REVIEW ARTICLE

Lichenological Practices for Monitoring Atmospheric Pollution and Climate Change in India

Rajesh Bajpai[1]*, C.P. Singh[2], D.K. Upreti[1]

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Abstract

Global changes due to climate and anthropogenic disturbance are an increasing threat to biodiversity, ecosystem functions and services worldwide. The better understanding of the magnitude of these impacts, the primary drivers, mechanisms, and ecological consequences at the individual, population, community, as well as ecosystem scales are urgently needed. Several experiments and observational studies, including long-term monitoring conducted in the world to address the global change-related questions across multiple scales.

Since lichens are the primary colonizers on the lithosphere and have numerous functional roles in ecosystem including nutrient cycling and pollution sensitivity. The sensitivity of lichens for the microclimatic changes may be used to estimate the ecological continuity of forest and to established network to monitor climate change. The peculiarity of lichens are to evaluate air quality, and effective as early-warning system, to evaluate glaciers retreat, accumulation of pollutants (metals, metalloids, PoPs, radioactive substances and pesticides) in terrestrial ecosystems was studied since long time. The sensitivity of lichens to environmental change has resulted in their wide use as indicator for pollution monitoring and to identify forest habitat for biodiversity protection.

One way to overcome these challenges is to simplify coordination and standardisation of methods and sampling protocols is needed for the beginners. Here we provide some methods for field and laboratory experiments will be use in order to stimulate standardised data collection and further relationship between air pollution and global change projects in scientific disciplines. Additionally, the methods provided in the chapter, is to increase our knowledge to understand responses of lichens in relation to air pollution and climate change at regional as well as global level to expedite the quality of work in near future.

Keywords: Biomonitoring, Ecosystem, Measurements, Pollutants.

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Introduction

The lichens are the outcome of a mutualistic symbiosis of two or more organisms; one heterotroph that is mycobiont or fungal partner (Ascomycota or Basidiomycota) coupled with one autotroph that is photobiont or photosynthetic partner (cyanobacterium or green alga). In some cases the lichen contains both alga and cyanobacterium (Hale, 1983). Among these, the fungus provides a home for the alga, and the alga supplies food to the fungus.

Lichens are extremely diverse plant group, occupying ecological niches on varied physical and biological substrates such as soil, rocks, branches and bark of trees as well as on man made artifacts. Lichens have been referred to as "extremeophiles" because they have the ability to endure the conditions ranging from the arctic to hot deserts, and alpine regions to sea level (Hill and Hawksworth, 1984). Lichens are often very plentiful in areas where vascular plants are not, such as the Arctic tundra, intertidal zones and mountain peaks (Brodo *et al*., 2001). Lichens were first recognized as organisms sensitive to high concentrations of gaseous pollutants such as sulfur dioxide (Rose and Hawksworth, 1981). Lichens have been used as indicators of urban pollution and point-source emissions from uncontrolled combustion sources and are also found to act as accumulators of elements, such as trace metals, metalloid sulphur, nitrogen and organic pollutants.

The lichens air pollution monitors being widespread, permit a higher sampling density that would be practical for comparatively expensive physicochemical methods, which can compensate for the high variability of biological data. It is important to realize that lichens can never directly replace technical equipment for the measurement of air pollution. Though, they do enable rapid survey of large areas acting as a natural sampler which subsequently provide an alarm signal indicating air pollution levels that can affect various organisms as well as identifying areas which should be monitored by physicochemical means.

[1]Lichenology laboratory, Plant Diversity Systematics and Herbarium Division, CSIR-National Botanical Research Institute, Lucknow-226001, INDIA

[2]AED/BPSG/EPSA, Space Applications Centre, ISRO, Satellite Road, Ahmedabad, Gujarat, India

Corresponding Author: Dr. Rajesh Bajpai, Lichenology laboratory, Plant Diversity Systematics and Herbarium Division, CSIR-National Botanical Research Institute, Lucknow-226001, INDIA; Mobile: +91-94 522 93746; Email: bajpaienviro@gmail.com

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It is well known that the nature and impact of environmental pollutants is changing continuously, and hence if lichen data are to be used to monitor or formulate regulatory decision regarding air pollution levels, we need to know what levels are damaging to lichen and which gaseous pollutants (or other substances) are the primary or contributing cause of the observed damage or distribution change. Integrated monitoring programmes are clearly essential in conjunction with physiochemical measurements and are already being carried out in some countries, where lichenologists are carrying out such studies investigating lichen diversity as an indicator of atmospheric levels of gases along with the measurement of trace metals.

The environmental pollution is usually measured with instruments but, during the last three decades several living organisms have been used in detecting the complex effects of

environmental pollution as biomonitors. Leading to these context lichens are recognized as the best indicator of air pollution and can be utilized as a 'tool' for monitoring different atmospheric pollutants (Haffner *et al*., 2001).

More than 2000 published account on lichen and environmental studies are available from different parts of the world (Shukla *et al*., 2014) indicating use of lichens in estimating the air quality. This becomes possible because of wide geographical distribution of lichens enabling one to adopt sampling strategies with a relatively high density of sampling points while considering the nature of air pollution phenomenon greatly enhancing the data quality.

The aim of this paper is to provide some basic approaches to assess air pollution and climate change studies in a single platform along with some basic characteristics of lichens useful for proper collection and curation. Our intention is also that the methods provided in this work will be widely used in order to stimulate standardised data collection and further collaborative projects of different disciplines.

Approaches

Passive approaches

- *Floristic survey*

The compilation of species present in an area and the distribution information for those species are termed as floristic survey. The accurate list of species of an area is essential for understanding the flora of the area which provides ecological information about the unit and necessary information for determining appropriate species for biomonitoring. The value of floristic studies in lichen biomonitoring projects has recently been reviewed by Wetmore (1988). Lichen floristic studies require the expertise of specially trained lichenologists because lichens possess many unique characteristics and it is necessary for their proper and authentic identification. Quality assurance (QA) and quality control (QC) standards must be met, as most important for further studies. An additional QA/QC requirement is that, adequate peer review of research and reports, preferably by other lichenologists should be done meticulously. Prior to the field work start, previous lichen studies on the vegetation, geology, climate, air quality, lichen occurrences, biotic and abiotic properties of the study area should be kept in mind. In the present scenario the surveyed species needs to be geotagged using a best quality GPS system for future prospects.

- *Collection*

The assemblage of lichens is a valuable tool for comparing current lichen floras, community structure, and elemental concentrations with herbarium samples collected in the past. If such comparisons are to be made, it is essential that well-trained experts make the field collections easier. Because of their relatively small size, many lichens may not be noticed by inexpert naturalists, and even individuals with initial training in lichenology will not be sensitive to the range of microhabitats in which lichens occur. Poorly trained individuals are not sensitive to understated morphological differences that distinguish different species or populations in vanishing. It is important to be aware of relatively obscure species as well as the more obvious ones, since some of the obscure ones may be more sensitive to air pollution.

The collection tools required in kit are basically chisel both for rock (narrow edge) and bark (wider edge), good quality hammer, secateur, 10x/30x hand lens, GPS, blotting paper, field book, brown paper packets or old newspapers for wrapping collected specimens (Fig. 1a-d).

The lichens generally collected along with their substratum, those lichens loosely attached to the substratum are scraped out by

Fig. 1: Tools used in collection of lichens **a**. chisel and hammer **b**. Secateur, **c**. GPS, **d**. Eyepiece.

the sharp knife. In case of saxicolous lichens, a small piece of rock is collected, if not possible then apply water on it and after few minutes its scrap out especially for foliose lichens. A care should be taken to collect intact thallus at least the margin should be clearly visible. In case of corticolous lichens once should try to collect superficial bark to avoid damage to the trunk. Lichens growing on loose soils require prompt impregnation of the soil with glue/fevicol after removal to insure that the specimen does not disintegrate during transport. A spray bottle of water is helpful to provide moisturise to umbilicate type of lichens for their easy removal from the substratum. Lichen collection from monument surface is slightly different; to avoid any type damage to the monuments in such cases samples should be collect from nearby rocks or abandoned building walls and sometime taking good photograph of lichens for identification. A hand lens is essential to examine reproductive structures (isidia, soredia, apothecia, lirelliae and perithecia), particularly with crustose lichens where different species may appear similar. The collected specimens are transferred to paper or blotters packets and avoid wrapping into polythene bags. If specimens with wet bark, then it be kept in plant press and tied tightly otherwise bark gets curled up when it dries, this curled bark make problem in keeping specimens into herbarium. A voucher specimen is taken from a study site at a particular time and deposited in an established publicly accessible herbarium. A collected voucher specimens, appropriately prepared and identified, is an essential part of the quality assurance of any floristic study.

- *Curation and Herbarium deposition*

The curation and herbarium deposition of lichens is totally different and easier as compared to higher plants. Specimens mounted on hard sheet and cover in a special type of acid free specimens paper packet preserved in boxes (Fig. 2). In lichens, no need of positing, fumigation it's because of due to presence of different chemicals in thallus of lichens they prevent all type of infections. But proper drying of specimen is important for long term deposition. However, the naphthalene balls may be kept in the boxes containing lichen specimens to avoid insect infection in the boxes. The herbarium collections and data deposited in any herbaria can be used to measure the effect of pollution and climate change and phenological events in future.

Pedicino *et al*. (2002) studied historical variation in $6^{13}C$ of leaf of herbarium specimens to decipher past environments since the leaves offer promise for assessing plant environmental response over the last several hundred years. Similarly, the records of lichen specimens collected in the past from a particular area and its comparison with the present diversity (re visit of the sites) can provide an assessment of important changes on plant communities and ecological parameters which can be correlated with climate change. Changes in lichens at the community or population level are used as sensitive indicators of the biological effect of pollutants. Presence/absence or dominance of a species or a group of species may provide valuable information about the alterations in the air quality of an area due to air pollution or due to microclimatic changes (Bajpai *et al*., 2016a). The historical data of the species also provide valuable information in selection of indicator species for long term monitoring of an area.

- *Identification*

The identification of lichen specimens starts at the time of collection as they can be classified based on their substratum viz. corticolous (lichens growing over bark); ramicolous (lichens growing over twigs); lignicolous (growing over dead wood); saxicolous (growing over rocks); muscicolous (growing over mosses); terricolous (growing over soil); foliicolous (growing over leaves); fabricolous (growing over clothes); vehicolous (growing on vehicles). After

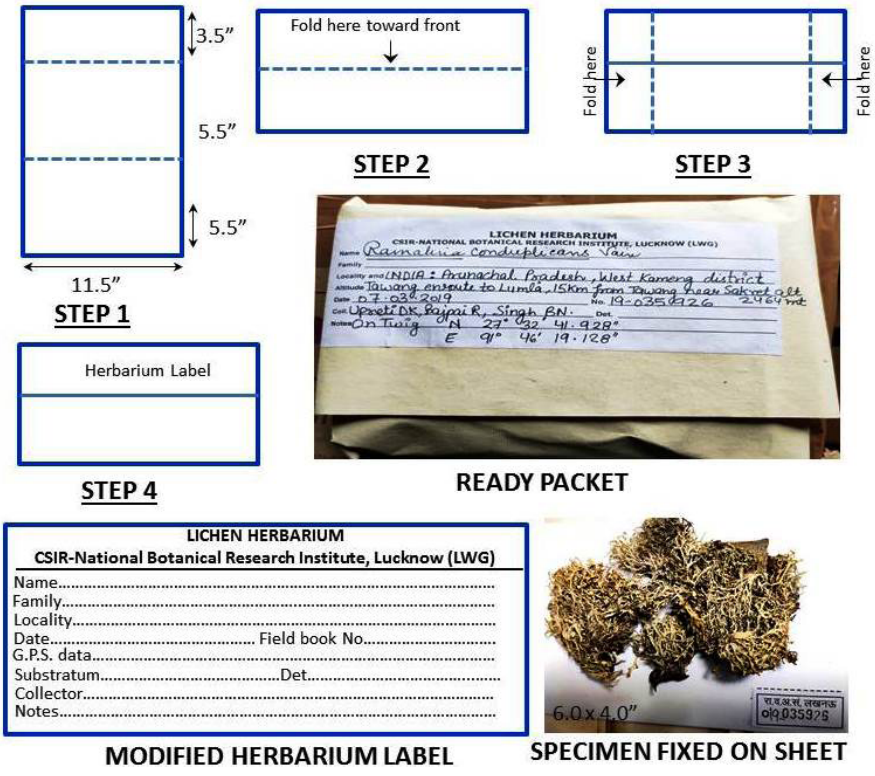


Fig. 2: Methods for preparing herbarium packets.

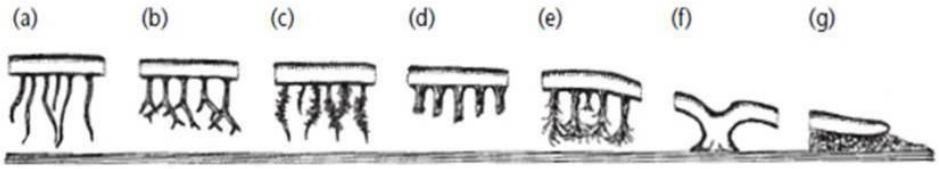
sample collection in the field, the specimens are bought back to the laboratory for further morphological, anatomical and chemical identifications. The Stereo-zoom dissecting microscope is used for observing the morphological characteristics such as thallus colour; growth forms viz. leprose (powdery to finely granular), crustose (crust like circular patches attached to the substratum throughout its underside by hyphae), squamulose (squamules like and is intermediate between crustose and foliose growth forms), foliose (leaf-like, usually dorsiventrally oriented with a distinct upper and lower surface), dimorphic (having two growth forms, the primary thallus is horizontal and is called thallus horizontalis, while the secondary thallus is vertical and is called thallus verticalis (podetia), fruticose (bushy, shrub-like, beard-like or stalked or they are either ± erect or ± pendent) (Fig. 3a-g); vegetative propagules (such as soredia, isidia, phyllidia, campylidia etc); attachment organs (such as rhizines, hapters, cilia, rhizinomorphs); reproductive organs (such as

Fig. 3: Different growth forms a. Crustose b. Foliose c. Fruticose d. Leprose e. Squamulose f. Placoid g. Dimorphic.

perithecia, apothecia, stroma). A compound and/or Phase contrast microscope (PCM) is used for studying the anatomical characters.

Lichens most commonly reproduce vegetatively (asexually) by soredia and isidia. The isidia are elongated outgrows from the thallus that break off for dispersal. Whereas soredia are small groups of algal cells surrounded by fungal filaments. They accumulate in soralia that sit upon the thallus surface. Both structures - soredia and isidia consist of algae and fungi and can be dispersed with help of wind, water or animals, so as to grow into young lichens in suitable conditions. Lichens also reproduce sexually like fungi, developing different kinds of fruiting bodies, which are spore-producing structures. The spores are made in special sacks termed as asci and supported by paraphyses. While the fruiting body is disc like and spore-generating tissue lies open to view, it is called apothecium. If the fruiting body is enclosed in a spherical structure and open through a pore, it is called perithecium. In Lichen family Graphidaceae, the apothecium is generally sessile or immersed in thallus called lirellae (Fig. 4-5).

Normally, thin free-hand cut sections of the fruiting body and thallus are used for carrying out anatomical studies. At least three separate slides of same specimens are prepared for conducting various tests. The sections were mounted in distilled water for studying the colour and the crystals of epihymenium, hymenium, subhymenium, hypothecium and to measure the ascus and ascospores size and colour of medulla. The lactophenol cotton blue (add 20.0 gm phenol crystals and 20.0 gm lactic acid and 20.0



Rhizines: a. simple, **b.** dichotomously branched, **c.** squarose, **d.** tufted, **e.** confluent, **f.** holdfast, **g.** hypothallus

Fig. 4: Type of rhizinines, isidia and soredia.

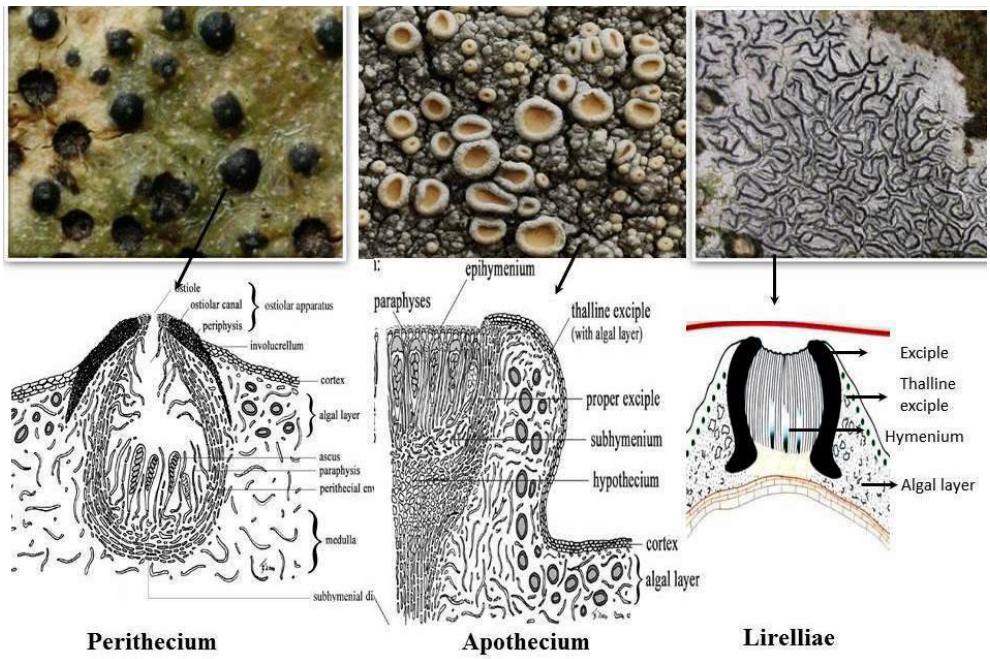


Fig. 5: Type of fruiting bodies and their vertical section.

ml distilled water) was used for staining the fungal tissue to study their organization with the fruiting body.

Apart from, morphological and anatomical identifications the chemistry of lichens also plays an important role in identification of taxa. The spot test, thin layer chromatography and microcrystallography are common methods for initial identification of chemicals. The common spot test reagents not only indicate where particular compounds are located in sectioned thalli, but may also give a clue to the chemical nature of the substance. Colour tests were performed by chemical reagents by applying it on the thallus and/or medulla which may results in change in colour that may be noted down. A positive change should be denoted by a positive (+) symbol, followed by colour change, while no change in colour was denoted by a negative (-) symbol. The following chemical reagents are used for colour spot test.

- *K test*: 10-25% aqueous solution of potassium hydroxide (70 gm KOH + 200 ml., distilled water), applied to cortex, medulla and part of fruiting body turns yellow or then red. For confirmation of reagent, medulla of *Parmelinella wallichiana* (Taylor) Elix & Hale, turns red by applying K.
- *C test*: A freshly prepared aqueous solution of calcium hypochlorite or bleaching powder (1 part calcium hypochloride + 2 part distilled water), turns red or yellow. For confirmation medulla turn rose red after applying C on *Parmotrema tinctorum* (Nyl.) Hale.
- *KC test*: At a particular spot of thallus, K reagent was applied first and immediately followed by C reagent. The colour turns yellow with usnic acid presence, blue with dihydoxy dibenzofurans, while red colour with C- depsides and depsidones which undergo rapid hydrolysis to yield a *m*- dihydroxy phenolic.
- *PD test*: Solution of *para*-phenylenediamine was prepared by dissolving 1.0 gm of *para*-phenylenediamine and 10 gm of sodium sulphite in 100 ml of distilled water with 1.0 ml of a liquid detergent. For confirmation of reagent medulla of *Flavoparmelia caperata* (L.) Hale, turns orange red.
- *I test*: 1.0 gm of iodine was dissolved in 300 ml. water with 2.0 gm of potassium iodide. The reagent keeps well for several days and is to be renewed when colour fades (confirmatory test tissue paper turns bluish - black after applying I).

The thin layer chromatography (TLC) technique is used to separate non-volatile mixtures. At present the TLC is performed on commercially available pre coated silica gel plate (MERK, cat. No. 1.05554.0007 size 20×20 cm). The standard size of plate is 15x20 cm and 2 cm at below make a pencil line for loading of lichen samples. The silica layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent (Solvent A: Toluene: Dioxane: Acetic acid, 180:60:8 ml or Solvent B: Hexane: Diethyl ether: Formic acid, 130:100:20 ml or Solvent C: Toluene: Acetic acid, 200:30 ml or Solvent D: Cyclohexane: Ethylacetate, 75: 25 ml or Solvent G: Toluene: Ethylacetate: Formic acid, 139:83:8 ml) known as the mobile phase is drawn up the plate via capillary action. After the experiment, the spots are visualized simply by projecting ultraviolet light (for lichenoxanthones) onto the sheet and the sheets are treated with 10% of H_2SO_4 and oven dried for 5 minutes in pre heated oven at 110-120°C to find out most of the organic compounds.

To quantify the results, the distance travelled by the substance being considered is divided by the total distance travelled by the mobile phase (Fig. 6). This ratio is called the retardation factor (Rf), for examples if the spot 'A' travelled 1.7 cm from the baseline while the solvent had travelled 5.0 cm, then the Rf of Á' is (Rf. 1.7/5.0) = 0.34. Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. The Rf can be match with literature of Elix (2014). For this reason, chemists usually apply a sample of a known compound to the sheet before running the experiment as control samples.

The micro-crystallography technique allowed definitive recognition of individual lichen acids on a routine basis. In this technique a small fragment of lichen to be investigated was placed on the middle part of a microscopic glass slide and one-two drops of acetone are dripped on to the fragment by dropper. Lichen substances if present gets dissolved in the solvent and extracted on the slide as residue in a ring form around the fragment as soon as the solvent evaporates. The thallus fragment was blown off. A micro-cover glass was placed over the residue and a drop of one of the crystallizing fluids (G.E. = Glycerol: acetic acid, (1:3); G.A.W.= Glycerol: ethanol: water, (1:1:1); G.A.OT = Glycerol: ethanol: ortho-toluidine, (2:2:1); G.A. = Glycerol: ethanol: aniline, (2:2:1); G.A.Q.= Glycerol: ethanol: quinoline, (2:2:1) was placed at the edge of the cover glass, the fluid gradually seeps. The slide is then heated

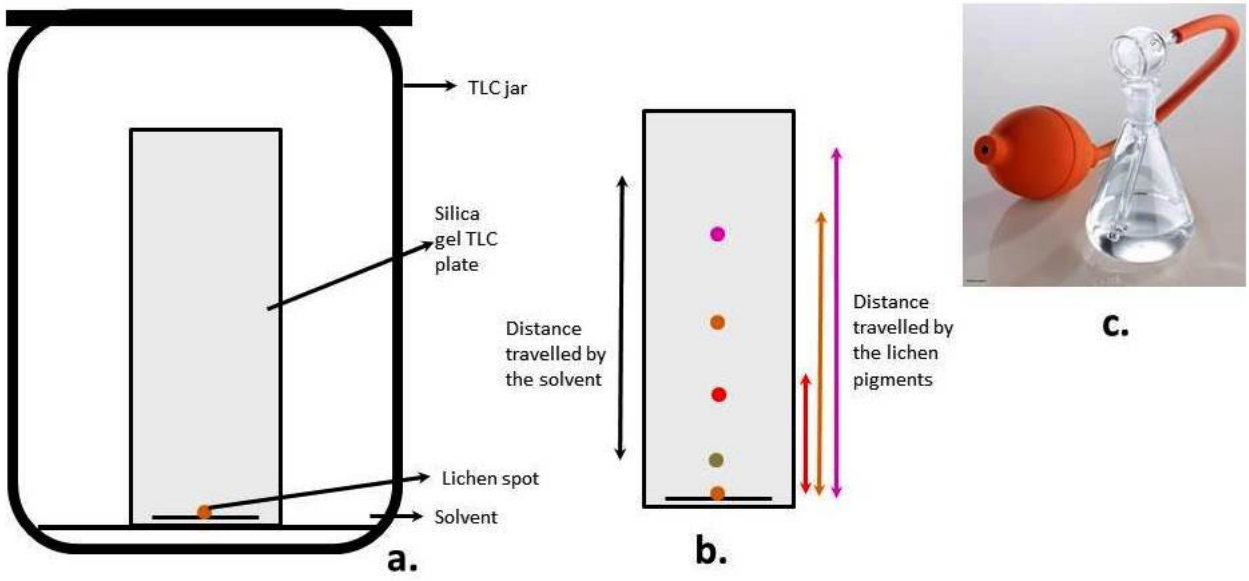


Fig. 6: Thin layer chromatography apparatus, **a**. TLC jar containing solvent and TLC plate, **b**. Developed TLC plate, **c**. Sprayer.

gently over sprit lamp. The residue dissolves in the fluid and lichen substances gradually crystallize into their characteristic shapes on cooling (Fig. 7). These crystals are observed under low power of microscope and identified by comparison with the photographs or line diagram published by Asahina (1950, 1952). The proper identification of any lichen specimens at species level both the TLC and sport test is necessary.

Zone mapping

Roth (1988) and Schulmeister (1996) carried out passive monitoring studies and develop zone maps. The method distinguishes areas with varying degrees of pollution. A large diversity of species is desirable because it is an indication of favourable conditions for the survival of many species, including humans. However, intensive effects of abiotic factors, for occurrence of nitrogen compounds or other pollutants with eutrophicating effects, may favour growth of certain specialized (nitrophilos) species. Thus the increase of these specialized species must be evaluated negatively, since the factors which cause the increase of specialized species disturb the ecosystem. The parameter for estimating the level of environmental stress, which is a diversity value, is obtained by the number of lichen species and their frequency per area.

Lichen zone mapping is a method used to indicate the severity of pollution with reference to distance from sources reflected by the number of species present or absent. The detailed physical investigation of epiphytic vegetation of cities or of larger areas around factories (point sources) can be used to segregate the area into three, four or more major lichen zones. Herzig *et al.* (1989) classified the zones corresponding to degree of injury to lichen flora and level of total air pollution as (Lichen desert-Critical air pollution; Inner struggle zone-Higher air pollution; Outer struggle zone-Medium air pollution; Transition zone-Lower air pollution and normal zone - very low air pollution). Further we can calculate IAP (index of atmospheric purity) index of the area. The occurrence of each species can be GPS tagged and plotted on a map to provide an idea about the overall picture of the lichen distribution in the area. Such distribution map helps to determine the distribution of a species that has changed in the area, in the course of time.

Quadrat

Quadrat sampling is a common tool to study ecology, especially biodiversity of an area. Commonly, a series of squares (quadrats) of a set size are placed in a habitat of study area, and the species within those quadrats are identified and recorded. Passive quadrat sampling (done without removing the organisms found within the quadrat) can be either done carefully, sorting through each individual quadrat by hand or more efficiently, can be done by taking a photograph of the quadrat for future analysis. Abundances of organisms found at the study site can be calculated using the number found per quadrat and the size of the quadrat area. The quadrate type may be varied according to the objective and the purpose of the study. The quadrats are in different types it may be List Quadrate (only listing the names of different species growing in the quadrat); List-Court Quadrate (records the number of individuals of each species represented in each quadrat); Chart Quadrate (record the position and areas covered by twigs, mats or tufts of grasses, mosses on the graph paper, these graphs help to compare any change in structure of community in future); Clip Quadrate (record the biomass or weight of each species, all individuals are collected (but when the weight of a particular organ, e.g., twigs or leaf are to be determined only the concerned organ is clipped and its fresh or dry weight is recorded); Nested quadrates (a series of quadrats, laid one over the other with gradually increasing size). The size of quadrates to be used in a given community is determined by constructing a species area curve.

Through the study of quadrates it is also emphasized to check the community compostion of lichens in the areas because community composition provides distinct evidence of climate driven effect on species diversity (Insarov and Schroeter, 2002). The small scale observations involves altitude or distances from seashore aims to determine the relation between climatic factors and lichen diversity (species diversity and community composition). Since the sensitive physiology of lichens, changes in temperature or water availability lead to shift in the lichen communities therefore, repeated monitoring of the lichen community indicators provides an early warning of response to climate change. Lichen community composition combined with type of forest vegetation and environmental data suggest causes for variation in the communities. The richness and abundance of species can be correlated with climate value of the area.

The lichen communities are also sensitive to landscape structure, land use context and forest management. The forest lichen communities respond to primary climate variables such as precipitation and temperature and to geographical gradients such

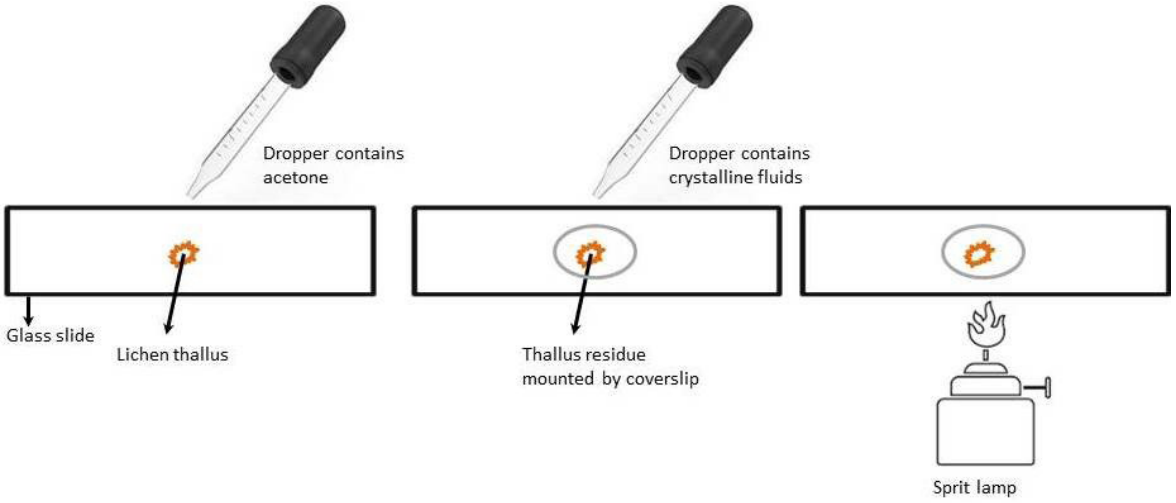


Fig. 7: Micro crystallography apparatus.

as elevation and latitude that integrate climate factors. Some of the lichen bioindiactor communities are listed in Table 1.

Multi-summit approach (MSA) for long term ecological monitoring

The long term ecological studies not only enhance our understanding of the relationship between vegetation and environment, but essential for authenticating responses of global climate change. Such type of periodical studies helps to distinguish between pathways, causes and mechanisms of vegetation change (Pickett *et al*., 1987). MSA for permanent monitoring indicate different stages of succession and also generate hypotheses on its pace and causes. The MSA have become an essential tool for monitoring vegetation and particularly emphasis on establishing long term monitoring changes in mountain vegetation that are early indicators of climate change because mountain ranges play a significant role in influencing the regional and global climate. Further, being governed by low temperature, high-altitude regions of the world are more responsive to the changing climatic conditions and hence, better indicators of the same (Grace *et al*., 2002; Bajpai *et al*., 2018). In recent year MSAs, in Indian Himalayan states such as Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh were established with support of Space Applications Centre (ISRO), Ahmedabad for long term climate change assessment under Himalayan Alpine Dynamics Research Initiative (HIMADRI) programme. In this programme, permanent plots were established throughout alpine regions and lichen diversity was analyzed (Fig. 8). The plot protocol determines the way in which current or future samples are taken. Methods for marking and mapping plots must be identified with current options for establishing coordinates for study sites including topographic maps and global positioning systems.

The past researches prove that the growth and reproduction of plant communities in mountain ranges are mainly controlled by temperature that gives rise to steep ecological gradients and narrow ecotones and any minor change in temperature may lead to change in tree line and nival zones. Further the types of organisms and their functional traits may lead to explore some hidden treasure of changes. At high altitude regions the lichens and bryophytes

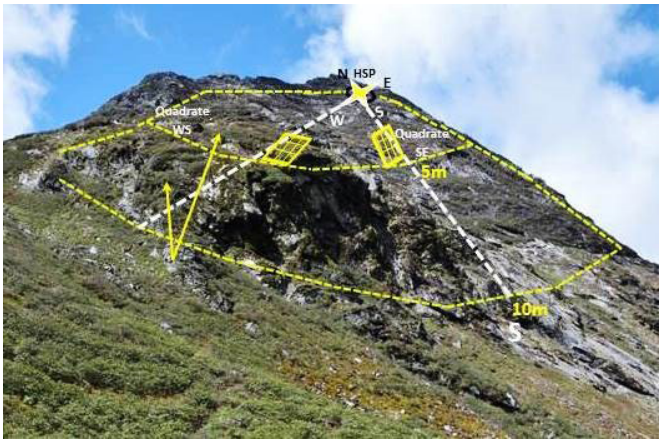


Fig. 8: Multi summit approach established in Arunachal Pradesh (Bajpai *et al*., 2018).

Table 1: Lichen bioindicator communities.

S.N.	*Community*	*Species of the lichen genera*	*Indication*
1.	Alectorioid	*Alectoria, Sulcaria, Ramalina*	Better air quality
2.	Calicioid	*Dibaeis, Baeomyces, Calicales taxa*	Ecological continuity of the old growth forest
3.	Cyanophycean	*Collema*, *Leptogium*	Forest age and continuity forest ecosystem function
4.	Dimorphic	*Cladonia*, *Cladia*, *Stereocaulon*	Undisturbed soil ecosystem
5.	Graphidioid	*Graphis*, *Diorygma*, *Opergrapha, Sarcographa, Phaeographis*	Open thinned out forest
6.	Lecanorioid	*Lecanora, Lecidella, Biatora*	Well illuminated environmental conditions/ forest expose of well light and wind
7.	Lecideoid	*Lecidia, Protoblastedia, Haematomma, Schadonia, Bacidia, Buellia*	Indicate exposed illuminated area
8.	Leprarioid	*Lepraria, Chrysothrix, Cryptothecia*	The *Chrysothrix* appears first after forest fire and indicate dry, rough barked trees of moist and dry habitats
9.	Lichinioid	Members of family Lichinaceae	Presence of calcareous/acidic substratum
10.	Lobarian	*Lobaria, Sticta, Pseudocyphellaria, Peltigera*	Moist shady forest /sensitive pollution indicators/long forest continuity
11.	Parmelioid	*Bulbothrix, Flavoparmelia, Parmotrema, Parmelia, Punctelia* and other genera of family Parmeliaceae	Thinned-out forest with more light and moist condition exhibit dominance of this community.
12.	Peltuloid	*Peltula, Endocarpon, Zahlbrucknerella*	Stable rock substratum
13.	Pertusarioid	*Pertusaria*	Old tree forest with rough bark trees
14.	Physcioid	*Physcia, Pyxine, Dirinaria, Heterodermia, Phaeophyscia*, *Rinodina*	Pollution tolerant/ their presence indicate a nitrophilous environment.
15.	Pyrenulioid	*Anthracothecium, Pyrenula, Lithothelium, Porina*	Young and regenerated forest and smooth thick barked tree forest
16.	Teloschistacean	*Caloplaca, Letroutia, Brigantiaea, Xanthoria*	Indicate high UV irradiance
17.	Umbilicoid	*Umbilicaria, Dermatocarpon*	Stable rock substratum along with high UV irradiance and low temperature
18.	Usnioid	*Usnea, Bryoria*	Old forest and better air quality
19.	Xanthoparmelioid	*Xanthoparmelia*	Indicate stable productive landscape

are growing abundantly over boulders, shrubs and some time on soil. The presence of lichens and bryophytes along with other organisms at high alpine areas of Indian Himalayan regions (IHRs), the Space Applications Centre (SAC-ISRO), Ahmedabad earlier established permanent plots in the IHRs and continued for transects based diversity assessment.The transects placed in representative vegetation in each region using satellite imagery and local expertise, to observe past (if available) and present data of diversity and in future this data can be used to study the shift (Fig. 9). The functional traits and presence of lichens and bryophytes in open and close canopy may play a significant role in the study of treeline position.

Remote sensing

The airborne, hyperspectral, and space borne multispectral remote sensing datasets can be employed to map distribution patterns at various scales which can be used for the assessment of lichen diversity. Remote sensing practices help to map spatial distribution, identifying spatiotemporal changes in the distribution and analyzing the disturbance patterns among the major lichen community (Nordberg and Allard, 2002).

The use of Digital Elevation Models (DEMs) and GIS (Geographic Information Systems) technologies can help in analyzing the spatial pattern for lichens. The field information collected through the field plots can be visualized and correlated for better understanding of the relationship of lichens along with its surrounding environment. Predictive modeling provides the potential suitable habitats for the major lichen communities and also can be used with various climate datasets to predict the distribution pattern due to climate change. The study will lead to assess large scale patterns of responses with broad species representation like air pollution data, diversity and distribution and community composition towards understanding current and future importance of climate change on species performance and diversity.

In Indian context, very few studies are available on the use of geographic information systems and lichens to map air pollution (Bajpai *et al*., 2014, Singh *et al*., 2016). Waser *et al*. (2007) developed several models to predict lichen species richness on soil, rocks and trees in Swiss Pre-Alps following a gradient of land use intensity combining remote sensing data and regression models. Though, the remote sensing techniques cannot replace lichen survey altogether, however, these methods provide information that is remotely similar to field samples and which would allow to considerably reduce extensive field surveys (Cousins and Ihse, 1998). The presence of lichen dominated communities in the Alpine region and mapping their distribution directly by matching image (narrowband) pixel spectra with the reference (hyperspectral) spectra of lichens using a matched filtering algorithm. Such techniques have been widely used in mineralogical and lithological studies and recently been applied for the mapping of lichens (Casanovas *et al*., 2015). Furthermore, the concept of spectral species can be explored further, using hyperspectral imageries at high spatial resolution.

Lichenometry

The method of lichenometry developed and used by Beschel (1973), and since then it has been widely applied in dating recently exposed rocky substrates in the world (Armstrong, 2005). In Indian context, Joshi and Upreti (2010); Bajpai *et al*. (2016b), Bisht (2018), Bisht *et al*. (2018a,b) performed lichenometric studies in some major Indian Himalayan Glaciers to study the glacier retreat. Based on the methods applied, lichenonometry may be direct and indirect. The direct lichenomerty deals with observations on individual lichen species at repeated intervals over time while, indirect lichenomerty is a correlation established between the size of the thallus and the surface age, based on lichens growth measurements from surface of known age e.g.: Graves's stones, stone wall/ manmade artifacts or including tree trunk of known age (Fig. 10a,b).

Both direct and indirect methods can be used by the researchers to get accurate data in filed and correlate with exposure date of the surface by the following ways:

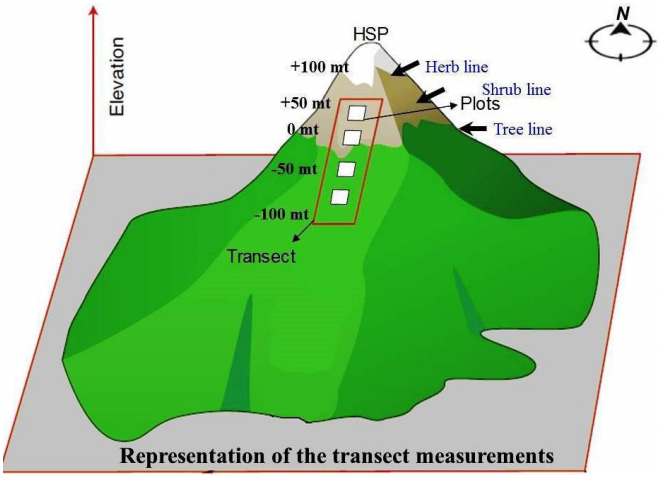


Fig. 9: Transect study initiated in Indian Himalayan Regions (Courtesy: CP Singh).

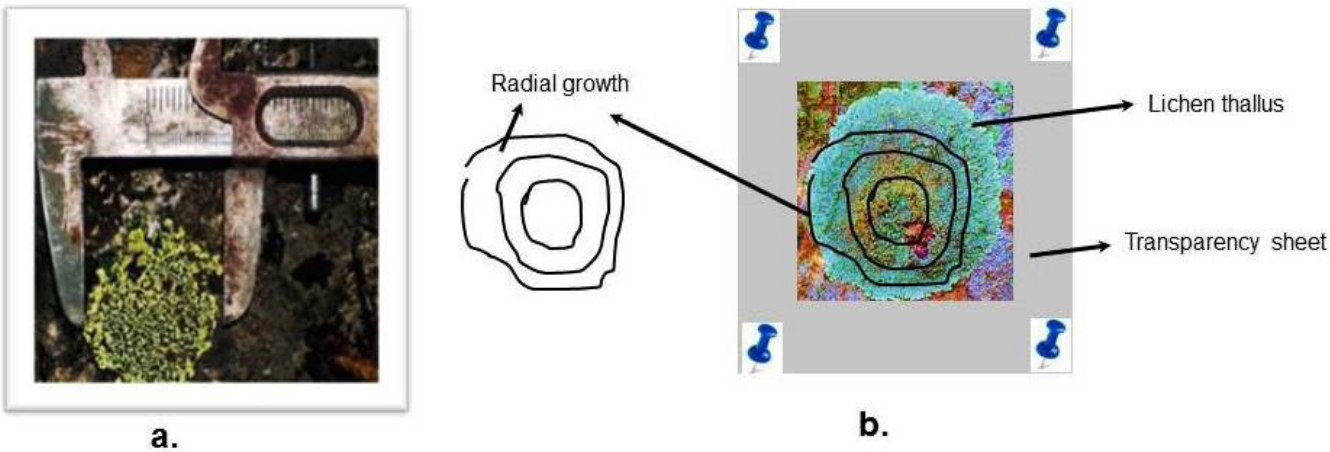


Fig. 10: Lichenomerty **a**. *Rhizocarpon geographicum* diameter by vernier scale **b**. radial growth measurements of known diameter of thallus.

- *Longest axis measurements*: According to Bull *et al*. (1995), the longest axis can be measured from edge to edge along the greatest diameter of the largest lichen thallus on the surface. In this measurement the role of hypothallus is important to differentiate overlapping of two or more thallus.
- *Mean of longest thallus in a single substratum*: The mean of the largest thallus diameter on a single substratum is assumed as the largest orbicular growth of the lichen. It is also emphasized the rapid colonization and optimal microenvironments.
- *Size frequency measurements*: The mean of about 100 to 500 lichen thallii, and the dates use to estimate the longest thallus or age of sub populations of lichens in the data set.
- *Percentage cover*: Percentage cover is the ratio of the area of lichen covering the surface to the total exposed surface area.
- *Development of dating curve*: A graph of lichen thallus size can be plotted against age of the surface on which it grows.
- *Photographic Method*: The photographs are taken over a period of time, the photographs are adjusted in a way that the millimeter scale included in each photograph can be reproduced at the actual size of lichen thallus (measured in pixels). Original and repeat photographs are viewed side by side to ensure measurements were made in the same locations.
- *Area measurement of lichen thallus*: Lichen growth rates can also be calculated by measuring the area of the thallus. Rydzak (1961) traced the outlines of thalli on transparent sheets and then retraced these at a later date. The surface area of each traced thallus can be measured; the procedure is repeated in the next period of measurement and the growth can be calculated in mm.

Innes (1988) provided a list of micro-and macrolichens that can be used in lichenometric studies. The macrolichens *Stereocaulon*, *Usnea*, *Ramalina* have mainly vertical or fruticose growth form, therefore they are irrelevant in the lichenomentry. The *Umbilicaria* species are special with their umbilicate thallus form, which means that they are attached to the substrate by a central umbilicus. However, the remaining species have a more or less typical centrifugal pattern of growth and may be recommended for dating purpose. Some microlichens, such as, the species from the genera *Aspicilia, Acarospora, Lecanora, Rhizocarpon* and *Xanthoria* can be recommended for dating of rock substrates. The most often used species in lichenometric studies is *Rhizocarpon geographicum*, because of its bright colour easily recognizable in the field, circular growth form and worldwide distribution in alpine areas.

In alpine environments, *R. geographicum* attains a slow growth rate of 0.2 mm/year (Hansen, 2008) and lives up to a considerable age. Morphologically, this lichen comprises discrete areolae that contain *Trebouxia* as algal partner, located on a fungal medulla, which is attached to the substratum and extends into a black algal-free marginal zone around the thallus called hypothallus. Primary areolae near the edge of the hypothallus may develop from free-living algal cells on the substratum that are trapped by the hypothallus, whereas secondary areoles may develop from zoospores produced within the thallus, thus ultimately resulting in the radial growth of *Rhizocarpon*. Due to their slow growth rate and uniform growth size, lichens help in dating the exposure time of the sequences of the rock forming glacier moraines due to retreat of the glacier thus providing the approximate time of glacier retreat. The lichenometry appears to be superior to many other techniques; it attempts to date glacial deposits in the most accurate way. The technique is easy, cheap and can be applicable to date surfaces less than 500-years-old where radiocarbon dating is least efficient.

Active approaches

Transplantation

Richardson (1992) reviewed the use of lichen transplants to assess air quality in urban environment and to monitor contaminants in air and water. The healthy lichens are transferred from an area where they occur naturally to test area. The changes in physiology, morphology and element accumulation provide data that can be correlated with extend and impact of pollution in an area. Most of the transplantation experiments have been performed with corticolous lichens particularly of foliose habit because they are generally more tolerant to gaseous or airborne pollutants. The twigs having lichens or lichen bearing bark are fixed in 20x20 cm cardboard with any fixative (Araldite), and are placed or hanged on trees and if trees are absent, the electric pole or other objects (Fig. 11).

Allen (2017) established a new method to transplant lichens for recolonization and proposed a need to reinforce, reintroduce and translocate species for conservation purposes.

Open top chambers (OTC)

The open-top chambers are the best currently available experimental technique for developing functional relationships useful for predictive purposes (Shriner *et al*., 1990). Open-top chambers used in the field and experimental designs for using open-top chamber systems include pollutant-free and ambient

Fig. 11: Prepared Transplant board for transfer into polluted area.

chambers as well as non-chambered control plots to estimate any chamber effects. The OTCs are made-up of polyvinyl chloride (PVC) plastic film enclosed by hexagonal frame constructed by aluminium (Fig. 12). The OTC approach for long-term climate change monitoring with lichens in Indian Himalayan region are initiated recently. Due to the unhurried growing rate and sensitive nature, lichens can be utilized as an indicator of climatic changes, on which they are growing as well as kept in OTC for continuous observations. Other parameters like lichen diameter, qualitative and quantitative estimation of secondary metabolites in high altitude lichens may be performed in OTC and their comparison with control may also lead to correlate with rising temperature as well as against high UV radiations.

Dust load

The dust interception and its accumulation in different plant species not only depends upon the sources and amount of pollutants in the environment but also depends on morphological characters of plants too. Dust may also act as a secondary stress, and allow penetration of toxic metals or phytotoxic gaseous pollutants in to the internal part of the organism. Effects of dust on natural communities may alter the competitive balance between species in a community. The most extensive evidence for the effects of dust on plant communities has come from studies on epiphytic lichens. Gilbert (1976) studied the effect of dust on lichens and described the effects of limestone on epiphytic lichen communities form distinctive zones around sources of sulphur dioxide pollution. He found zones surrounding a lime dust source and resulted that heavily dusted trees had few lichens, but this was followed by a zone containing lichens that are normally saxicolous i.e. *Caloplaca decipiens*, *Catillaria chalybeia*, *Lecanora calcarea*, *L. campestris, Lecidella scabra* and some species of *Bacidia* and *Micarea*. Kaupii (1980) stated that dust from fertilizer factories also affect lichens and revealed that the application of fertilizer dust to *Hypogymnia physodes* and *Cladonia stellaris* caused a temporary increase in net photosynthesis and an increase in the number of algal cells in the thalli. The measurement of dust is an important parameter to observe the qualitative as well as quantitative particulate matter in the area. The amount of dust was calculated by taking the initial and final weight of Petri plate in which the samples were washed.

$$W = \frac{W_2 - W_1}{A}$$

Where, W = Dust content (mg/cm^2), W_1= Weight of Petri plate without dust, W_2 = Weight of Petri plate with dust A = Total area of thallus in cm^2

Accumulation valuation

The various heavy metals, metalloids, pesticides, persistent organic pollutants (POPs), radionuclide are widely accumulated in lichens through active as well as passive methods. These pollutants accumulated in lichen thallus through absorption as well as adsorption beyond their physiological needs. The pollutants accumulated in lichens can be analyzed with following methods.

- *Metals and metalloid analysis*

The oven-dried (70°C) lichen samples grounded to fine powder and digested (0.25 g) in HNO_3: H_2O_2 (3:1 v/v). After digestion the volume will make up to 5 ml by Milli Q water. The samples diluted 10 times and the concentration of elements like Fe, Zn, Co, Ni, Cu, Se, Mn, As, F, Cr, Pb will analysed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500 ce). The standard reference materials of metals/metalloids (E-Merck, Germany) used for the calibration and quality assurance for each analytical batch. Analytical data quality of metals/metalloids can be ensured with repeated analysis (n=5) of quality control samples, and the results thus found within (±2.82) the certified values. Recovery of Fe, Zn, Mn, Cu, Co, Se, Cr, Pb, F and As from the samples can be found to be more than 98%, as determined by spiking of samples with a known amount of elements.

- *Persistent organic pollutants (PoPs) analysis*

Persistent Organic Pollutants are chemical substances having high persistence, bioaccumulation, transfer and diffusion in the environment. The PoPs are basically Polycyclic aromatic hydrocarbons (low, medium and high molecular weight), pesticides and chemicals containing only carbon and hydrogen. The PoPs are carcinogenic and stranger to living beings. The persistent organic pollutants (PoPs) can be estimated following the procedure of Environmental Protection Agency -EPA 8310 (USEPA, 1986). Lichen samples (0.5 g) extracted in 100 ml of Dichloromethane (Merck, AR) for 16 hours using a Soxhlet apparatus. The extract passed through anhydrous sodium sulphate (Qualigen, AR) to remove moisture and then concentrated to 2 ml under vacuum in Buchi rotary evaporator purified on a silica gel (100/200 mesh size, Qualigen) column using hexane according to the EPA method 3630. The purified extract solvent exchanged to acetonitrile (Merck, AR) and final volume can be made to 2 ml in umber coloured volumetric flask. Samples stored in dark at 4°C till the analysis of PAHs.

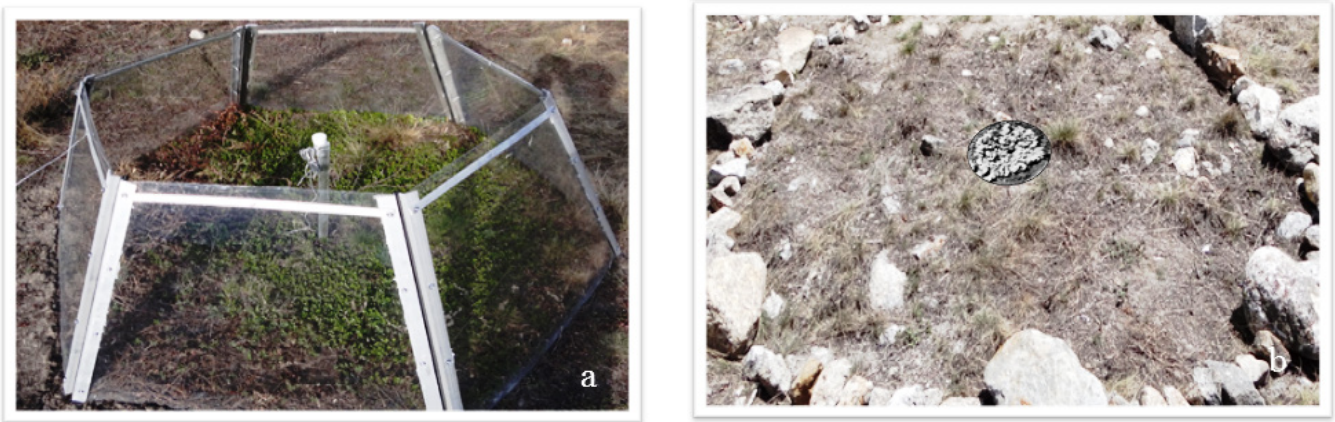


Fig. 12: Open Top Chamber (OTC) **a**. In side **b**. Outside as control.

The PoPs can be separated using reverse phase C-18 column (250 nm × 4.6 mm, 5 µm particle size; Waters) on a HPLC consisting 515 pump (Waters milford, MA, USA) and UV-visible detector (2487, Waters) and eluted through 70% (v/v) acetonitrile at flow rate of 1.5 ml min^{-1} at 27°C. The chromatogram recorded at 254 nm and processed using the software Empower™. The identification and quantification are performed by using the respective PoPs standards procured from Sulpelco, USA. The limit of detection for individual PoPs ranged between 10-30 ng g^{-1}.

The bioaccumulation factor (BAF) can be calculated at monitoring sites by the applying the formula of Chang et al. (2005) as:

BAF = (Concentration of pollutants in thallus / concentration of pollutants in substratum); the factor class represented by a range of variations i.e., 1-2=A, 3-4=B, 5-6=C, 7-8=D, 9-10=E and more than 10=E class. The lower class indicated that the species do not have hyperaccumulation capacity in the natural state.

- *Carbon, nitrogen concentration and carbon discrimination analysis*

The carbon and nitrogen concentration of lichen samples can be analyzed by an elemental analyzer (EA 1108, Carlo-Erba-Milano, Italy) with an analytical precision of 0.1%. The stable C isotopic ratio can be measured with an isotope ratio mass spectrometer (CONFLO interface, Thermo, MAT Bermen, Germany) operating in continuous flow mode after the combustion of the samples in an elemental analyzer (EA 1108, Carlo-Erba-Milano, Italy). Samples weighted by using a high precision Ultra Micro Balance and the percentage composition are calculated based on Carlo Erba Elemental Standards B2005, B2035, and B2036, with an error of <1%. Standards of ammonium sulphate (IAEA-N1 and IAEA-N2) for nitrogen, and sugar (IAEA-CH6) and graphite (EIL-32) for carbon were used for calibration.

- *Radionuclide analysis*

Apart from metals, metalloids (inorganic) and organic pollutants lichens have an ability to accumulate the radioactive substances like Cesium, Thorium, Cobalt, Plutonium, Radium, Radon, Uranium and Strontium in their thalli in huge amount. The analysis of a radionuclides in the lichen thallus can be measured by following the procedure of USEPA (1986) and modified by Kirchner and Daillant (2002).

The radionuclides in lichen samples can be analyzed through gamma-spectrometrically using a large volume reversed electrode high purity germanium detector (Canberra, 55% relative efficiency). Unlike conventional coaxial detectors, this detector has a usable energy range which extends down to 5keV and thus enables to determine ^{210}Pb by measuring its 46.5 keV gamma line.

All samples ashed in a stove at 380°C. As the intensity attenuation of gamma radiation in matter considerably varies with its density, the ashed samples need mixing with appropriate amounts of wax and pressed to pellets of standard geometry (70 mm diameter, 5 mm height) and density (1.0 g cm^{-3}), for which the detector are calibrated using a multi-isotope standard. All samples should be sealed priority for measurements using an aluminum foil coated with plastic (thickness 0.1 mm) and stored for more than three weeks to ensure that ^{226}Ra and ^{228}Ra were equilibrated with their decay products ^{214}Pb and ^{212}Pb, respectively. Activity concentrations of ^{226}Ra were determined indirectly by ^{214}Pb, since the gamma decay energy of ^{226}Ra (186 keV) interferes with a ∂-line of ^{235}U which may also be present in the lichens. As time between sampling and analyses usually exceeded 2 months, the short lived gamma emitting isotopes ^{228}Ac and ^{234}Th were used to determine concentrations of its long lived precursers (^{228}Ra and ^{238}U, respectively).

- *Accumulation mechanism*

The lichens fulfil their water demands from the atmospheric moisture or dew water together with nutrient passively from their environment. Being long lived they cumulatively accumulate metals, metalloids, radionuclides, PoPs, through variety of mechanisms including particulate trapping, ion exchange, extracellular electrolyte adsorption, hydrolysis and intra cellular uptake in huge amount with any harm to self. Lichens have following characteristics features which play an important role in accumulation.

Rhizines: Species with rhizines have been observed to have higher concentrations of elements than species without rhizines, suggesting that rhizines can act as reservoir to store excess elements.

Isidia/soredia (asexual reproductive organs): The presence of vegetative structures on the cortex not only makes the surface rougher but also increases the surface area, thereby enhancing the particle entrapment compared to smooth, shiny surfaces.

Thallus type: The foliose species have higher element concentrations than fruticose species, as they have bigger lower surface area attached with substratum. The crustose species with areolate condition (cracks) between thalli accumulate also higher concentration of elements than the smoother thalloid species.

Lichen chemical substances: Many lichen secondary metabolites such as parietin and usnic acid, are known to chelate metals, suggesting that species with higher concentrations of secondary products may also have higher metal loads.

Apothecia (Sexual reproductive organs): Some species are known to concentrate lichen substances and elements in fruiting structures, hence species that produce many apothecia could be expected to contain greater amounts than those with few or none.

Colour of thallus: Some pigments are known to complex metals, for e.g. parietin complexes Pb while melanin complexes UV; thus species with such pigments could contain more elements. The darker coloured thalloid species contain higher levels of elements than the whiter, grey, green and thalloid taxa.

Hydration: The moisture levels of the thallus affect the K/Ca ratio because these two elements interact to regulate water balance in tissues, primarily through the action of calcium oxalate. Species that are tolerant of drier habitats are likely to have more Ca and a lower K/Ca ratio.

Size/area: Larger lichens have more surface area than small lichens, and could be expected to trap more elements and have higher concentrations. This appears to be the case with the parmelioid species, which have larger area than the other species.

Substratum: It is commonly thought that lichens growing on soil may have higher concentrations of soil elements than lichens growing elsewhere.

Exposure: Everything else being equal, it is thought that lichens on trees are more exposed to ambient wind carrying particulates than those growing on ground.

Detoxification: It is known that species able to precipitate metals and other elements with oxalate have higher tissue concentrations of metals than species that use cell wall carboxylation to detoxify the metals. *Diploschistes* is an example of the former, while *Xanthoria* is an example of the latter.

Thickness: Thinner lichens would be expected to have higher tissue concentrations than thicker ones because they have higher surface/volume ratios.

Pigment quantity: Not only would the presence/absence of pigments affect element concentrations, but also the quantity of pigments too. Higher pigment concentrations would obviously lead to higher element concentrations.
Thallus acidity: Lichens with a lower thallus pH have been found to have increased elemental uptake, thereby leading to higher tissue concentrations.
Thallus age: Older lichens will have higher elemental concentrations than younger ones because they have been exposed longer.
Necromass: Mat growing lichens accumulate dead tissue at the base, and probably accumulate higher concentrations of elements if this part of the mat is analyzed.
Length of exposure: A longer exposure, everything else being equal, will lead to higher tissue concentrations.
Element competition: Elements that are related in the periodic table often displace one another due to antagonistic effect. The final tissue concentration of some elements may therefore depend on other elements already present.
Ultraviolet light: Lichens exposed to ultraviolet light increase their concentrations of usnic acid, which, in turn, would complex more elements. Therefore, lichens in higher ultraviolet environments may have higher element concentrations than those in lower ultraviolet environments.

Physico-chemical approaches

Physiological analyses detect alteration to the normal functioning of an organism or any of its parts. There are many techniques available to determine physiological changes resulting from air pollution; the more commonly used ones are listed here.

Photosynthetic pigment analysis

About 0.5 g of lichen sample need to be ground to powder with acid washed sand, 25 mg calcium carbonate and 5 ml chilled acetone (80% Merck, Analytical grade) on ice in dim light. The slurry obtained then need to be transferred to a 10.0 ml centrifuge tube, vigorously shaken and centrifuged at 10,000 rpm for 10 min. The supernatant was then decanted, kept in the cold and pellet re-suspended in 1.5 ml chilled acetone (80%) and centrifuged as above. The supernatant was then combined, made to known volume and analysed using a UV scanning spectrophotometer. The pigments content (Chl *a*, Chl *b*, total chlorophyll and carotenoid) can be calculated from absorbance values at 663 and 645 nm according to the equation of Arnon (1949). The total carotenoid content can be calculated according to Parsons *et al*. (1984) from absorbance values at 480 and 510 nm (Fig. 13). The chlorophyll degradation can be measured by methodologies developed by Ronen and Galun (1984). In this method the ratio of 435/415 nm will be observed by lichen samples (0.05 g) dipped in 5 ml of dimethyl sulfoxide (DMSO, Merck, analytical grade) overnight and determined with a spectrophotometer.

$$\text{Chlorophyll a} = \frac{[\,12.7\,(A_{663}) - 2.63\,(A_{645})\,]}{1000 \times W} \times V$$

$$\text{Chlorophyll b} = \frac{[\,22.9\,(A_{645}) - 4.68\,(A_{663})\,]}{1000 \times W} \times V$$

$$\text{Total Chlorophyll} = \frac{[\,20.2\,(A_{645}) + 8.02\,(A_{663})\,]}{1000 \times W} \times V$$

$$\text{Carotenoid} = \frac{[\,7.6\,(A_{480}) - 1.49\,(A_{510})\,]}{d \times 1000 \times W} \times V$$

$$\text{Protein} = \frac{A_{700} \times 2.5\ (\text{BSA standard})}{W} \times V$$

Fig. 13: Steps involves in calculation of pigment.
(Whereas A = Absorbance reading at spectrophotometer, W= weight of samples, V= Final volume in ml, d= length of light path (d=1); all values are comes in mg g^{-1} fresh weight).

Protein analysis

The protein concentration of homogenates can be determined according to the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as the standard and calculations can be made from absorbance values at 700 nm. As per this method following are the reagents prepared for quantification of protein in a lichen samples (Fig. 14).

Reagent A (2.0 gm NaOH + 500 ml Distilled water + 10.0 gm Na_2CO_3)

Reagent B (0.5% $CuSO_4.5\,H_2O$ in 1% sodium potassium tartrate) 0.5 gm $CuSO_4.5\,H_2O$ + 1.0 gm sodium potassium tartrate (SPT) + 100 ml distilled water

Reagent C 50 ml reagent A + 1 ml Reagent B

Reagent D 1 part of folin phenol + 2 part of distilled water

Antioxidative enzyme analysis

About 50 mg fresh weight of lichen thalli need to be homogenized in 0.5 ml of 50 mM potassium phosphate buffer pH 7.5, containing 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone. The homogenate was then centrifuged at 15,000 rpm for 10 min and the supernatant was used for SOD and CAT analysis. For APX, 1 mM DTT and 2 mM ascorbate were also added to homogenization buffer.

SOD activity can be measured spectrophotometrically as described by Beyer and Fridovich (1987). The assay mixture (1 ml) contains 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 µM nitro-bluetetrazolium, 1% (w/v) triton X-100, and the extract. The reaction starts by addition of riboflavin (1.3 µM) and the mixture was then exposed under photon flux of 50µmol m^{-2} s^{-1} at room temperature for 7 min. In this assay, 1 U of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%.

CAT activity can be assayed in mixture (1 ml) containing 100 mM phosphate buffer pH 7.0, 6 mM H_2O_2, and extract. The decomposition of H_2O_2 are monitored at 240 nm (Cakmak and Marschner, 1992). CAT activity calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

APX activity can be determined by monitoring the decrease in A290 using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. The assay mixture (1 ml) contained 100 mM potassium phosphate buffer

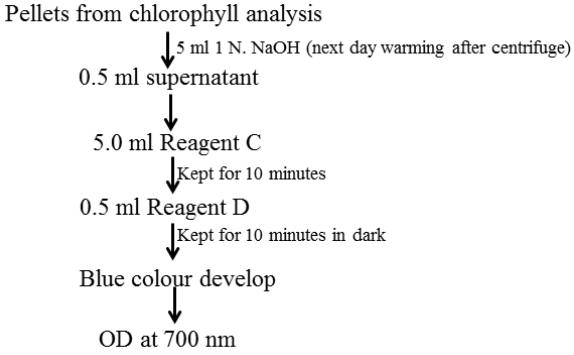


Fig. 14: Steps involves in analysis of protein.

pH 7.0, 0.5 mM ascorbate, 0.4 mM H_2O_2, and extract (Nakano and Asada, 1981).

Chlorophyll fluorescence (Fv/Fm) analysis

A PAM 2000 Chlorophyll Fluorometer (Walz, Effeltrich, Germany) can be used to measure chlorophyll fluorescence in lichens. Lichen samples need to be kept in dark for 5 min before the fluorescence measurements will be taken. The minimum fluorescence level with open PSII reaction centers (F*o*) was measured by a weak red measuring beam, followed by a saturation light pulse to determine the maximum fluorescence (F*m*) level with closed PSII reaction centers. Variable fluorescence F*v* is the difference between F*m* and F*o*, and the chlorophyll fluorescence is represented by F*v*/F*m*.

Amino acids/ Mycosporine-like amino acids (MAAs) analysis

The Pico-tag method can be used for the estimation of amino acid (AAs) on HPLC system (Bidlingmeyer *et al*., 1984). Three hundred milligrams of homogenized lichens sample will be hydrolyzed in 10 mL 6 N HCl for an hour at 150°C in an oven and than filtered for further analysis. Ten microliters of samples and standard (2.5 µmol mL^{-1} in 0.1 N HCl) were dried in a vacuum oven at 55°C for 30 min at 75m Torr. The samples re-dried twice by adding 20µL of re-drying solution (Ethanol: Triethylamine: Water, 2:1:2). Then the samples are derivatizated by adding 20 µL of derivatization reagent (Ethanol: Triethylamine: Water: Phenyoisothiocynate, 7:1:1:1) and again vacuum dried. These samples are diluted to 1 mL with Pico-Tag sample diluent and filtered (0.22 µm syringe filters). The separation was carried out at 40°C using a Pico-Tag amino acid C18 column (3.9×15 cm; 5µm). For each sample, 20 µL of extract was injected and the column was eluted at 1 mL min^{-1}, with an optimized gradient established using solvents A (0.14 M sodium acetate, containing 0.05% triethylamine and 6% acetonitrile, and maintain pH 6.40) and B (60% acetonitrile in water). A step-by-step gradient was used with an increase of proportion of solvent B until it reached 46% during 10 min, followed by an increase up to 100% in 5 min, with a flux of 1 mL min^{-1}. The column was then cleared and optimized to 100% A for 8 min at 1 mL min^{-1}. The amino acids analyzed by this procedure and expressed in mg Kg^{-1} fresh weight. Chromatograms were integrated using Empower 2 HPLC software v 6.0.

The cyanobacterial lichens synthesize a unique class of chemical substances known as mycosporine-like amino acids (MAAs) which has strong ultraviolet (UV) absorption between 300 and 360 nm. Analysis of MAAs can be done with 0.5 g of fresh lichen sample which is extracted with 2.0 mL of methanol overnight at 4°C. The whole extract was then centrifuged (5000×g, 10 min) and the supernatant transferred to a 2-mL Eppendorf tube. Thereafter, the supernatant was evaporated to dryness using a vacuum evaporator and reconstituted with 0.5 mL ultrapure water. This solution was then vigorously shaken with 200 µL of chloroform to remove non-polar compounds. The resultant solution was centrifuged (5000×g for 5 min) to separate the water phase which was subsequently filtered through 0.2-µm pore sized cellulose syringe filter for UHPLC analysis. The liquid chromatographic analysis of MAAs will be performed on a UHPLC DIONEX-Ultimate 3000 system coupled to photodiode array (PDA) detector set at the scanning range of 260-380 nm. The separation of MAA compounds will be achieved using a RP C18 column (4.6 × 250 mm, 5 µm) (Thermo Fischer Scientific Acclaim™) with a gradient elution of solvent A (methanol) and solvent B (0.1 vol % formic acid in MilliQ water) at constant flow rate of 0.8 mL min^{-1}. Solvent B kept constant at 90% for 6 min then decreased to 80% for next 3 min followed by a decrease to 0% within 4 min. Then the system was re equilibrated with 90% solvent B for 4 min. Definite sample volume (20 µL) can be injected into the UHPLC system using a Rheodyne injection port equipped with a 20-µL loop. Whole instrumental processes controlled by Chromeleon software (Thermo Fisher Scientific).

A triple quadruple mass spectrometer equipped with an ESI source (API-4000, ABSciex) can be used for the identification of MAA compounds in the UHPLC-PDA-based fractional extracts of lichens. The isolated fractional solutions introduced into the ESI-MS/MS using a Harvard apparatus (Holliston, MA, USA) model 11 plus syringe pump at a flow rate of 20 µL min^{-1}. The source temperature (TEM), ion spray voltage (IS), nebulizer gas (GS1) and turbo gas (GS2) are operated at 150°C, 5500 V, 16 psi and 28 psi, respectively. The declustering potential (DP), entrance potential (EP), collision gas (nitrogen, 99.99%) collision energy (CE) and cell exit potential (CXP) were thoroughly optimized and finally maintained at 44 V, 10 V, 4-8 psi, 25-44 psi and 10 V, respectively. The whole instrumental processing of ESI-MS/MS was controlled by Analyst™ software (Version 1.6, ABSciex, Foster city, CA, USA).

Electrolyte conductivity analysis

The variation in electrolyte conductivity (EC) measured by placing a piece of lichen thallus in deionized water is a simple test to check the integrity of the plasma membrane enclosing lichen cells. In damaged cell membranes, permeability is altered and electrolyte leakage occurs, mainly K^+ ions which are the most abundant (Marques *et al*., 2005). Each sample will be soaked for 1 h in 50 mL of deionized H_2O. The electrolyte conductivity of the water (expressed µS cm^{-1} at a normalized temperature of 25°C) was measured before and after lichen immersion using a conductivity meter (Crison Basic 30).

Nitrogenase analysis

Lichens with cyanobacterial phycobionts are capable of "fixing" atmospheric nitrogen, converting it into a usable form of nitrogen to the plants of an area. Lichens, may be an important contributor of nitrogen to the ecosystems in which they occur. The atmospheric pollutants have been shown to affect nitrogenase activity levels. Measuring nitrogenase activity is fairly simple and cheap. Since nitrogenase enzyme converted into acetylene followed by ethylene thus, nitrogenase activity levels are reflected in the amount of ethylene that is produced. Levels of ethylene and acetylene can be measured on a gas chromatography for estimation of nitrogen fixed in the organism.

Respiration (Gas Exchange) analysis

A significant decrease in respiration rates of lichens exposed to increasing pollutant levels has been demonstrated repeatedly in the literature (Eversman, 1980; Fields and Clair, 1984). The most common methods of measuring respiration rates employ an oxygen electrode to measure O_2 absorption or an infrared gas analyzer to measure CO_2 evolution in the absence of light.

Conclusion

Owing to the vast geographical area of India, the number of studies so far conducted on lichens in relation to the pollution monitoring is quite meagre. It is hypothesized that apart from a number of complex and costly techniques available for monitoring air pollution and climate change. However, the presented practices helps to familiarize some easy and low cost techniques which would not only reduce considerably the extensive field survey but also help to obtain simple data for developing air pollution and climate change models.

Moreover, the methods provided definitely helpful to the students, researchers and scientists in terms of diversity assessment and bioindication to evaluate changes in climate and pollution load in the study area for long term perspective in the region as well as short term assessment.

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