis between a fungal heterotroph and an algal/cyanobacterial autotroph, have a propensity to accumulate atmospheric nitrogen (N) (Munzi et al., 2019; Wolseley et al., 2006). Moreover, some lichens have the potential to accumulate large amounts of other environmental elements, such as heavy metals and are considered hyper-accumulators (Osyczka and Rola, 2019). Accumulation of atmospheric N compounds and metal ions may occur through both dry and wet deposition (Root et al., 2021; Zhang et al., 2021), while through surface complexation, bio-mineralisation and dust trapping for metals are also possible (Anderson et al., 2022). Due to the lack of a root system or protective epidermis, lichens readily absorb both nutrients and pollutants through their thallus surfaces, sometimes even exceeding the threshold required to maintain their physiological functioning, which can ultimately change the community composition of lichen species and

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their physico-chemical properties (Çobanoğlu, 2020; Herzig et al., 1989; Oishi, 2019; Wolseley et al., 2006).

Due to their unique biochemistry, including susceptibility to toxins, lichens are sensitive to changes in air quality and are a useful tool for monitoring changes in the environment and ecosystem health (Oishi, 2019; Ristić et al., 2021; Yang et al., 2023). Although there are possibilities for lichens to cope with extreme environments by enhancing their resistance to toxins, the disappearance of sensitive lichens with only a slight increase in pollution level is a common phenomenon (Farkas et al., 2022). Thus, assessment of the abundance and diversity of particular lichen species (either sensitive or tolerant) is commonly used to gauge the severity of pollution on the local or regional scale. Moreover, the evaluation of changes in physico-chemical and biochemical responses of lichens is a reliable technique for tracking their integrity and functioning (Frati and Brunialti, 2023). Studies focused on biomonitoring have highlighted the deterioration of lichen thallus health, including photosynthetic abilities, chlorophyll pigmentation, escalation in membrane damage and oxidative stress caused by pollution (Abas, 2021). Such understanding of the connection between environmental contaminants and physico-chemical responses may offer new possibilities in using lichens for evaluating the impacts of pollutants on terrestrial habitats.

The world's environment has undergone significant changes, notably in terms of air pollution (Feng et al., 2017). It is now well established that the Indo-Gangetic Plain (IGP) is a hotspot for atmospheric ammonia (NH_3) (Kuttippurath et al., 2020; Van Damme et al., 2018). Alongside agricultural emissions, vehicular emissions, fossil fuel burning, forest fires, household combustion and construction work, among others, contribute to high levels of air pollution in this region, which includes fine particulate matter ($PM_{2.5}$), a range of N species, carbon species, sulfur species and greenhouse gases (Perera, 2018; Van Damme et al., 2015). Moreover, atmospheric blocking by the Himalayas can lead to stagnant or recycling airflow in the IGP, where atmospheric concentrations accumulate within the mixing layer, exacerbating pollution levels (Móring et al., 2021; Pawar et al., 2021; Tomlinson et al., 2025).

Recent desk-based meta-analysis studies warn that Himalayan forests may exceed 'critical loads' for atmospheric nitrogen deposition and 'critical levels' for atmospheric NH_3 concentrations (Delves et al., 2023; Ellis et al., 2022). This suggests that increasing emissions of N-based pollutants in the region represents a significant threat to biological diversity, especially for forest health and lichen bioindicators. However, there is a research gap on the physiological reactions and responses of lichens to N and heavy metal exposure in their natural Himalayan habitats. Previous assessments have relied on the transfer of information from heavily researched European and North American temperate environments, which may not be comparable ecologically.

Studies of potential lichen bioindicators are lacking in South Asia, with information restricted to a limited number of situations from some parts of India, Sri Lanka and Pakistan (Bajpai et al., 2023; Dilrukshi et al., 2024; Edirisinghe and Athukorala, 2024; Firdous et al., 2017; Gupta et al., 2016; Pulak Das et al., 2013). It should be noted that not all such bioindicator properties are targeted for NH_3 and wider N air pollution. The use of thallus physico-chemical properties and responses in lichens is crucial to create robust field-based references for the Himalayan forests of Nepal. This is needed as a basis to monitor environmental stresses within naturally variable habitats and micro-climatic conditions in the absence of large-scale air quality monitoring (Pradhan et al., 2025a).

To address these issues, the present study quantified the thallus N, ammonium (NH_4^+), and metal ion contents in two potentially sensitive Himalayan lichens: *Usnea* spp. Dill. Ex. Adans. (1763) and *Hypotrachyna* spp. (Vain.) Hale (1974) from two distinct forests of Nepal, along with monitoring of atmospheric NH_3 at the same locations from where the lichens were sampled. We further examined the lichen signatures of physico-chemical responses to pollution and impacts related to oxidative stress, which can be used in bioindication to monitor the integrity of ecosystem health. Our analysis is based on measurements from two contrasting transects in the forest zone of Nepal (2200–3200 m above sea level (m a.s.l.)).

Our hypotheses were:

i) there is a significant impact of atmospheric NH_3 on thallus physico-chemical properties and responses,
ii) there is a significant impact of thallus N (under different levels of air pollution) and metal ions on the physico-chemical and oxidative responses of the lichens,
iii) there is a significant impact of environmental co-variables on responses of lichens and
iv) there is a consistent response in both indicator species.

2. Materials and methods

2.1. Study area

This study was carried out in two distinct forests; Ganeshdevi Bandevi community forest of Chandragiri, Kathmandu (KTM) (Central Nepal, peri-urban environments; 27°40'17.53"N 85°12'28.81"E to 27°39'59.46"N 85°12'21.96"E) and Ghorepani-Ponhill forest of Annapurna Conservation Area (ACA), Myagdi (Western Nepal, rural environment adjacent to the village; 28°24'11.78"N, 83°41'51.02"E to 28°23'57.72"N, 83°41'25.69"E) (Fig. 1A-C). In both cases, atmospheric NH_3 and lichen measurements were conducted along a sub-1 km transect away from postulated urban pollution sources in KTM, and near postulated rural pollution sources in ACA (Fig. 2). Additionally, total N deposition and its components were simulated using the South Asian application of the European Monitoring and Evaluation Programme with Weather Research and Forecasting (EMEP-WRF) model (Wang et al., 2025). This model implementation combines the EMEP model (Simpson et al., 2012) with the WRF model (Skamarock et al., 2019), applied over South Asia using 0.11° × 0.11° resolution. The initial projection with a 0.33° × 0.33° resolution was carried out using the global emissions dataset from the Hemispheric Transport of Air Pollution (HTAP) v.2 product for 2010 (Galmarini et al., 2017; Janssens-Maenhout et al., 2015) and 2015 WRF meteorology (Powers et al., 2017; Skamarock et al., 2019). The projection of 0.11° × 0.11° resolution using the same emissions data with 2018 WRF meteorology was used for this work. The details of the model configuration can be found in (Ellis et al., 2022).

The KTM site is located at an elevation of 2200–2550 m a.s.l. and is dominated by evergreen *Quercus semecarpifolia* forest with dominant *Usnea*, *Ramalina*, *Hypotrachyna*, *Parmotrema*, and *Heterodermia* lichen genera. This site was expected to receive a large amount of air pollution from massive infrastructure development and vehicular movement in the Kathmandu Valley. The modelled total N deposition is 16.33 kg N ha^{-1} yr^{-1}, where the wider KTM area experiences modelled deposition in the range of 10.94–17.64 kg N ha^{-1} yr^{-1} (Fig. 1D). The simulated NH_3 concentration for the same 0.11° × 0.11° grid square is 6.27 µg m^{-3}, where the wider KTM area experiences estimated ambient NH_3 concentrations in the range of 5.04–6.67 µg m^{-3} (Fig. 1F). Further, the ACA site is located at an elevation of 2700–3200 m a.s.l. and encompasses the world's largest primary *Rhododendron* Forest in Ghorepani, Poonhill area. The sampling site is dominated by mixed *Rhododendron* spp. and *Q. semecarpifolia* forest. The dominant lichen genera of this forest are *Usnea*, *Ramalina*, *Parmotrema*, *Hypotrachyna*, *Cladonia*, *Leptogium*, *Physcia*, *Bryoria*, *Lobaria* and *Heterodermia*. Simulated total N deposition for the relevant 0.11° × 0.11° grid is 24.24 kg N ha^{-1} yr^{-1}, where the wider ACA experiences modelled deposition in the range of 2.08–37.09 kg N ha^{-1} yr^{-1} (Fig. 1E). The simulated NH_3 concentration for the same grid square is 4.90 µg m^{-3}, where the wider ACA experiences estimated ambient NH_3 concentrations of 0.25–6.77 µg m^{-3} (Fig. 1G).

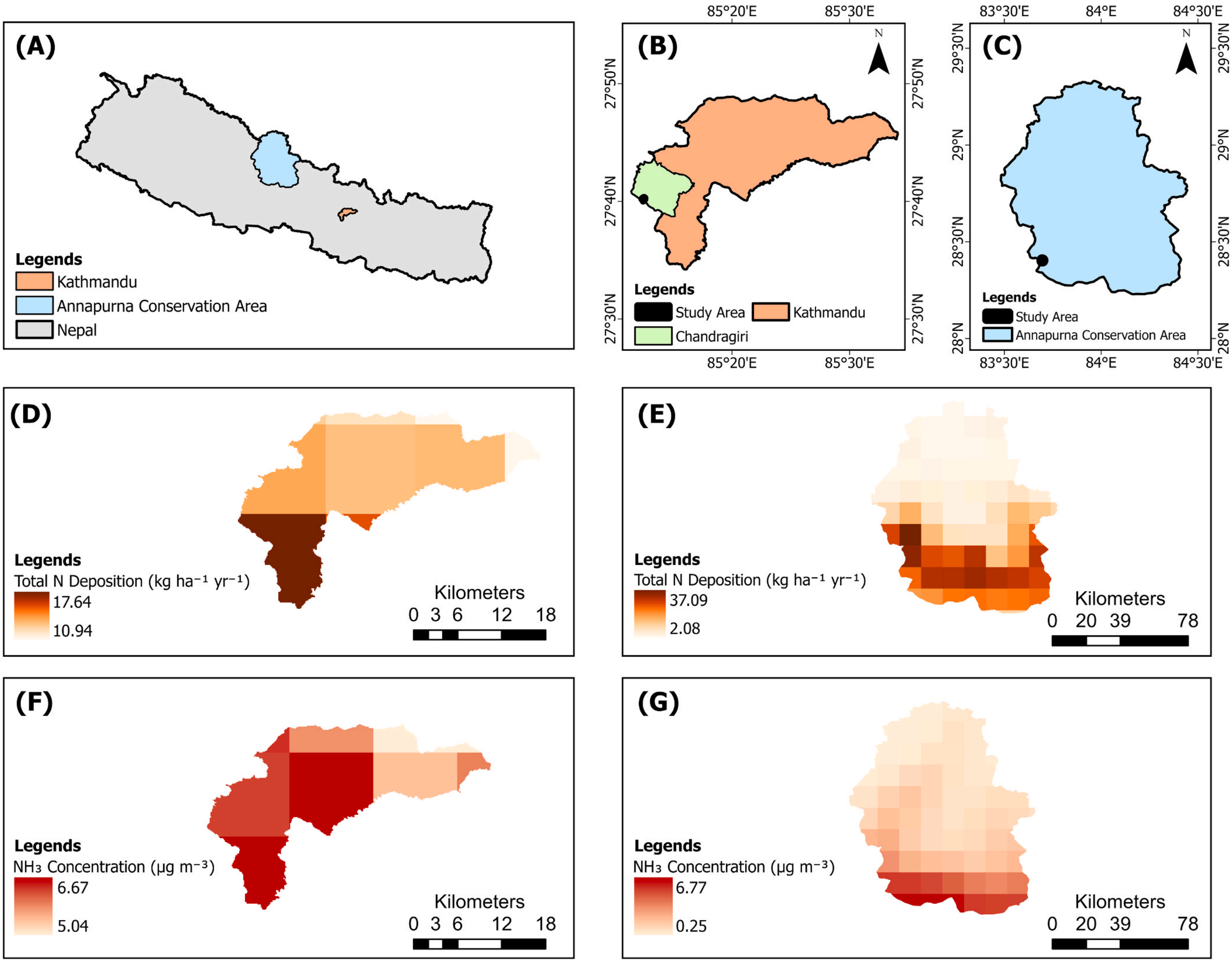

Fig. 1. Study area. A) Map of Nepal showing study area, B) Chandragiri in KTM, C) Ghorepani in ACA, D) Modelled total N deposition in KTM, E) Modelled total N deposition in ACA, F) Modelled ambient NH_3 concentration in KTM and G) Modelled ambient NH_3 concentration in ACA. The model estimates using the EMEP 2010 emission and WRF Chemistry for 2018 (Ellis et al., 2022).

2.2. *Measurement of atmospheric NH_3*

Atmospheric NH_3 concentrations were measured in triplicate sets at five locations in each transect (Fig. 2) using Adaptive Low-cost Passive High Absorption (ALPHA©) passive samplers (Tang et al., 2001). Operational constraints meant that it was only possible to conduct these measurements after the lichen samples were collected (Section 2.3), with NH_3 monitoring conducted for 12 months from January to December 2023. Strictly, the measurement of atmospheric NH_3 after the collection of lichen samples limits direct causal inference on results interpretation. However, based on extensive monitoring of NH_3 at other locations (Tang et al., 2021) and noting the mainly agricultural origin of NH_3 emissions, we expect the relative spatial patterns of NH_3 concentrations across sites to remain similar, which provides a reasonable basis for comparison and data interpretation. The temporal variation in atmospheric NH_3 has a very strong seasonal trend in other parts of the world and concentrations follow similar monthly trends across years (Tang et al., 2018) because of the direct influence of agricultural activities on NH_3 emissions. The atmospheric NH_3 monitoring in this study includes the same seasonal period, i.e., September-October months of the following year of lichen samples collection – which had comparable environmental conditions – thus providing a basis for interpretation of relationships between lichen parameters and atmospheric NH_3 concentration. Atmospheric NH_3 in general shows characteristic seasonal patterns, with modest differences between years (Tang et al., 2018; Van Damme et al., 2021). Atmospheric NH_3 concentrations in the Himalayan Forest have exceeded critical ecological thresholds across seasons (Deshpande et al., 2025). The measured data gives an indication of how NH_3 concentrations varied along each transect, for which the data are considered representative.

All analyses were conducted following the methodology described in (Deshpande et al., 2024). The ALPHA samplers are very sensitive and allow measurement of NH_3 concentration in the range 0.03–100 μg m^{-3}. After field exposure, the ALPHA samplers were analysed at the National Centre for Sustainable Coastal Management (NCSCM), Chennai, India, using an SA 5000 wet chemistry analyser (Skalar Laboratory Automation, The Netherlands). The ALPHA samplers' field-calibrated sampling rate of 3.24×10^{-3} mm^3 hr^{-1} under UK conditions (Martin et al., 2019) was adjusted to 4.31×10^{-3} mm^3 hr^{-1} under local Himalayan conditions using the ideal gas law (mean annual temperature: 24 °C, mean annual atmospheric pressure: 80 kPa). Other supporting measurements of wet-deposited N are to be reported in future publications.

2.3. *Collection of lichens, bark and soil samples*

Samples of *Hypotrachyna* spp. and *Usnea* spp. were collected in

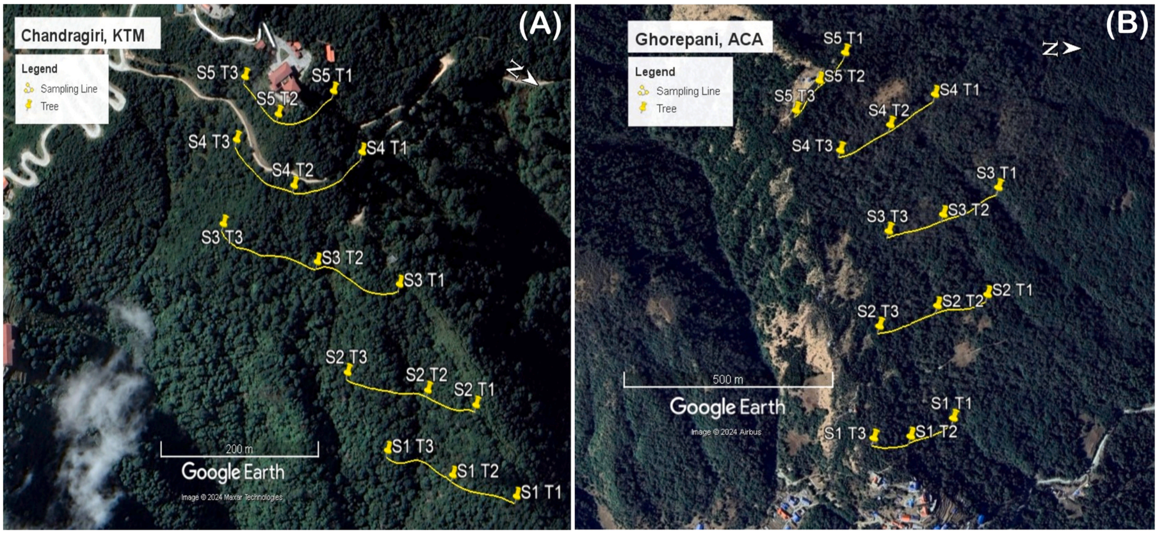

Fig. 2. Details of sampling lines and trees in each 1 km transect: A) Chandragiri, KTM and B) Ghorepani, ACA. Five positions along each transect were taken based on increasing elevation (S1-S5), with three replicated trees (T1-T3) at each elevation. Triplicated sets of ALPHA samplers were placed near each sampling line for the atmospheric NH_3 monitoring. At KTM, the nearest built-up urban area is 2–3 km east of the S1 sites. At ACA, the village of Ghorepani (approximate population of 1000 local inhabitants) is 100 m east of the S1 sites.

September 2022, based on random stratified sampling from the trunks of *Q. semecarpifolia* in KTM and from the trunks of *Q. semecarpifolia*, *Rhododendron arboreum*, *Betula utilis*, *Magnolia champaca*, *Pinus wallichiana* and *Abies* sp. in ACA. The selection and combination of three common species as a single sample of each genus (*U. nepalensis*, *U. orientalis* and *U. himalayana* for *Usnea* spp., and *H. cirrhata*, *H. nepalensis* and *H. excesta* for *Hypotrachyna* spp.) were based on their sensitivity to atmospheric pollution (Munzi et al., 2023; Pradhan et al., 2025b) and the most common lichen species of the region (Baniya and Bhatta, 2021). At each study site, a transect of five sampling lines was established to cover a vertical distance of 100–200 m, equivalent to an approximate total horizontal distance of 1 km. For each sampling line, sampling was conducted on three trees, within 100–200 m intervals (Fig. 2). A total of 15 trees were sampled for each of the main transects (Supplementary Table S1 & S2).

Sampling of lichens on the trunks of each sampled tree was conducted using five 10 cm × 10 cm quadrats (within a vertical ladder of overall dimension 10 cm × 55 cm), within which the lichen species were selected and collected. These were then stored in a sterile zip-lock bag for transfer to Kathmandu University, Dhulikhel, Nepal, for laboratory analyses. Moreover, bark and soil samples were collected for chemical characterisation. In addition, tree diameter at breast height (DBH) (DBH tape, Forestry Suppliers, Inc.), crown cover (Densiometer, James C. Doster) and tree height (clinometer, SUNTO PM-5/360 PC OPTI) were measured on-site. The collected lichen samples were taxonomically identified to the genus level precision at the Central Department of Botany, Tribhuvan University, Nepal. The subsequent analyses (Table 1) were carried out from October-December 2022.

2.4. Lichen abundance-cover score

The abundance of lichens was quantified from the quadrat sampling using the modified Braun-Blanquet Score method. The ladder of five 10 cm × 10 cm quadrats was placed on the trunk of the tree to assess the percentage cover of each lichen thalli in each quadrat (Roper, 2018). The score 0 was assigned for taxa absent from the quadrat, 1 for taxa representing < 5 % cover, 2 for taxa representing 5–25 % cover, 3 for taxa representing 25–50 % cover, 4 for taxa representing 50–75 % cover and 5 for taxa representing 75–100 % cover.

Table 1
Summary of analysed parameters for lichens, soil and bark.

Parameters (Samples)	Acronym	Unit
Elevation (Area)	-	m a.s.l.
Aspect (Area)	-	-
Diameter at Breast Height (Tree)	DBH	cm
Crown Cover (Tree)	-	%
Height (Tree)	-	m
Slope (Area)	-	%
Abundance (Lichens)	-	%
Roughness (Bark)	-	-
Total Kjeldahl N (Lichens, Soil, Bark)	TKN	%
Ammonium (Lichens, Bark)	NH_4^+	mg L^{-1}
Calcium (Lichens, Soil, Bark)	Ca^{2+}	mg kg^{-1}
Manganese (Lichens, Soil, Bark)	Mn^{2+}	mg kg^{-1}
Zinc (Lichens, Soil, Bark)	Zn^{2+}	mg kg^{-1}
Nickel (Lichens, Soil, Bark)	Ni^{2+}	mg kg^{-1}
Lead (Lichens, Soil, Bark)	Pb^{2+}	mg kg^{-1}
Cadmium (Lichens, Soil, Bark)	Cd^{2+}	mg kg^{-1}
pH (Bark)	-	-
Electrical Conductivity (Lichens, Soil, Bark)	EC	µS cm^{-1}
Chlorophyll-a (Lichens)	Chl-a	mg kg^{-1}
Chlorophyll-b (Lichens)	Chl-b	mg kg^{-1}
Total Chlorophyll (Lichens)	Chl-total	mg kg^{-1}
Chlorophyll Degradation (Lichens)	Chl-degradation	-
Chlorophyll Fluorescence (Lichens)	Chl-fluorescence	-
Total Phenolic Content (Lichens)	TPC	mg GAE g^{-1} DW
2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity (Lichens)	DPPH	%
Catalase Activity (Lichens)	CAT	U CAT E min^{-1} g^{-1} DW

2.5. Nitrogen in lichens, bark and soil

The total concentration of N in lichen thalli, bark and soil were analysed using the Macro Kjeldahl method (Keeney et al., 1992). In brief, 1 g of lichen thalli, soil and bark samples were subjected to digestion in sulfuric acid and hydrogen peroxide solution, and a final calculation was made after titrating with a boric acid indicator. In addition to this, the NH_4^+ ion concentration in undigested lichen thalli was quantified using the ion-selective electrode method, for which 200 mg of lichen thalli were extracted with 20 mL of ultra-pure water and stabilised for one hour. The concentration of NH_4^+ ions was measured through an ion-selective NH_4^+ electrode (EDT, UK).

2.6. Heavy metals in lichens, soil and bark

The tri-acid extraction method (i.e., in a mixture of nitric, sulfuric and perchloric acids) with a slight modification was used to determine the concentration of metal ions (calcium (Ca^{2+}), manganese (Mn^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}), cadmium (Cd^{2+}), and lead (Pb^{2+}) accumulated in lichen thalli, soil, and bark (Sghaier et al., 2019). These metals are selected for the examination based on the fact that they are the most essential micronutrients and toxic pollutants for lichen epiphytes. In brief, 1 g of each lichen, soil and bark samples were subjected to 25 mL of a tri-acid mixture (sulfuric acid: nitric acid: perchloric acid; 4:10:1). The mixture was heated initially to 80°C for 30 min and later to 110°C for 10 min to achieve complete dissolution. In case of the appearance of a brown colour, 1 mL of the acid mixture was added, and heating was continued until the colour no longer appeared brown to ensure complete digestion. After that, the analysis volume was adjusted to 50 mL by adding deionised water with 0.5 % nitric acid, and the entire solution was filtered before metal ion analysis using the direct air-acetylene method in an Atomic Absorption Spectrometer (AAS) (GBC, SAVANTA, Australia). The minimum detection limit of the instrument for these metal ions is 0.001 ppm. Further, the source of metal ions accumulation in lichens, whether they are from natural, anthropogenic or long-range transport, was explored using an enrichment factor (EF) analysis (Varrica et al., 2022) for which the concentration of Mn in Earth's crust was used as a reference element for source normalisation based on these considerations: i) comparatively high natural abundance, ii) easily determined by conventional techniques, iii) mostly derived from soil sources and iv) presumably has limited metabolic values and impacts in lichens (Bargagli, 1998).

2.7. Soil and bark characteristics

In addition to N and metal ions, the pH and EC of bark and soil samples were determined using standard methods. In brief, for the determination of EC and pH in bark, 2 g of bark samples were dissolved in 20 mL ultrapure water and left overnight before measurement. Moreover, for the determination of soil pH, 10 g of soil was dissolved in 10 mL ultrapure water and left for 1 hr before measurement. For the determination of EC in soil, 5 g of soil was dissolved in ultrapure water and mixed into a slurry and the mixture was then filtered through Whatman filter paper before measuring EC. The pH and EC were measured using a standard pH meter (pH 50 VioLab, XS, Italy) and a standard EC meter (COND 7, Vio Set, Italy), respectively. Moreover, the bark roughness categories (high roughness (a), moderate roughness (b), and smooth (c)) were quantified based on the smoothness characterisation of crevice depth, using a method adopted by previous investigators (Buba and Danmallam, 2019).

2.8. Physico-chemical responses in lichens

2.8.1. Electrical conductivity

The electrical conductivity (EC) as a measure of cell membrane integrity of lichen samples was measured by a standard EC probe (COND 7, Vio Set) by dissolving 2 g of lichen samples in 20 mL of deionised water for 35 min. Deionised water was used as a reference (Paoli et al., 2011).

2.8.2. Chlorophyll contents, chlorophyll degradation and chlorophyll fluorescence

The chlorophyll-a, chlorophyll-b, total chlorophyll, and chlorophyll degradation in lichen samples were examined by the modified dimethyl sulfoxide (DMSO) method adopted by previous investigators (Boonpragob, 2002; Ronen and Galun, 1984). In brief, 15 mL of DMSO with 2.5 % polyvinylpyrrolidone (PVP) was used to dissolve 20 mg of lichen thalli, and the mixture was then incubated at 65°C for three hours. After centrifuging the reaction mixtures for 10 min at 5000 rpm, the optical density (OD) at 665, 648, 435, and 415 nm was measured using a Shimadzu-1800 UV–visible spectrophotometer. Chlorophyll a, b, total chlorophyll, and chlorophyll degradation were calculated using the following formulas.

$$\text{Chlorophyll-a (mg kg}^{-1}) = 14.85OD_{665} - 5.34OD_{648}$$

$$\text{Chlorophyll-b (mg kg}^{-1}) = 25.48OD_{648} - 7.36OD_{665}$$

$$\text{Total Chlorophyll (mg kg}^{-1}) = 20.34OD_{648} + 7.49OD_{665}$$

$$\text{Chlorophyll degradation (Chlorophyll a/phaeophytin)} = OD_{435}/OD_{415}.$$

Further, on-site measurement of chlorophyll fluorescence as photosystem II damage (variable fluorescence to maximum fluorescence; Fv/Fm ratio) in lichens was performed using a standard fluorescence meter (Opti Science, USA). For the determination of the Fv/Fm ratio, each lichen thallus was dark-adapted for 15 min prior to analysis and OD was measured (Paoli et al., 2010).

2.8.3. Phenolic content

The collected lichen thalli were dried at room temperature and ground using an electric grinder. Two-gram powder samples were mixed with 20 mL of absolute acetone and maintained overnight at room temperature on a shaker at 350 rpm. The solution was then filtered at room temperature and used for phenolic content and DPPH radical scavenging analysis. This approach was chosen to avoid thermal degradation of sensitive lichen metabolites. Further, we have focused this study on quantitative analysis of total phenolics as a physico-chemical response rather than identifying specific chemical composition of lichen species, for which we combined major lichens as a single sample for analyses. For the total phenolic content (TPC), one millilitre of lichen extract (1 mg mL^{-1}), 200 μL of Folin-reagent, and 1600 μL of distilled water were combined (Singleton et al., 1999). After 5 min, 600 μL of 7 % sodium carbonate (Na_2CO_3) and ultrapure water were added to make 8 mL. A UV–visible spectrophotometer was used to measure the absorbance at 765 nm, after 30 min of incubation.

2.8.4. Radical scavenging potential

The radical scavenging potential of lichen extracts was examined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In brief, 50 μL of lichen extracts of various concentrations (10–160 μg mL^{-1}) were added to 150 μL of DPPH solution (100 μM) and incubated for 30 min. In a 96-microplate reader (Spectrostar nano, BMG, Labtech), absorbance was measured at 517 nm (Pradhan et al., 2024). The reference and positive controls were Gallic acids and methanolic DPPH, respectively.

$$\%\text{DPPH Scavenging} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} * 100\%$$

2.8.5. Catalase activities

For enzyme extraction, two grams of fresh lichen thalli were ground with 20 mL extraction buffer [0.3 M potassium phosphate buffer, pH 7.5, adding 0.5 mM ethylenediaminetetraacetic acid (EDTA)]. The extracts were centrifuged at 15,000 rpm for 20 min, and the supernatant was utilised in the enzymatic assay. The first-line oxidative response analysis, as catalase activity, was performed to check the environmental stresses of lichens (Singh et al., 2010). The absorbance at 240 nm on a UV spectrophotometer was used to measure catalase (CAT) activity (mM H_2O_2 decreased min^{-1} g^{-1} FW), and a reduction in absorbance was recorded over time for 30 min. In a reaction mixture, 1.5 mL of potassium phosphate buffer (pH 7.0), 0.5 mL of 75 mM H_2O_2, 0.2 mL of enzyme extract, and 0.8 mL of double-distilled water (DDW) were used. The final results were expressed as a unit of catalase equivalent per minute per gram fresh weight (U CAT E min^{-1} g^{-1} FW) of lichen extracts.

2.9. Statistical analyses

All chemical analyses were carried out in triplicate, and results are presented as mean±standard error (mean±SE). The significant difference in the median concentration of atmospheric NH_3, thallus N, NH_4^+, metal ions, and physico-chemical and oxidative responses between sampling lines in 1 km transects in each site was analysed by Kruskal-Wallis Pair-wise comparison tests. The results were significant for $P < 0.05$. Further, the Mann-Kendall test was performed to analyse the trend of atmospheric NH_3 concentration, and thallus NH_4^+, N, metal ions concentration, and physico-chemical and oxidative stress parameters with elevation. The trends were significant for $P < 0.05$ and negative and positive trends were indicated by τ-values. The correlation among different lichen variables was analysed using Pearson's product-moment correlation test. Linear regression analyses were performed to examine the impact of atmospheric NH_3 on lichen's physico-chemical properties and responses. A multiple regression analysis was performed to test the significant impacts of N, NH_4^+, and metal ions in the physico-chemical and oxidative responses of lichens, for which a variance inflation factor (VIF) of less than 5 was considered. Principal Component Analysis (PCA) was performed to analyse the contribution of major variables in soil, bark, and lichens. In addition, linear regression analysis was performed to check species-specific N, NH_4^+ and metal ions concentration in thallus and associated physico-chemical and oxidative stress responses.

3. Results and discussion

3.1. Atmospheric ammonia

The annual average atmospheric NH_3 concentration ranged from 3.01 to 4.84 μg m^{-3} and 2.51–4.74 μg m^{-3} in KTM and ACA, respectively (Fig. 3). The average concentrations varied slightly between sampling locations but were comparable with the modelled data (i.e., modelled values of 6.27 μg m^{-3} in KTM and 4.90 μg m^{-3} in ACA, respectively; see Fig. 1F & 1 G). Although NH_3 concentrations showed a slight decreasing trend with increasing elevation at ACA, the relationship was not statistically significant ($P > 0.05$) at either site. Moreover, there is no location-wise variation in NH_3 concentration ($P > 0.05$) (Supplementary Table S3).

At the ACA transect, the highest NH_3 concentration occurred at the second sampling location, rather than immediately adjacent to the village as might have been expected. This might be related to local air mixing patterns, while we note that there was a contrasting dispersion pattern of NH_3 in the other locations further away from the source (Flechard et al., 2011; Leith et al., 2004; Loubet et al., 2009). Although all sampling locations were away from the local emission sources, the contrasting pattern of NH_3 dispersion and the low concentration in the second sampling line of the KTM site might be due to the canopy shading effect of trees around the ALPHA location (Wilson and Skeffington, 1994). Further, the atmospheric NH_3 in most polluted urban areas can be depleted by conversion to particulate matter (Ehrnsperger and Klemm, 2021; Viatte et al., 2020; Wang et al., 2023). A study also suggests urban roadside NH_3 could be lower than at suburban sites due to immediate conversion to particulate matter (Huang et al., 2021). If this effect is significant, there may be a compensatory effect for NH_3 that is converted to particulate matter at the urban KTM site, which is heavily polluted due to vehicular emissions and agricultural activities in the Kathmandu valley.

In temperate regions of the world, the average annual concentration threshold of atmospheric NH_3 as a critical level is set as 1 μg m^{-3} for lichen epiphytes (Cape et al., 2009), although similar thresholds have not been determined empirically for tropical and subtropical regions (Ellis et al., 2022). Our measurements showed the exceedance of the United Nations Economic Commission for Europe (UNECE) threshold for lichen bioindicators in our study area. A recent meta-analysis also provided the annual atmospheric NH_3 concentration of 0.87 μg m^{-3}, 1.44 μg m^{-3} and 2.76 μg m^{-3} as lower bound threshold, mean safe threshold and upper bound threshold, respectively, for lichen bioindicators (Ellis et al., 2022). Our measurements show the exceedance of thresholds in both sites, indicating the risk of atmospheric NH_3 pollution in the region.

The impacts of excess NH_3 might have significant impacts on community structure, physico-chemical properties, chemistry and responses of N-sensitive lichens like *Usnea* and *Hypotrachyna* (Munzi et al., 2023; Pradhan et al., 2025b). Nonetheless, there could be additional effects of other N-based pollutants, such as oxidised N compounds, in these habitats. These findings point to the need for more robust and comprehensive measurement of total N deposition in the region (including the chemical speciation between different N forms) for better understanding the spatial extent of the lichens' responses and resilience.

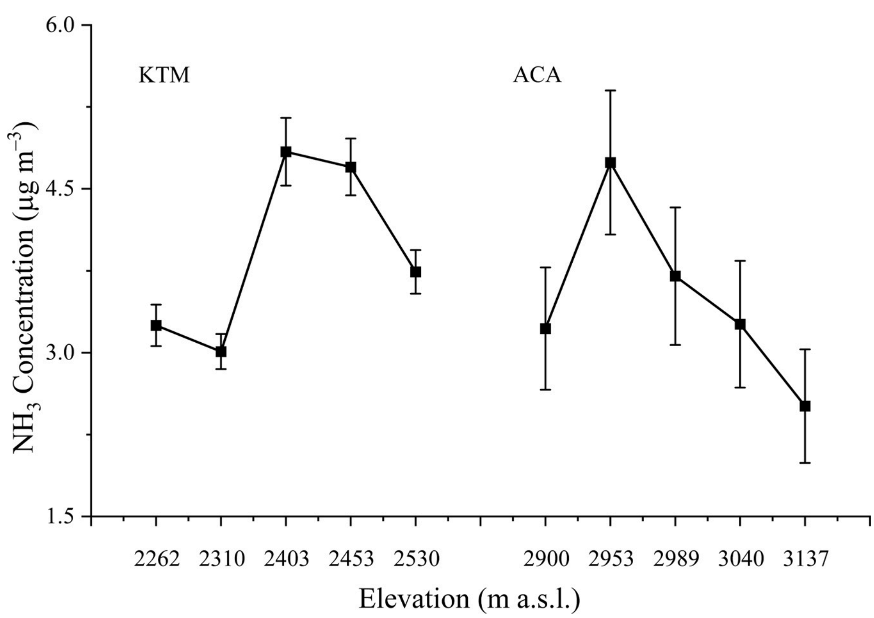

Fig. 3. Mean annual atmospheric NH_3 concentration at different elevations within a 1-km transect of Chandragiri, KTM and Ghorepani, ACA. The error bar shows standard errors. The measurements were carried out monthly in five different elevations from January to December 2023.

Notwithstanding the possible reasons for the second sampling location at the ACA transect having the highest NH_3 concentration in this transect, this provides useful information, as it is shown below how this sampling location was also associated with several indicators of N pollution impact (see further below).

3.2. Lichens abundance-cover

The abundance-cover of the selected lichen species was higher in the rural forest of ACA (33–72 % *Usnea* spp. and 52–74 % *Hypotrachyna* spp.) as compared with KTM (17–65 % *Usnea* spp. and 38–65 % *Hypotrachyna* spp.). The sampling-line-wise variation was observed for the abundance of lichen species in both sites. The increase in lichen abundance with sampling lines from lower to higher elevation was observed (Fig. 4A and Supplementary Tables S1 & S2). However, there is only a significant trend for increasing abundance of *Hypotrachyna* spp. with increasing elevation in ACA ($P < 0.05$). It has been suggested that the availability of moderate N in the atmosphere can favour lichen growth and abundance (Aragón et al., 2019), which might be the case for the notable abundance of lichen species in our study sites. The abundance, composition and distribution of epiphytic lichens are influenced by characteristics of the host tree, surrounding ecosystem and landscape (Buba and Danmallam, 2019). It is also well established that the abundance of epiphytic lichens and their diversity are influenced by microclimatic conditions such as humidity, temperature and light, and structural factors such as canopy cover, canopy structure, and shrub layer (Király et al., 2013).

The functional traits of lichens can be used as a proxy for environmental change indicators (Bajpai et al., 2025). Although the lichen species for this study were selected based on the community structure of commonly available species in study sites, the details of functional traits – which could serve as pollution indicators in the region – will be examined in future publications. It has been suggested that the abundance and condition of lichens reflect the condition of a forest (Naincy et al., 2024). Furthermore, studies suggest that the diversity of lichens is directly influenced by the elevation (Baniya et al., 2010; Rai et al., 2011). Our study also revealed the influence of canopy cover and elevation on the abundance of lichens at both sites (see Section 3.9 below). This could be a reason for the noteworthy but contrasting abundance of the examined lichens on the trees along the sampling lines. Thus, to characterise the responses of lichens to atmospheric pollution and other environmental factors, further emphasis was given to the assessment of lichens' thallus physico-chemical properties, such as thallus concentration of N and heavy metals, and alternation of physico-chemical and oxidative responses that could serve as proxies for the impact monitoring on species at the ecosystem level.

3.3. Thallus ammonium and nitrogen content

The thallus NH_4^+ concentration ranged from 1 to 1.65 mg L^{-1} in *Usnea* spp. and 1.09–3.99 mg L^{-1} in *Hypotrachyna* spp. and thallus TKN ranged from 0.36 % to 0.98 % dry mass (DM) in *Usnea* spp. and

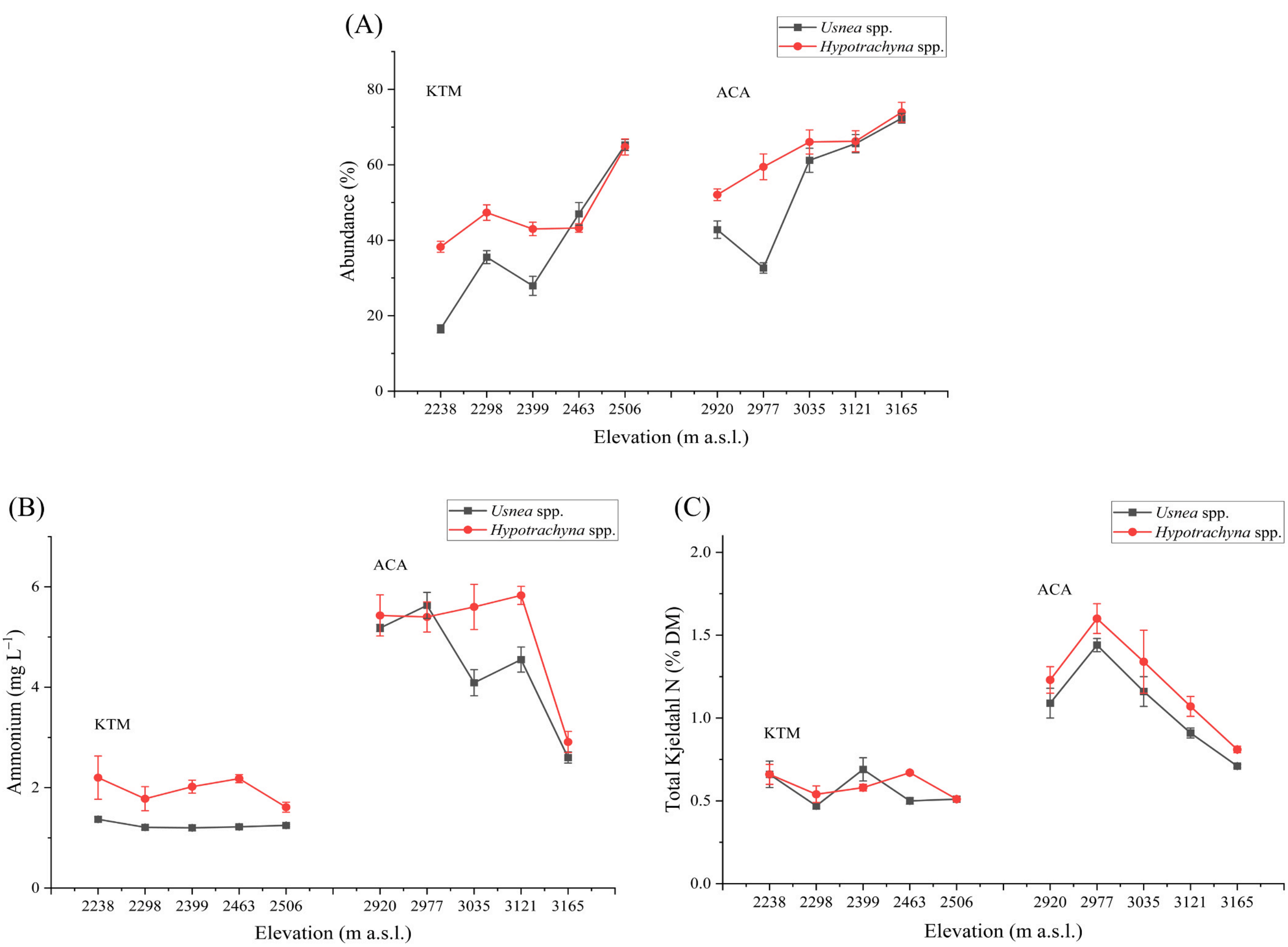

Fig. 4. Abundance, thallus NH_4^+ content and thallus N content in lichens from sampling lines along elevation in a 1-km transect. A) Abundance of lichens in KTM and ACA, B) Thallus NH_4^+ concentration in lichens from KTM and ACA and C) Thallus TKN in lichens from KTM and ACA. Values are means for three trees at each elevation, with error bars showing the standard errors.

0.36–0.82 % DM in *Hypotrachyna* spp. from KTM. Similarly, thallus NH_4^+ ranged from 2.16 to 6.70 mg L^{-1} in *Usnea* spp. and 2.01–7.01 mg L^{-1} in *Hypotrachyna* spp. and thallus TKN ranged from 0.67 % to 1.57 % DM in *Usnea* spp. and 0.72–2.04 % DM in *Hypotrachyna* spp. from ACA (Table 2). Among the two sites, the highest NH_4^+ and TKN concentrations were observed in *Hypotrachyna* spp. from ACA. In general, the decrease in thallus NH_4^+ and TKN concentration was observed with increasing elevations (negative τ value). However, there is no statistically significant trend in thallus NH_4^+ and TKN concentration with elevation ($P > 0.05$) (Fig. 4B & 4 C). The highest thallus TKN and NH_4^+ concentrations at lower elevations and near point sources, and the lowest concentration at higher elevations, might be due to the contrasting dispersion and distribution pattern of pollutants in the sampling sites due to both local emissions and long-range transport. Furthermore, a sampling line-wise variation in thallus concentration of both NH_4^+ and TKN was observed for both species from both forest transects ($P < 0.05$) (Supplementary Table S4), which suggests variability in local microenvironmental conditions affecting the accumulation of N compounds.

The contributing factors for the highest accumulation of NH_4^+ and TKN in ACA could be the influence of household emissions, local transport, long-range transport, wind patterns, and enhanced dry deposition. The high thallus NH_4^+ and TKN concentrations in lichens from sampling line 2 (S2) of ACA might be the result of uneven dispersion and deposition of pollutants emitted from nearby households. Furthermore, variations in lichen growth form, thallus size and shape affect how species accumulate pollutants (Osyczka et al., 2018; Rola et al., 2016) – which possibly explains the lower accumulation of NH_4^+ by *Usnea* spp. compared to *Hypotrachyna* spp. in our case. While it would be expected that fruticose lichens like *Usnea* gather a considerable amount of nutrients in their thalli, recent research revealed that fruticose lichens can acquire less N as compared to foliose lichens (Susan et al., 2017). A study on *Everniastrum* spp. from the Indian subcontinent also revealed a similar pattern of thallus N concentration and subsequent effects on chemical responses (Rai et al., 2022). Comparatively, the hyper-accumulation potential of *Hypotrachyna* spp. could be related to its growth form, structure and surface area (Chettri et al., 1997; St. Clair et al., 2002). Since *Hypotrachyna* spp. has a leafy branching system with a large surface area exposed to the atmosphere, a higher accumulation of N is conceivable.

Although lichens from both locations indicate moderate thallus N content and variation among sampling lines, no visible morphological damage was observed in any of the sampled lichens. However, it is crucial to characterise thallus physico-chemical properties as early warning bioindicators for the potentially increasing toxicity in lichens due to environmental pollutants. This is because, if those pollutants keep accumulating, the accumulation threshold will be breached and morphological and physiological damage will occur, eventually leading to mortality. Thus, these measurements help indicate the beginning of toxicity even before actual damage occurs to lichen bioindicators. We further characterised the impacts of N accumulation in lichen responses as discrete physico-chemical properties such as cell membrane integrity, chlorophyll content, photosynthetic ability, and oxidative stresses.

3.4. Heavy metals in lichen thalli

A considerable amount of heavy metals in lichens from both sites were observed. Among the analysed heavy metals, the concentration of Ca (1673–17906 mg kg^{-1} in *Usnea* spp. and 4571–20741 mg kg^{-1} in *Hypotrachyna* spp.) was highest in KTM, followed by the concentration of Mn, Pb, Zn and Cd, respectively (Table 2 and Fig. 5A-E). Similarly, the concentration of Ca (4936–26305 mg kg^{-1} in *Usnea* spp. and 7350–26786 mg kg^{-1} in *Hypotrachyna* spp.) was highest in ACA, followed by the concentration of Mn, Pb, Zn, Ni and Cd, respectively. Consistent findings in both sampling sites indicate a noteworthy abundance of metals contributing to thallus physico-chemical properties. Comparatively, the highest concentration of metal ions was observed in *Hypotrachyna* spp. collected from the ACA. Our findings are in line with the results of previous work (Bergamaschi et al., 2002), a similar study conducted in the Himalayas of Nepal, where *Hypotrachyna* spp. consistently showed higher concentrations of the examined trace elements. Notably, we detected Ni in our study only in the lichens collected from ACA, and a high amount of Pb was observed in the lichens from KTM (Table 2).

In general, we found a decrease in thallus heavy metal concentration with increasing elevation at both sites (negative τ value; see Fig. 5A-E) and a significant difference in the median concentration of heavy metals was observed between the sampling lines in the same forest transect (Supplementary Tables S5 & S6). Statistically, the decreasing trend in heavy metal concentration with increasing elevation was only significant for the concentration of Mn in *Hypotrachyna* spp. from KTM ($P < 0.05$) (Fig. 5C). However, this is consistent with the overall trends for several of the heavy metals at both sites. The high thallus concentration of heavy metals in lower elevations (first and second sampling lines) near pollution sources in both sites signifies that the abundance and deposition of metal ions in lichens depend on local environmental factors, especially distance from pollution sources. The accumulation of Ni only in the lichens from the ACA transect is suggestive of household emission sources (Genchi et al., 2020) and the high amount of Pb in the lichens from the KTM transect is suggestive of vehicular emission sources (Hussain et al., 2018). The high concentration of Ca in lichen species from both sites might be due to the dust originating from vehicular emission, household emission and constructional activities near the sites. Further, occasional wildfires and regular household combustion might contribute to dust emissions, especially in the ACA site.

Metal ions play an important role in the physiological and metabolic functioning of lichens, involving both symbionts (Rola et al., 2022). Certain lichen species can withstand or even store substantial quantities of potentially toxic metal ions inside their thalli, and they are frequently used in metal ion biomonitoring and biogeochemical prospecting (Bargagli and Nimis, 2002). However, excessive deposition may cause severe damage through changes in thallus physico-chemical properties

Table 2
Thallus NH_4^+, TKN and metal ions in lichens. All chemical analyses were carried out in triplicate, and results are presented as mean ± standard error (n = 45).

Parameters	Variables of thallus concentrations	Mean value along each 1 km transect (±SE)			
		Chandragiri, KTM		Ghorepani, ACA	
		Usnea spp.	*Hypotrachyna* spp.	*Usnea* spp.	*Hypotrachyna* spp.
N compounds	NH_4^+ (mg L^{-1})	1.25 ± 0.02	1.96 ± 0.10	4.41 ± 0.18	5.04 ± 0.21
	TKN (% DM)	0.57 ± 0.02	0.59 ± 0.01	1.06 ± 0.04	1.21 ± 0.05
Metal ions	Ca^{2+} (mg kg^{-1})	6789.48 ± 707.11	10491.09 ± 619.80	14175.49 ± 1103.79	18414.77 ± 877.68
	Cd^{2+} (mg kg^{-1})	1.55 ± 0.14	1.12 ± 0.08	0.83 ± 0.06	1.10 ± 0.11
	Mn^{2+} (mg kg^{-1})	47.56 ± 4.06	60.11 ± 2.20	22.79 ± 2.19	27.37 ± 0.93
	Ni^{2+} (mg kg^{-1})	-	-	7.59 ± 0.56	15.60 ± 1.28
	Pb^{2+} (mg kg^{-1})	45.81 ± 3.60	32.13 ± 2.87	13.30 ± 1.45	14.61 ± 1.40
	Zn^{2+} (mg kg^{-1})	9.58 ± 0.83	8.54 ± 0.72	13.51 ± 0.99	20.02 ± 1.35

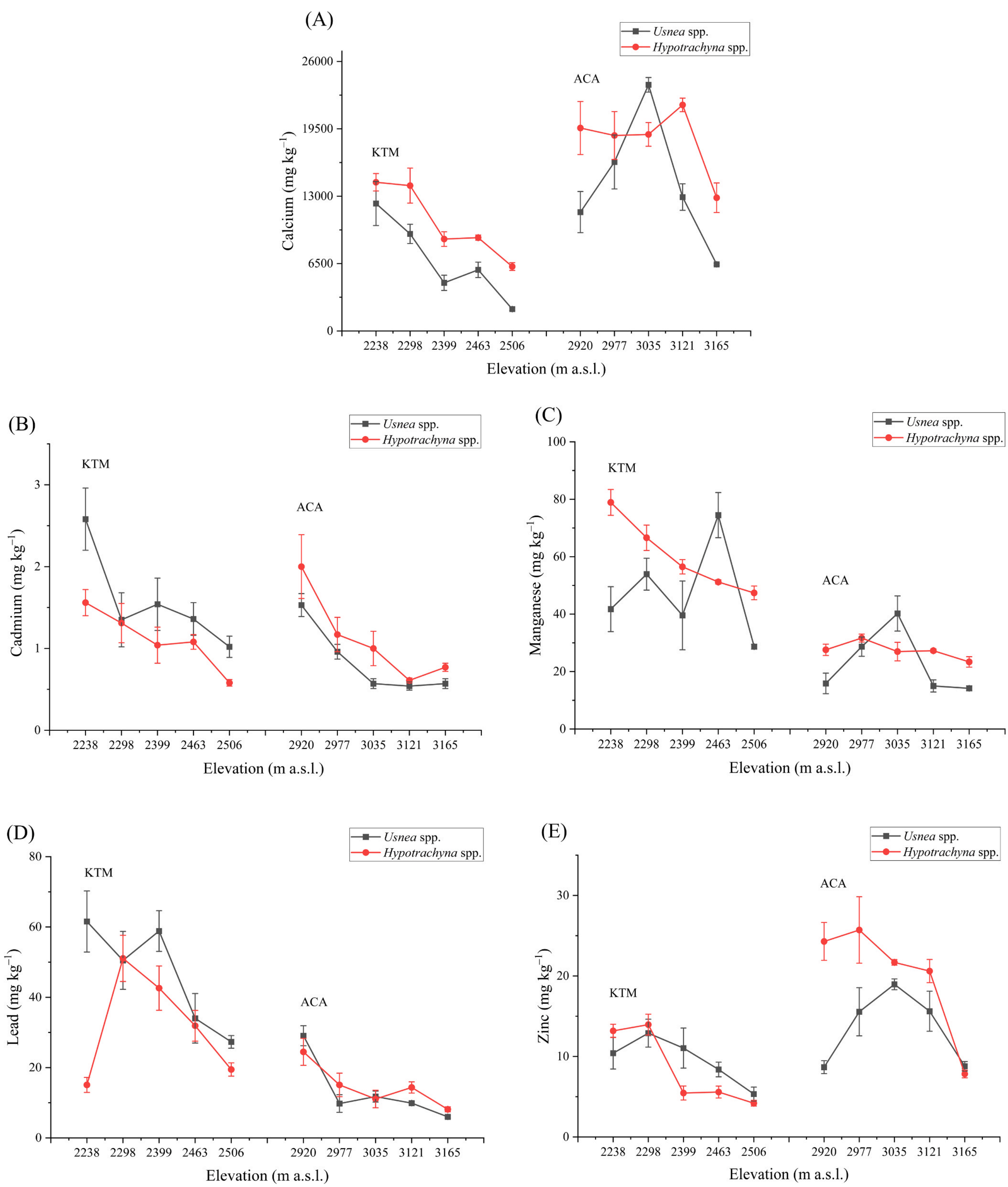

Fig. 5. Metal ions in lichens from sampling lines along elevation in a 1-km transect. A) Calcium, B) Cadmium, C) Manganese, D) Lead and E) Zinc. Values are means for three trees at each elevation, with error bars showing the standard error.

that ultimately undermine the ecosystem services that lichens can provide. Our previous findings suggest the comparatively low concentration of heavy metals in *U. cornuta* and *Ramalina intermedia* collected from the pristine forest of Western Nepal (Pradhan et al., 2025a). Thus, these present findings suggest that the accumulation of heavy metals in lichens from pollution gradient forests is relatively higher.

Further, a range of metal toxicity over physico-chemical properties and oxidative stress of lichens has been reported in different studies. For example, Mn triggers chlorophyll breakdown and some heavy metals, such as Ni lead to the generation of reactive species that cause oxidative stress in lichens (Kováčik et al., 2018). One study suggests that the high concentration of metals in lichens from urban areas results in decreased chlorophyll-a, indicating less photosynthetic efficiency and an increase in lipid peroxidation and electrolyte conductivity, indicating cell membrane injuries in the sensitive lichen *Flavoparmelia caperata* (Majumder et al., 2013). Further, a recent study has suggested the contrasting impacts of heavy metals on physico-chemical responses of foliose lichen *Pyxine cocoes* in India (Gupta et al., 2025). Thus, examining metal ions and their subsequent impacts on lichen's physiology could give a comprehensive overview of the usability of lichen epiphytes for bioindicators of metal ion toxicity in the forest ecosystem.

Although the accumulation of metal ions on lichen thalli is a natural phenomenon due to the ability of lichens to accumulate nutrients directly from the atmosphere, the excessive heavy metal accumulation could lead to severe toxicity and may cause morphological and physiological damage (Bačkor and Loppi, 2009a). Thus, assessment of the increasing accumulation of heavy metals in lichens could serve as an early warning bioindication for the habitat. While the amount of metal accumulated by lichens is species-dependent and relates to its morphological and structural features (Chiarenzelli et al., 1997), the uptake and release of metal ions and trace elements could be influenced by several factors such as bark pH, duration of exposure to microclimatic conditions and type of pollutants present in the surrounding environmental settings (Bačkor and Loppi, 2009b). We further use the thallus concentration of metal ions as a signature of physico-chemical and oxidative stresses in lichens and see the impacts of other environmental variables on the accumulation of heavy metals in lichens.

3.5. Physico-chemical and oxidative responses

3.5.1. Electrical conductivity

The EC measured in this study for the KTM transect varied between 30 and 74.60 μS cm^{-1} in *Usnea* spp. and 31.30–66.80 μS cm^{-1} in *Hypotrachyna* spp. Similarly, in ACA, the EC ranged from 101.60 to 223 μS cm^{-1} in *Usnea* spp. and 110–229.20 μS cm^{-1} in *Hypotrachyna* spp. (Table 3). A statistically significant decreasing trend of EC along sampling lines was observed in the *Usnea* spp. collected from the KTM transect ($P < 0.05$) (Supplementary Figure S1). Further, the significant difference in EC between the sampling lines in a 1-km transect (in both sites) testifies the important role of the local environment when interpreting pollution effects (Supplementary Table S7). The relatively high EC in lichens collected from sampling line 2 (S2) of ACA signifies the elevated pollution effects and uneven dispersion of pollutants from near sources, as mentioned above for atmospheric NH_3.

The high EC in lichens from ACA suggests a degree of physiological stress on their vitality. Generally, the increase in EC in lichens under high pollution stress is attributed to membrane damage and ion leakage, which compromises cell membrane integrity (Munzi et al., 2009; Osyczka and Rola, 2019). There is evidence of an elevated EC due to N and heavy metal stress in lichens from different parts of the world (Chowaniec et al., 2023; Daimari et al., 2021). Our recent study also revealed a comparable EC in *U. cornuta* from a very remote forest in Nepal, which might, however, be affected by the tree species composition – which was collected from needleleaf *Pinus wallichiana* trees (Pradhan et al., 2025a). Although our present study suggests the probable effects of N compounds, heavy metals and microclimatic conditions on cell membrane integrity, there is no literature available for this part of the world to compare their impact on lichen physico-chemical properties over time.

Table 3
Physico-chemical and oxidative responses in lichens. All values are presented as mean ± standard error of triplicated chemical analyses (n = 45).

Variables	Mean value along each 1 km transect (±SE)			
	Chandragiri, KTM		Ghorepani, ACA	
	Usnea spp.	*Hypotrachyna* spp.	*Usnea* spp.	*Hypotrachyna* spp.
EC (μS cm^{-1})	45.52 ± 1.79	48.08 ± 1.54	151.34 ± 5.05	156.67 ± 4.93
Chlorophyll-a (mg kg^{-1})	23.74 ± 1.06	18.68 ± 0.79	11.79 ± 0.84	12.89 ± 0.67
Chlorophyll-b (mg kg^{-1})	7.23 ± 0.62	6.62 ± 0.49	2.75 ± 0.34	2.91 ± 0.25
Total Chlorophyll (mg kg^{-1})	30.97 ± 1.01	25.30 ± 0.91	14.55 ± 1.09	15.81 ± 0.73
Chlorophyll Degradation	1.11 ± 0.02	1.02 ± 0.02	0.93 ± 0.02	0.86 ± 0.01
Chlorophyll Fluorescence	0.67 ± 0.01	0.62 ± 0.01	0.18 ± 0.02	0.17 ± 0.01
Phenolics (mg GAE g^{-1} DW)	161.72 ± 6.42	198.46 ± 8.65	80.96 ± 4.38	91.68 ± 4.36
DPPH Scavenging (%)	67.87 ± 1.50	65.04 ± 2.21	32.09 ± 2.21	38.58 ± 2.38
Catalase (U CAT E min^{-1} g^{-1} DW)	0.46 ± 0.03	0.49 ± 0.04	1.35 ± 0.08	1.50 ± 0.09

3.5.2. Chlorophyll pigmentation, degradation and fluorescence

Our study revealed the highest chlorophyll concentration (chlorophyll-a, chlorophyll-b, and total chlorophyll) in *Usnea* spp. collected from the KTM transect, and the least in the same species collected from the ACA transect. The total chlorophyll ranged from 20.71 to 41.86 mg kg^{-1} in *Usnea* spp. and 11.56–34.68 mg kg^{-1} in *Hypotrachyna* spp. from KTM. Similarly, total chlorophyll ranged from 4.56 to 30.98 mg kg^{-1} in *Usnea* spp. and 8.98–26.24 mg kg^{-1} in *Hypotrachyna* spp. from ACA (Table 3). Comparatively low concentration of chlorophyll was observed in lichens from ACA, in the site with the highest amount of thallus NH_4^+, TKN and metal ions. An increasing trend in chlorophyll content in lichen samples with increasing elevation in both sites indicates the impact of local environmental setup on the accumulation of pollution and integrity of lichen health (Supplementary Figure S1 and Supplementary Table S7). The comparatively low chlorophyll content in lichen samples from sampling line 2 (S2) in both sites indicates the uneven distribution of pollutants and their impacts on lichen physico-chemical properties and responses. The maintenance of a considerable amount of chlorophyll pigmentation in lichens is an indicator of good ecosystem health. Increased chlorophyll pigmentation can be a result of the moderate availability of N, which acts as a nutrient for lichen growth, including the photobiont population. However, the availability of N greater than the critical load might pose a risk to lichen physiology and result in decreased chlorophyll content (Zarabska-Bożejewicz, 2020).

Chlorophyll degradation is a parameter used to analyse lichen physiology, which is expressed as the phaeophytisation quotient (ratio of chlorophyll-a to pheophytin-a). A low ratio implies degradation and stress, while a high ratio indicates a healthy situation, with actual values for a healthy situation characteristic according to different species. It is crucial for lichens to maintain chlorophyll levels to survive in harsh environmental conditions. The largest chlorophyll degradation was observed in *Usnea* spp. collected from KTM, with the least degradation observed in *Hypotrachyna* spp. collected from the ACA, suggesting the species-wise variation. The chlorophyll degradation ranged from 0.87 to 1.44 in *Usnea* spp. and 0.44–1.26 in *Hypotrachyna* spp. from KTM. Similarly, chlorophyll degradation ranged from 0.65 to 1.46 in *Usnea* spp. and 0.77–0.93 in *Hypotrachyna* spp. from ACA (Table 3). It is suggested that the degradation ratio is around 1.4 for healthy lichen thalli and decreases with increasing stresses (Karakoti et al., 2014). Thus, our results revealed a comparatively higher level of vulnerability of *Hypotrachyna* spp. against photosynthetic abilities. The increasing trend of chlorophyll degradation in *Usnea* spp. with increasing elevation from both sites indicates the impacts of local environmental setup, along with a decrease in pollution level (Supplementary Figure S1 and Supplementary Table S7). There is research in different parts of the world to suggest a decrease in chlorophyll degradation in *Usnea* spp. and

Hypotrachyna spp. with an increase in pollution levels (Alia et al., 2019; Rai et al., 2022; Rangel-Osornio et al., 2021; Santos et al., 2022).

Chlorophyll fluorescence is one of the widely used methods for assessing damage in the photosynthetic ability of the lichens and can be used as a proxy for evaluating the severity of impacts on lichen health (Paoli et al., 2011; Piccotto et al., 2011). In this approach, the ratio Fv/Fm indicates the stress on the lichens, where Fv is the variable fluorescence and Fm is the maximum fluorescence. Our results revealed the ranges of chlorophyll fluorescence in lichens (0.59–0.74 in *Usnea* spp. and 0.46–0.75 in *Hypotrachyna* spp.) from KTM. Besides, chlorophyll fluorescence ranged from 0.01 to 0.55 in *Usnea* spp. and 0.01–0.43 in *Hypotrachyna* spp. from ACA (Table 3). The highest chlorophyll fluorescence was observed in *Usnea* spp. from KTM – suggesting the least impact of pollutants on lichens' photosynthetic abilities on this site. A significant increasing trend of chlorophyll fluorescence in both *Usnea* spp. and *Hypotrachyna* spp. along elevation in ACA suggests the decreasing pollution gradient and its impacts on photobiont vitality in lichens at lower elevations closer to pollution sources (Supplementary Figure S1 and Supplementary Table S7). Overall, the higher impacts on photosynthetic pigments and abilities (i.e., lower Fv/Fm values) were observed in the rural ACA site. Although the lichens were highly abundant in the ACA site, severe damage to the photosystem II in lichens from this site might explain the impacts of dry weather conditions and low water availability in the site, along with possible impacts of comparatively higher deposition of N and heavy metals from local emission sources.

3.5.3. Phenolic content

Phenolics are a class of secondary metabolites/lichen acids that can act against harmful reactive species, such as reactive N and oxygen species that have detrimental impacts on lichen health (Hajam et al., 2023; Holger et al., 2014). Our results revealed the highest TPC in *Hypotrachyna* spp. from KTM and the least in *Usnea* spp. from ACA. The phenolic contents varied from 98.78 to 309.64 mg GAE g^{-1} DW in *Usnea* spp. and 93.98–296.33 mg GAE g^{-1} DW in *Hypotrachyna* spp. from KTM. Similarly, it varied from 51.71 to 190.59 mg GAE g^{-1} DW in *Usnea* spp. and 44.59–159.78 mg GAE g^{-1} DW in *Hypotrachyna* spp. from ACA (Table 3). The low concentration with no significant trend of TPC in lichens from ACA ($P > 0.05$), indicates the physiological stress over lichens from this area, which may have suppressed the production of phenolics (Supplementary Figure S2 and Supplementary Table S7).

The production and upkeep of phenolics and lichen acids support the cellular defence against environmental stresses (Goga et al., 2020; Pratyusha, 2022; Shrestha and St Clair, 2014). It is expected that the production of lichen acids enhances the lichen's ability to cope with environmental stresses by protecting them against cellular damage (Ndhlovu et al., 2024). It is well established that the lichens spend their carbon skeleton to finely manage the N excess by converting it into amino acids (Greaver et al., 2023). So, a declined concentration of carbon-based secondary metabolites, such as phenolics, in lichens with increased concentrations of excess nutrients, such as N, could serve as a useful preliminary indicator and proxy measure for evaluating the extent of N stress on lichens. The extraction and characterisation of secondary metabolites from these lichens might provide a perspective for ecological studies. However, it should be noted that there could be influences of environmental conditions, seasonal variations and species-specific affinity on the production and availability of secondary metabolites in specific lichen species. Further, there could be influences of solvent polarity on the quantification of phenolics. Thus, to fully understand the influences of specific secondary metabolites that contribute to the total phenolics, the identification of the chemical composition of each studied lichen species is crucial.

3.5.4. DPPH radical scavenging activities

The ability of different lichen extracts to scavenge free radicals is one of the key indicators of their potential to counteract oxidative stress (Gülçin et al., 2002). In this study, we assessed the ability of lichen extract on DPPH free radical scavenging – which is crucial for understanding the potential of lichen extracts to act against reactive species. Our results revealed the highest DPPH radical scavenging by the extract of *Usnea* spp. collected from KTM, and the least was observed in the extract of the same species collected from ACA. The DPPH scavenging ranged from 50.48 % to 80.91 % in *Usnea* spp. and 42.58–84.09 % in *Hypotrachyna* spp. from KTM. Similarly, it varied from 9.29 % to 62.46 % in *Usnea* spp. and 16.37–69.07 % in *Hypotrachyna* spp. from ACA (Table 3). The increasing trend of DPPH radical scavenging with elevation (Supplementary Figure S2 and Supplementary Table S7) signifies the local pollution gradients and their impacts on lichens' biochemical properties. Several pieces of literature describe the use of different *in vitro* methods based on electron transfer and hydrogen atom transfer in radical scavenging processes in lichens (Holger et al., 2014). However, there is no literature available for this part of the world to compare the results. Our results illustrate the use of DPPH free radical scavenging activities for the determination of antioxidant properties in lichens, which could serve as a probable proxy for the stress of pollutants on lichens' health.

3.5.5. Catalase activities

Reactive oxygen species (ROS) and reactive N species (RNS) are often produced as a consequence of metabolic processes, including respiration and photosynthesis (Delmail et al., 2013), and are heightened by stressful situations such as a lack of nutrients, xenobiotic exposure, or desiccation and/or rehydration (Mandal et al., 2022). The first line of oxidative defence against harmful reactive species generated by various environmental factors is crucial for maintaining lichen health and integrity. The production of CAT is a signature of environmental stresses on sensitive lichens and can be used as a proxy for their impact on lichen physiological integrity (Rajput et al., 2021). Our study revealed the highest CAT activities in lichens from ACA, which varied from 0.34 to 2.34 U CAT E min^{-1} g^{-1} FW in *Usnea* spp. and 0.42–2.42 U CAT E min^{-1} g^{-1} FW in *Hypotrachyna* spp. Similarly, the CAT activities varied from 0.16 to 0.90 U CAT E min^{-1} g^{-1} FW in *Usnea* spp. and 0.17–1.02 U CAT E min^{-1} g^{-1} FW in *Hypotrachyna* spp. from KTM (Table 3). The high production of CAT is expected when there is oxidative stress over lichens' physiology and functioning. Thus, a decrease in CAT activities with increasing elevation in our case signifies the local gradients and stress in lichens possibly due to high thallus N and heavy metals concentration (Supplementary Figure S2 and Supplementary Table S7). Our results indicate that the highest stress on lichens occurs at sites with elevated levels of thallus N and heavy metals. A previous investigation suggests enhanced CAT activities due to Cd toxicity (Santos et al., 2022). Thus, this oxidative stress-related parameter could serve as a proxy for analysing the impacts of N and heavy metals accumulation in lichen physico-chemical properties to better understand their usability for bioindication studies. To characterise the impacts of microclimatic conditions, atmospheric NH_3, thallus N and thallus metal ions accumulation on physico-chemical and oxidative stress responses of lichens, we further analysed the associations among variables (See Sections 3.6, 3.7 and 3.9).

3.6. Influences of atmospheric ammonia on thallus physico-chemical properties

Variation in the annual mean concentration of atmospheric NH_3 was observed in both sites. However, a significant correlation between atmospheric NH_3 and thallus N concentration was only found for *Hypotrachyna* spp. from the ACA site (Fig. 6). Apart from this, NH_3 had no significant relationships with thallus physico-chemical properties or the responses of both lichen species of either site (Supplementary Table S8). This contrasting pattern of correlations and likely NH_3 effects might be due to the dispersion and deposition pattern of the pollutant, resilience of lichens and the effects of microclimatic conditions such as elevation,

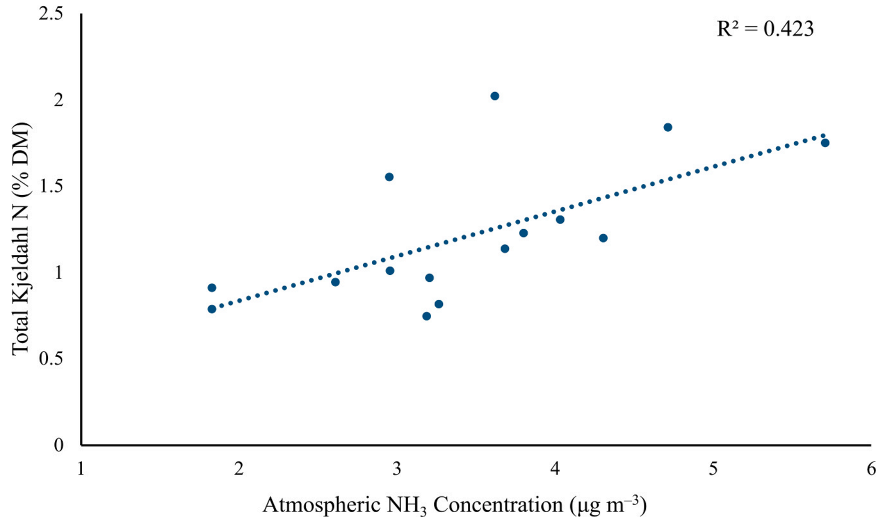

Fig. 6. Linear regression plot for atmospheric NH_3 vs TKN in *Hypotrachyna* spp. from ACA. The analysis was carried out for the triplicated samples of ALPHA and lichens from each location of similar elevations across five different locations within a 1-km transect (n = 15).

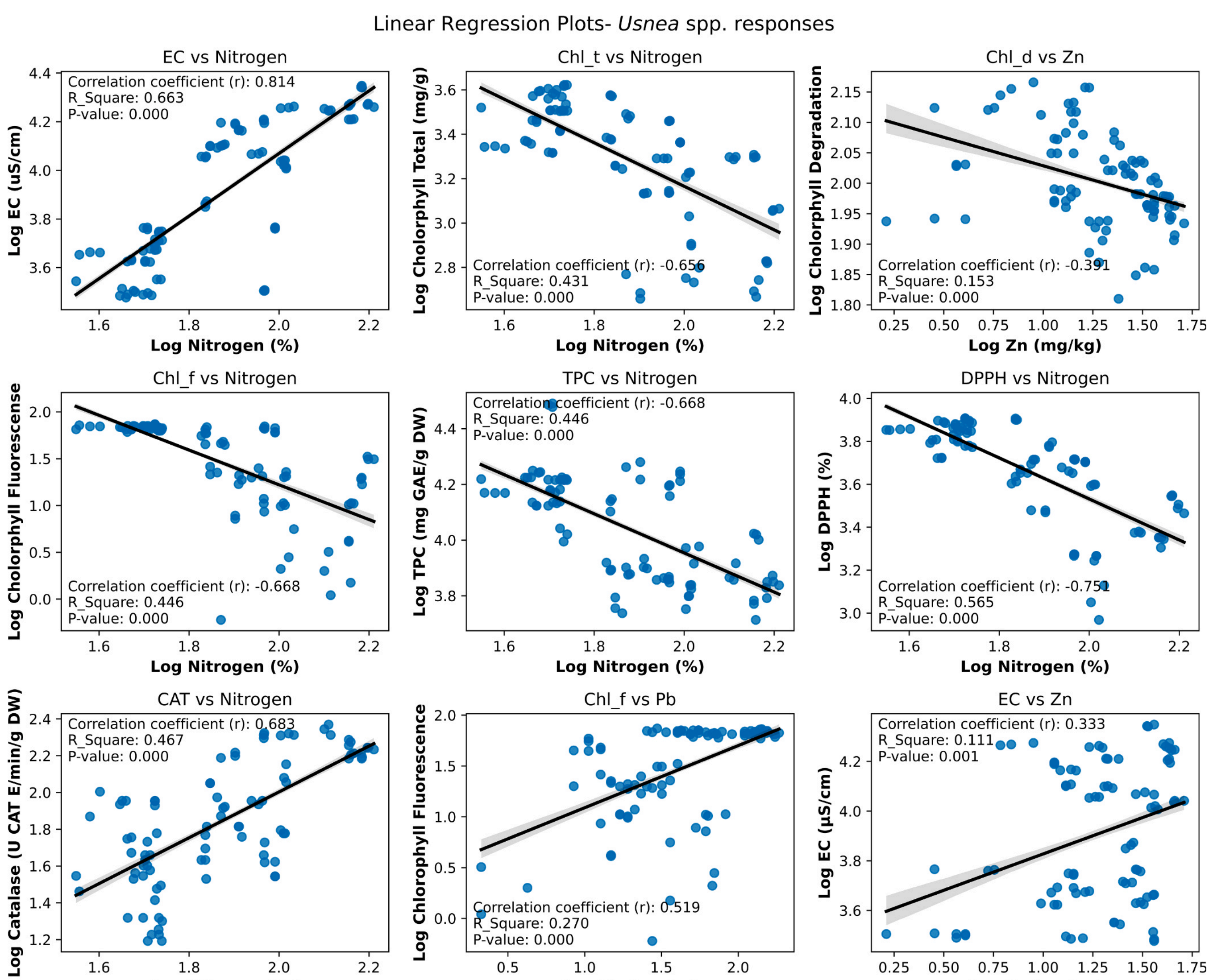

Fig. 7. Regression plots for the *Usnea* spp. variables. Each point represents a single chemical analysis (analysed three times) for one of three biological samples along each sampling line, for five sampling lines in each transect, for two 1-km transects (n = 90).

slope, wind flow, humidity, temperature, and tree characteristics like canopy settings and bark characteristics (Ansaldo et al., 2021; Frati et al., 2007; Morillas et al., 2021).

The impacts of atmospheric NH_3 on changing thallus physico-chemical properties, especially the concentration of thallus N, have been well reported from different parts of the world (Manninen et al., 2023). This can be expected due to both dry and wet deposition of N species, especially NH_3 in rural settings and NO_2 in urban environments (Greaver et al., 2023). The relationship of atmospheric NH_3 with thallus N concentration of *Hypotrachyna* spp. from the ACA site suggests the probable sources of reduced N in rural areas due to the use of agricultural fertilisers and household emissions due to biomass (for example: N-containing organic matter like cow-dung cakes and firewood) burning. However, this needs additional support from studies on sources of N species on the site.

3.7. Relationships between thallus physico-chemical properties and responses

The contrasting concentrations of N and heavy metals in thalli with different physico-chemical and oxidative responses observed in our examined lichens point to the interplay between different variables in maintaining lichen health against environmental stresses. We found that these also occur in a species-specific manner (comparatively higher in *Hypotrachyna* spp. than in *Usnea* spp). To assess the relationships among lichen variables, we performed correlation and regression analyses. The correlation coefficients of lichen parameters are presented in Supplementary Tables S9 & S10, regression coefficients along with VIF are presented in Supplementary Tables S11–13, and significant results on the relationship among thallus physico-chemical properties and responses are presented in Fig. 7 & 8.

The Pearson correlation analysis for *Usnea* spp. revealed the significant positive correlation between EC, TKN, NH_4^+ and Ca, and a significant negative correlation of EC with Pb. Further, in the context of *Hypotrachyna* spp., a significant positive correlation of EC with TKN, NH_4^+, Ca and Zn was observed, whereas a significant negative correlation between EC and Mn was observed. The regression analysis, including lichen variables of both sites, revealed the elevated EC in both the lichens that might be linked primarily to the significant concentration of N compounds and heavy metals, as evidenced by our results. Further, the correlation and regression analysis support the fact of reduced chlorophyll content with increased thalli N and Mn concentration in *Usnea* spp. and thalli concentration of Mn in *Hypotrachyna* spp. The relatively low chlorophyll degradation in our examined lichens

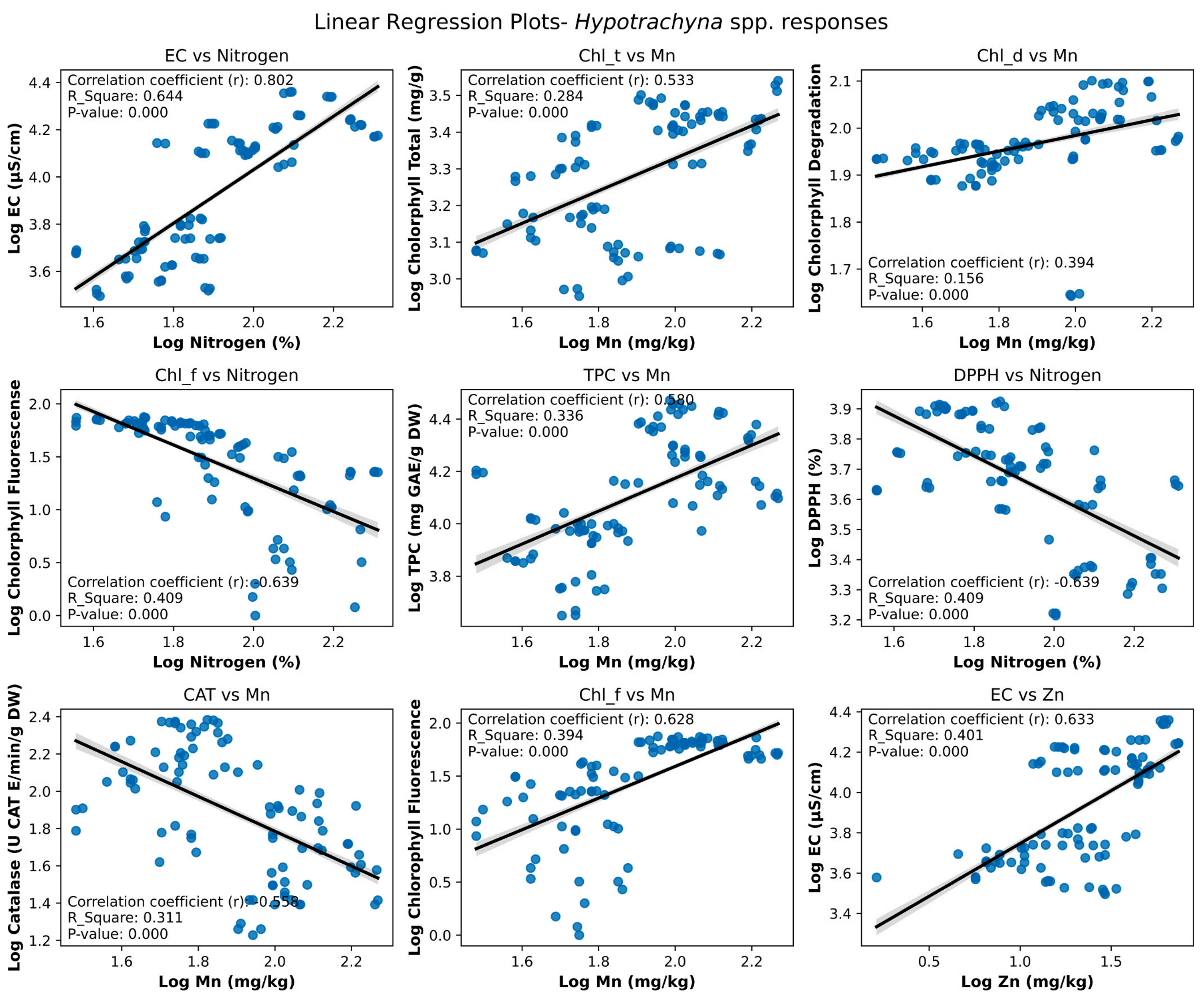

Fig. 8. Regression plots for the *Hypotrachyna* spp. variables. Each point represents a single chemical analysis (analysed three times) for one of three biological samples along each sampling line, for five sampling lines in each transect, for two 1-km transects (n = 90).

indicates the stress on lichen physiology that might be affected negatively by NH_4^+, TKN, Ca, Ni, and Zn in both species. On the other hand, a positive impact of thallus Mn concentration was observed for chlorophyll degradation. Further, the results revealed the significant positive relationships of Mn and Pb, and significant negative relationships of NH_4^+, TKN, and Ca with chlorophyll fluorescence. This suggests that the excess N is damaging photosynthetic performance. Conversely, it does not mean that Mn and Pb improved photosynthetic performance, but rather they might simply be related to an anticorrelation between these metal concentrations and N pollution levels due to the local pollution structure along the measured transects.

Further, our results revealed a significant negative correlation between phenolic content with NH_4^+, TKN, Ca, and Zn for both species. Further, a significant drop in TPC with the high thallus N concentration was observed in *Usnea* spp., and an increase in phenolics with high thalli Mn concentration was observed for *Hypotrachyna* spp. Overall, the decreasing pattern of TPC was observed with an increase in N and heavy metal concentrations. Moreover, a significant negative correlation between DPPH radical scavenging with NH_4^+, TKN, Ca and Zn was observed for both species. Although the lichen extracts showed noteworthy radical scavenging potential, a significant drop in DPPH radical scavenging with the high thallus N concentration was observed for both lichen species. Further, the significant positive correlation of CAT activities with NH_4^+, TKN, Ca and Zn was observed for both lichen species. Moreover, the significant positive relationship of augmented thallus N with CAT activities in *Usnea* spp. was observed. The overall result indicates increased CAT activities with augmented N and heavy metals in the lichen thallus.

The correlation between N and metal ions with stress-related parameters and intercorrelation between stress-related parameters indicates the alternation in thallus physico-chemical properties and the coaction of different chemical variables. The positive correlation of EC with catalase activities in both lichens signifies an enhanced first-line oxidative defence against stress caused by ion leaching and cell membrane damage (Marques et al., 2005), which was triggered by augmented thallus N and metal ions (with a positive correlation between TKN and EC in both the lichens). Further, the negative correlation of EC and CAT with chlorophyll content, chlorophyll fluorescence, chlorophyll degradation, TPC, and DPPH radical scavenging signifies the physico-chemical and oxidative damage in the examined lichens related to higher EC and cell membrane damage (Paoli et al., 2011). The positive correlation between EC, N and heavy metals was also evidenced by other findings (Carreras and Pignata, 2007; Munzi et al., 2009). Moreover, the positive correlation between chlorophyll content, chlorophyll fluorescence, chlorophyll degradation, TPC, and DPPH radical scavenging signifies the protective effects. Several studies presented the negative effects of N compounds and heavy metal deposition on lichen photobionts and photosynthetic activities (Garty et al., 2001; Karakoti et al., 2014; Rola et al., 2019; Tretiach et al., 2007). However, our finding contrasts a positive correlation of Pb on the photosynthetic ability of acidophytic lichen species *Cladonia*, as evidenced by a previous study (Rola et al., 2019). Our results also revealed comparatively greater stress in lichens from ACA (with high EC, CAT, and low chlorophyll content, TPC and radical scavenging potential), which suggests the moderate to severe impact on lichens' physiology.

These clearly showed the enhanced toxicity of atmospheric pollutants and the negative impact of thalli N and heavy metals on physico-chemical and oxidative responses in lichens from two different forests in Nepal. Although there is no such impact observed for atmospheric NH_3 on lichen's physico-chemical properties and responses other than thallus N concentration, the combined effects of metal ions and N on the physico-chemical functioning of lichens give a clear idea of the response potential of indicator species towards excessive accumulation of N and metals. Thus, it is crucial to fully understand factors influencing lichen susceptibility to pollutants, which could provide a wider context for pollutant toxicity on lichens before exceeding the critical level that may cause morphological and physiological damage. Furthermore, examining how contaminants interact to affect lichen responses sheds light on the severity of physiological function disturbances and compounding consequences.

3.8. Soil and bark characteristics

The physical environment, especially soil and bark characteristics, are important factors that directly/indirectly affect lichen physiology and vice versa (Ghiloufi et al., 2023; Welden et al., 2018). These are the substrates for lichens to grow and could have control over lichens' physiology. In order to characterise soil and bark, we determined pH, EC, TKN, and heavy metals in bark and soil samples collected from corresponding trees and their surroundings. A slightly acidic pH in soil and bark samples were observed for both sites. Moreover, a comparatively high TKN concentration was observed for the bark collected from KTM (0.95 %) and high TKN in soil from ACA (0.60 %) (Supplementary Tables S14 & S15). Further, a moderately higher concentration of Ca in the bark was observed in samples collected from ACA and a high concentration of Pb and Mn was observed in bark samples from KTM. Cd was not detected in both soil and bark samples of the KTM site. Further, the Ni was detected only in the bark samples from the ACA site, showing the aligned results with lichen samples. Still, Ni was detected in soil samples from KTM. In addition, a higher concentration of Mn in soil was observed in KTM and a high concentration of Pb was observed in soil samples from ACA (Supplementary Tables S16 & S17). There was no significant variation in soil and bark variables between sampling lines in both sites.

The contrasting patterns for the availability of heavy metals in soil and bark might be due to the variation in sources and deposition patterns; household emission and throughfall deposition were possible in ACA, and vehicular emission and throughfall deposition were possible in KTM. The availability of micronutrients in soil might contribute to the health of the soil in both sites, and presumably, there is no impact of soil heavy metals on the accumulation of heavy metals in lichens' thalli. Although the significant impacts of atmospheric NH_3 on bark and soil characteristics were not observed, there might be some degree of influence of bark on the deposition of N and heavy metals in lichens and vice versa (See Section 3.9).

3.9. Additional factors influencing lichen's responses

The synergistic effects of different environmental components are crucial for alternation in the physico-chemical integrity of lichens (Thakur et al., 2024). So, rather than acting alone, N with other pollutants (heavy metals in our case) might – in combination – have the greatest impact on lichen's health. The PCA analysis revealed the contrasting relationships of thallus N and heavy metals content with physico-chemical responses of lichens in our study sites. In the context of *Usnea* spp., a total of seven principal components (PCs) with 79 % component loadings were observed based on an Eigenvalue greater than one (Supplementary Table S18). Results revealed the positive relationships of TKN and Ni with CAT and EC, which were also enhanced with an increase in elevation (mainly for the ACA site). Additionally, crown cover and slope negatively impacted the TKN but were associated with elevated chlorophyll content, chlorophyll degradation, chlorophyll fluorescence, TPC, and DPPH (mainly for the KTM site). Further, negative impacts of heavy metals such as Mn and Pb on lichen abundance were observed, with these effects decreasing with increasing elevation (Fig. 9). In the context of *Hypotrachyna* spp., a total of nine PCs with 82 % component loadings was observed based on an Eigenvalue greater than one (Supplementary Table S19). Results revealed the negative relationships of TKN, NH_4^+, Ni and Zn with chlorophyll content, chlorophyll fluorescence, chlorophyll degradation, TPC, and DPPH (mainly for the KTM site). Still, positive relationships of TKN were observed for EC and CAT, which were negatively related to crown cover and slope

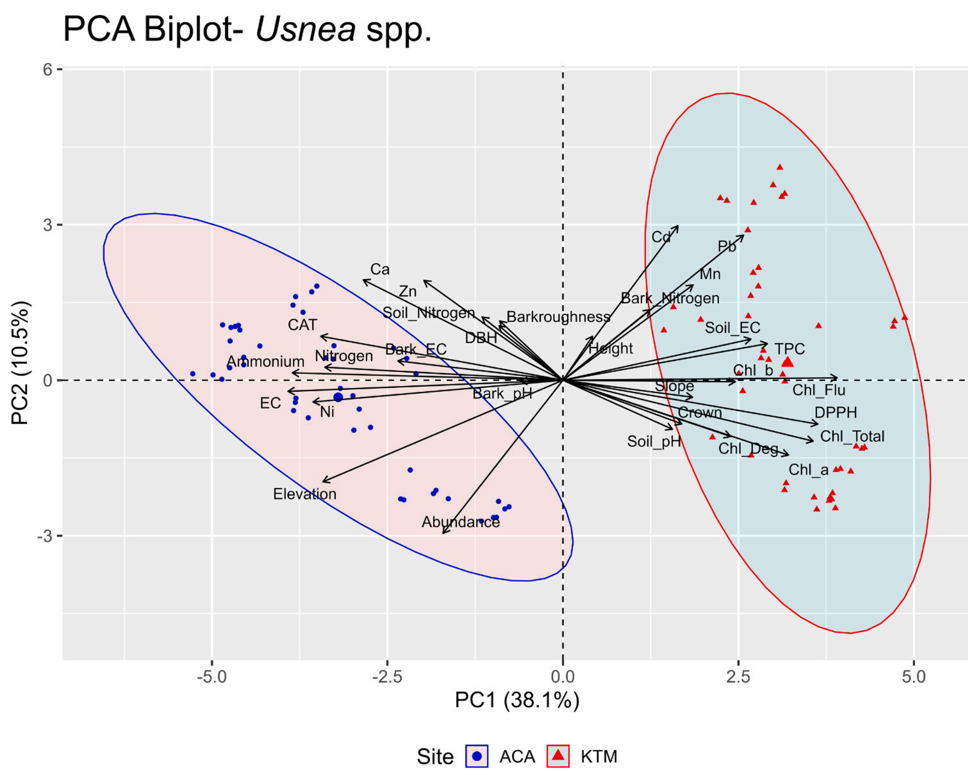

Fig. 9. Principal Component Analysis (PCA) biplot for *Usnea* spp. indicating site-wise variations in analysed lichens, soil and bark variables influencing thallus physico-chemical properties and responses.

(mainly for the ACA site). Overall, the greatest impact was observed for the *Hypotrachyna* spp. collected from the ACA (Fig. 10).

These results illustrate the site and species-specific relationships to N and elemental accumulation in lichen thalli and the combined effects of environmental variables such as slope, elevation, and canopy settings over physico-chemical and oxidative responses. Broadly, elevation, crown cover, and tree height were important factors affecting N and heavy metals accumulation and physico-chemical and oxidative

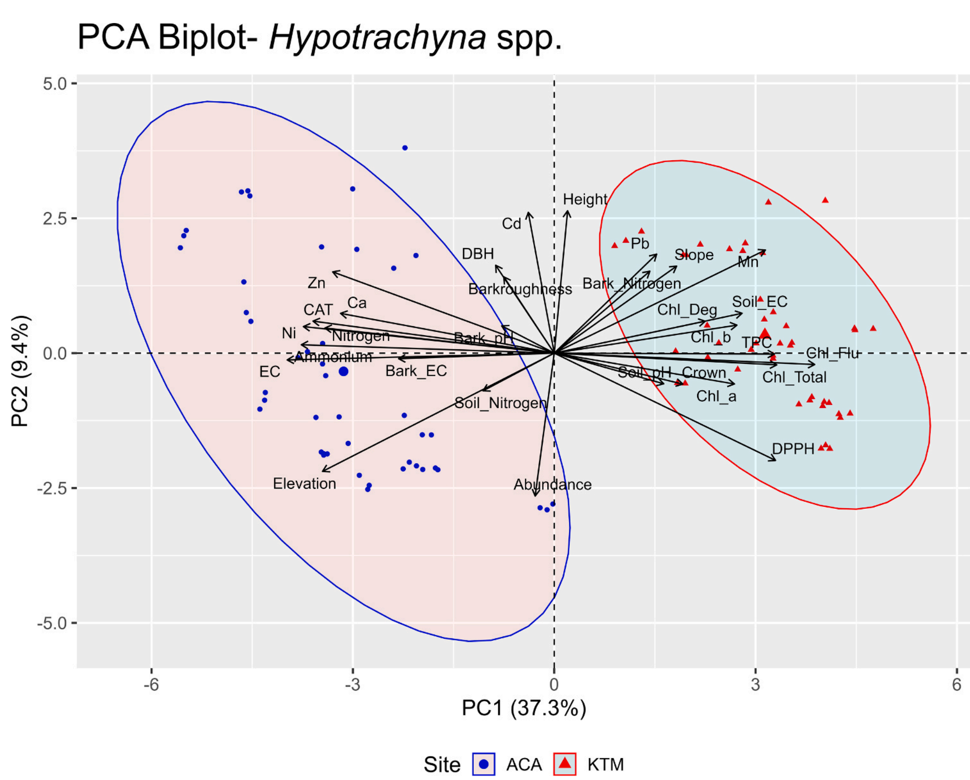

Fig. 10. Principal Component Analysis (PCA) biplot for *Hypotrachyna* spp. indicating site-wise variations in analysed lichens, soil and bark variables influencing thallus physico-chemical properties and responses.

responses in lichens. Our results are aligned with the previous study, where the significant negative impact of crown cover on the accumulation of N in lichens was observed (Esseen and Ekström, 2023). Further, a positive impact of bark pH and EC on thallus N, heavy metals such as Ni and physico-chemical responses such as EC and CAT were observed in lichen species. Thus, the tree characteristics and environmental factors should be taken into consideration to understand the functionality of lichens for pollution indicators.

Moreover, the abundance of lichens was highest in higher elevations, where the impact of pollution was presumably low (as lichens in the lower elevations get pollution directly from local sources) – signifying the visible impacts of pollution in the local gradient. Whilst the tree characteristics and microclimatic conditions also affect the abundance of lichens along with elevation, which is well supported by other studies (Calviño-Cancela et al., 2020; Di Nuzzo et al., 2022). Furthermore, no significant relationships between soil variables and lichens were observed, supporting the conclusion that there are no direct impacts of soil variables on the thallus physico-chemical properties and physiology. However, there might be some associated relationships between lichens and soil chemistry, which might be explored by throughfall monitoring, which was not in the scope of this current work.

Furthermore, various factors such as terrain, elevation, latitude, and geographic isolation have been identified as affecting tree populations and microclimate at the landscape level, which in turn impacts lichen communities (Asplund et al., 2014). In our study, due to the climatic and altitudinal variations, some disparities were observed for N and heavy metals accumulation and their impact on physico-chemical and oxidative responses in lichens from the two different sites. However, it is also crucial to understand the impact of other environmental variables, such as deposition sources, rain patterns, wind patterns, and tree characteristics, on pollutant accumulation and their interplay with physico-chemical responses. The PCA analysis revealed the sampling line-wise variation in lichen characteristics based on the analysed covariables, including both lichen's physico-chemical properties and physical environmental conditions (bark and soil characteristics) (Supplementary Figure S3). The samples taken from the first and second sampling lines, which were presumed to be most polluted in a 1-km transect, were greatly influenced regarding thallus physico-chemical responses compared to other sampling lines in the transect, supporting our findings on the trend of thallus N, heavy metal concentration and physico-chemical and oxidative responses.

Furthermore, the accumulation of N and heavy metals might be buttressed by the wind pattern. The wind pattern analysis through the ERA-5 database with a 9 km resolution (Muñoz Sabater, 2019) revealed the revolving wind pattern with low speed in the ACA area (Supplementary Figure S4). On the contrary, wind speed was higher in KTM, which might dilute air pollutants rapidly in the area, not favouring the accumulation. Furthermore, household emissions from near sources, uneven distribution and long-range transport of pollutants, especially heavy metals, might be other possible reasons for the greatest accumulation of pollutants in ACA (see Section 3.10 below). Further, the back trajectory analysis revealed the wind originating from the lower belts of Nepal and India that accumulated in Ghorepani, ACA, which might escalate the accumulation of pollutants in this area (Supplementary Figures S5 & S6) – supporting the high total N deposition in lichens from the area. A study also highlighted the role of wind and direction as a major contributor for dispersion of heavy metals in lichens from North India (Bajpai et al., 2010). Thus, it is worth examining the regional sources and deposition of pollutants in future studies.

3.10. Probable sources of heavy metals in lichens

Enrichment factor analysis revealed that long-range transport and anthropogenic inputs are major sources of heavy metal deposition in lichens from ACA. In contrast, anthropogenic and geogenic inputs were major sources of heavy metals deposition in lichens from KTM. Different studies have suggested that EF greater than 30 and 1.5 denotes metal ions from long-range transport and anthropogenic origin, respectively, and less than 1.5 denotes metal ions from natural/geogenic sources (Daimari et al., 2021). Our study revealed that the Zn accumulated in the lichens from KTM was mostly from natural sources (EF$<$1.5) and Ca and Pb from anthropogenic origin (EF$>$1.5 and $<$30). Moreover, Ca was from long-range transport mechanisms (EF$>$30) and the accumulation of the rest of the heavy metals were from the anthropogenic origin (EF$>$1.5 and $<$30) in ACA (Supplementary Tables S20 & S21). The source, transport pattern and speed of wind in ACA support the possible long-range transport of pollutants to the area. The open terrain at the ACA site may facilitate higher fluxes and deposition of nitrogen and heavy metals compared to the valley terrain at the KTM site. In addition, no household activities near the KTM site support the finding of the geogenic source of heavy metals deposition in lichens rather than anthropogenic and long-range transport sources. This signifies the substantial influences of anthropogenic activities, household emissions, and long-range transport of heavy metals and their deposition in lichens from ACA in comparison to lichens from KTM.

3.11. Species efficacy for application as bioindicators

The ability of lichens to tolerate pollution depends on thallus properties and chemistry, symbiosis, and the surrounding environment that shapes their functional traits to cope with environmental stresses (Matos et al., 2015). Thus, the selection of appropriate biomonitoring tools and lichen (whether sensitive or tolerant) species is crucial to resolve and understand the impacts of pollutants on the species to ecosystem levels. We examined two lichen species of different life forms with presumed distinct abilities in accumulating pollutants and subsequent physiological functioning. Although *Hypotrachyna* spp. showed a relatively higher accumulation of pollutants and a greater degree of physico-chemical and oxidative variability, the regression analysis revealed comparable abilities of both species toward N and heavy metals accumulation and consecutive physico-chemical and oxidative responses (Fig. 11). The significant correlation ($r = 0.98$) and regression (r^2=0.96) highlighted the analogous potential of both species ($P < 0.001$) for effective bioindication, which is consistent with our initial hypothesis. These two lichen species are very common in forested ecosystems of Nepal, which are mostly distributed in temperate and alpine climatic zones (Baniya and Bhatta, 2021; Baniya et al., 2010). Thus, the use of any of these species from the Himalayan Forest is much convenient and beneficial for bioindication studies based on thallus physico-chemical properties and responses. This approach offers countries in the region with emerging atmospheric pollutant hotspots the opportunity to utilise common lichen bioindicators as a cost-effective and accessible tool for monitoring ecosystem health and integrity.

4. Conclusions

This study revealed the exceedance of UNECE-recommended ecological thresholds of atmospheric NH_3 in study sites and highlights the urgency of comprehensive monitoring of pollutants along with assessment of ecological integrity for the conservation of biological diversity in the Himalayas. Our findings indicate the stress in the physico-chemical and oxidative integrity of lichens due to N and heavy metals accumulation, which is supported by comparatively higher effects on lichen's physico-chemical properties where background atmospheric NH_3 concentration and N deposition were high. This signifies the potential of lichens in accumulating those pollutants from the atmosphere. Further, the relationships between thallus metal ions and physico-chemical and oxidative responses indicate the suitability of using those proxies for understanding the impacts of environmental stress in bioindication studies. Also, the surrounding environmental and microclimatic conditions played a vital role in the thallus physico-chemical properties and responses. These partially support our first hypothesis

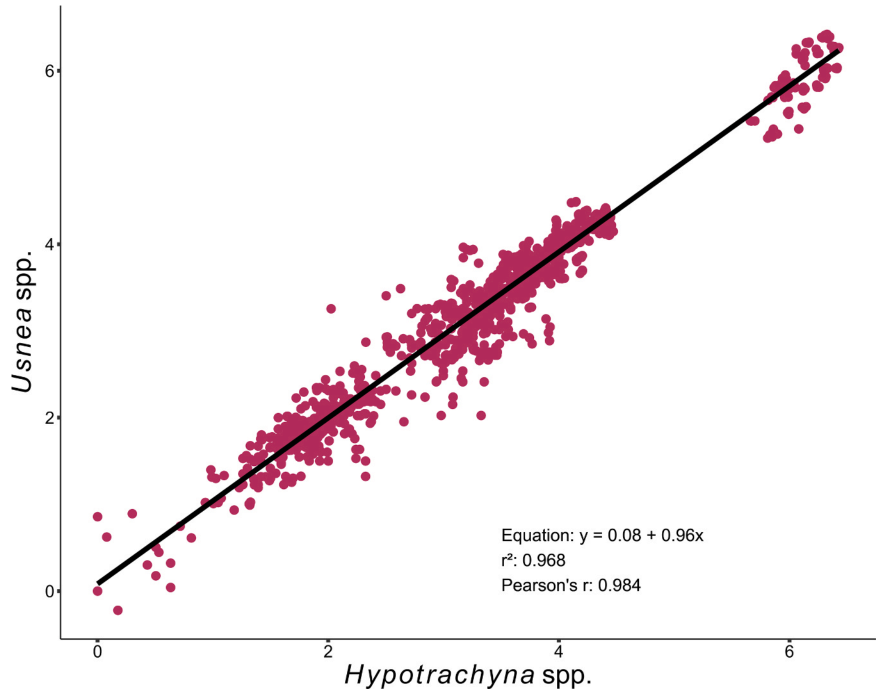

Fig. 11. Unweighted mean values of all measured lichen parameters (as listed in Table 1, only the lichen-based indicators are used here) visualised as a regression plot for *Hypotrachyna* spp. vs *Usnea* spp. ($P < 0.001$). Values of all variables were log-transformed for regression analysis.

and fully support second and third hypotheses. Thus, the characterising physico-chemical properties of thalli and their corresponding responses, together with environmental factors influencing them, are crucial in understanding the impacts of pollution on the integrity of lichen health, which in turn can serve as an indicator of ecosystem health.

This is the first study of its kind, characterising thalli physico-chemical properties and responses in two sensitive lichens from the Himalayan forests of Nepal, which can be used further to monitor ecosystem health by taking these covariables as pollution signatures in selective habitats. The similar efficacy of both the examined lichen species for N and metal ions accumulation and physico-chemical responses supports our fourth hypothesis. This study provides mechanistic insights into the responses of Himalayan lichens as pollution indicators in the natural environment, moving beyond the studies confined to temperate and boreal regions. The study also underpins the utilisation of lichens' response as proxies of environmental and pollution stress in (sub)tropical and Himalayan regions. Further studies encompassing a wider range of lichen species, their physico-chemical properties and responses are now recommended to better understand the sensitivity and tolerance of different lichen species from this region, which will provide comprehensive efforts for pollution monitoring and support biodiversity conservation. Moreover, the robust monitoring of atmospheric pollutants – especially different N species – would provide more comprehensive insights into the pattern of atmospheric deposition in lichens and their associated impacts.

CRediT authorship contribution statement

Suman Prakash Pradhan: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hirendra Bista:** Visualization, Software, Methodology, Formal analysis, Data curation. **Bishal Lamsal:** Visualization, Methodology, Formal analysis, Data curation. **Ajinkya G. Deshpande:** Writing – review & editing, Methodology, Formal analysis. **Matthew R. Jones:** Writing – review & editing, Validation, Methodology, Formal analysis. **Bishnu Prasad Pandey:** Validation, Supervision, Methodology. **Gothamie Weerakoon:** Writing – review & editing, Methodology. **Chitra Bahadur Baniya:** Visualization, Validation, Methodology. **Subodh Sharma:** Supervision, Project administration. **Mark A. Sutton:** Writing – review & editing, Validation, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119795.

Data availability

Data will be made available on request.

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