COMPOSITE SAMPLES WITH A SMALL SAMPLE SIZE REFLECT MEAN ELEMENT CONCENTRATIONS IN THREE LICHENS DIFFERING IN ELEMENT-SPECIFIC CONCENTRATIONS

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Abstract. The biomonitoring of atmospheric element deposition often uses a single composite sample to test the local mean lichen element concentrations, so as to reduce the expenditure. However, further study is needed to test the composite sample representativeness on the mean element concentration (CSRMC) and its lichen species specificity and element specificity. In this study, we compare the concentration differences of 55 elements between three lichens (*Evernia mesomorpha* Nyl., *EVM*; *Usnea aciculifera* Vain., *UAC*; *Ramalina sinensis* Jatta., *RSI*) from a remote forest site of Hebei, northern China. The results show that the differences of element concentrations between lichens have strong element specificity. Composite samples can well represent the local mean element concentrations in three lichens. CSRMC is the highest in *RSI* in comparison with *EVM* and *UAC*. It is usually ranking 1 for most elements in three lichens except Al, Ca, Hg, P and Si. Therefore, when we use the composite samples with a small sample size for lichen biomonitoring, it is necessary to carefully select lichen species and the number of composite samples according to the CSRMC of the elements of interest. This method is especially suitable for sites with small spatial heterogeneity in atmospheric element depositions.
Keywords: *biomonitoring, composite sample representativeness, local variability, species specificity, element specificity*

Introduction

The passive biomonitoring technique analyzes the level, transportation, and source–sink relationship of atmospheric element deposition based on the temporal and spatial variation of element concentrations in local organisms (Abas, 2021; Dołęgowska et al., 2021; Bajpai et al., 2022). This technique can be used in remote areas and extreme environments, and is suitable for monitoring atmospheric element depositions on large spatio–temporal scales. The shortcomings of traditional (instrumental) methods, including high cost, complicated operation, high technical level, and limited number of monitoring sites and items, can be well overcome by this technique. Lichen

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biomonitoring technique is a reliable tool in environmental forensics (Abas, 2021; Dołęgowska et al., 2021). Lichens are nutritionally dependent on dry and wet deposition, and their element concentrations, especially heavy metal concentrations, have a close positive correlation with the ambient level (Paoli et al., 2018; Dörter et al., 2020).

A composite sample composed of multiple individuals of a same species is often used to represent the mean local level of element accumulations at a specific monitoring point with reducing the analysis costs in the biomonitoring studies (Patil et al., 2011). For example, in the passive biomonitoring of atmospheric element deposition in Continental Europe using mosses, the researchers suggested selecting a 50 × 50 m^2 quadrat at each monitoring point and collecting 5-10 moss subsamples from this quadrat to form a composite sample (Fernández et al., 2015). Geiser (2004) suggested that a composite sample of lichens should be collected from each plot to represent the mean element concentration in lichen thalli, and replicates should only be made at plots with rich lichen materials to evaluate the variability of lichen element concentrations caused by the collection method. The composite sampling of a same lichen species is widely used in the passive biomonitoring studies (Barre et al., 2015; Hanedar, 2015; Kurnaz and Cobanoglu, 2017; Ratier et al., 2018) due to the following advantages. (1) It can reduce the workload and financial cost. (2) It can lower the influence of individual differences on element concentrations which are related to the age/size, morphology/physiology of lichens and the microtopography/microclimate conditions (Dołęgowska et al., 2021). (3) It can greatly lower the influence of species difference in element concentrations which is common across diverse ecosystems and biomonitors (Dołęgowska et al., 2021).

The composite sample representativeness on the mean element concentration (CSRMC) is necessary to be evaluated, particularly for the composite sample with a small sample size. A large size enables a high CSRMC theoretically and therefore was adopted by some researchers. For example, the composite sample is composed of 200-300 g dw lichen thalli (Cayir et al., 2007; Kurnaz and Cobanoglu, 2017). However, a much smaller size of composite sample has often to be adopted due to the poor population size of native lichens. In the relevant studies, a composite sample is often composed of 5-25 individuals or 5-20 g dw lichen thalli (Hanedar, 2015; Yavuz and Çobanoğlu, 2019; Jia et al., 2020; Wu et al., 2020).

The CSRMC can be evaluated with the probability that element concentration of a single composite sample falls within a threshold range. This range should not exceed the local variation of lichen element concentrations (expressed by coefficient of variation, CV). When comparing the deposition levels between sites on a large spatial scale, the local variation of lichen element concentrations must be lesser than the global variation, otherwise it will lead to false negative errors easily (Adams and Gottardo, 2012). The local variation generally does not exceed 30% in related studies. For example, in a forest ecosystem in Portugal, the local variation of 20 elements in *Flavoparmelia caperata* (L.) Hale and *Evernia prunastri* (L.) Ach. is 1-33% (Godinho et al., 2009). In a forest ecosystem in France, the local variation of 10 elements in *E. prunastri* is less than or equal to 30% (Ayrault et al., 2007). In a remote forest of Italy, the local variation of 18 elements in *Pseudevernia furfuracea* (L.) Zopf is 0.5-35.7% (Malaspina et al., 2014). In two remote sites of Tunisia, the local variation of 10 elements in four epiphytic lichens is 0.9-35.7% (Chahloul et al., 2022). In a remote site of China, the CVs of 42 elements in two lichens are 10-29% (Wu et al., 2020). In

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contrast, some researchers suggested that local variation should not exceed 25% (Frati et al., 2005; Malaspina et al., 2014). For the sake of conservative estimation, we adopted the local variability of ≤ 25%, set the threshold range of 0.75×mean ~ 1.25×mean, and evaluated the CSRMC by $P_{(mean \pm 0.25 \times mean)}$. A higher CV mean that an individual has a higher element uptake/release sensitivity to environmental fluctuations in space and time, and therefore a lower CSRMC.

CSRMC is rarely evaluated in the lichen biomonitoring studies, and its lichen species– and element– specificity are even less studied. It is unreasonable to expect an acceptable CSRMC in the polluted sites usually with high heterogeneity of atmospheric element depositions if it cannot be detected in a background site with a much lower one. Therefore, in a remote forest of Hebei, northern China (*Fig. 1a*, *b*), we collected 5 composite samples of three morphologically distinct lichens (*Evernia mesomorpha* Nyl., *EVM*, *Fig. 1d*; *Usnea aciculifera* Vain., *UAC*, *Fig. 1e*; *Ramalina sinensis* Jatta., *RSI*, *Fig. 1f*, *g*), and tested the concentrations of 55 elements (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Gd, Ge, Hg, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pb, Pr, Rb, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Tb, Th, Ti, Tl, Tm, U, V, Y, Yb, and Zn). The purposes of this study are as follows: (1) To study the species differences of element concentrations and their element specificity. (2) To analyze the intra–site variation of lichen element concentrations to evaluate CSRMC of composite sample with a small sample size and its species and element specificity.

Materials and methods

Study area and sampling point

Saihanba Machinery Forest Farm is a remote forest area of Yanshan Mountains in China, located about 283 km north of Beijing (*Fig. 1a*), and lies in the forest–grassland ecotone at the junction of Inner Mongolia Plateau, Greater Khingan Mountains and Yin Shan Mountains. The area is dominated by hills and lava fields, with an altitude of 1500-1940 m. The climate is cold temperate continental monsoon climate, with an average annual precipitation of about 438 mm. The main type of vegetation is forest–grassland, with the forest coverage as high as 80%. There are a number of epiphytic lichens here. The area is sparsely populated and is 50 km away from the nearest populated area, with few industrial and agricultural activities and low air pollution level (*Fig. 1b*).

Sampling point (42°26’7”N, 117°9’29”E) is set in the hilly area of a typical forest ecosystem. The human disturbance within around 10 km is mainly slight forestry activity, and the vegetation is mainly artificial forest dominated by *Larix gmelinii* (Rupr.) Kuzen and *Betula platyphylla* Suk.

Sample collection

On October 25th, 2016, a 50 × 1000 m transect line was set up on the sunny slope of a hill at an altitude of 1482-1507 m. Small altitude fluctuation was chosen because altitude changes will affect lichen element concentrations (Bačkor and Loppi, 2009). Three lichens (*EVM*, *UAC*, and *RSI*) were randomly collected on the transect line.

EVM, *UAC* and *RSI* are selected as the biomonitors due to the following considerations. (1) They are widely distributed with high biomass in northern China (Wei, 2020), and therefore are expected to become the readily available biomonitors in the vast

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northern China. (2) The applicability of *EVM* and *RSI* as biomonitors has been confirmed (Bennett and Wetmore, 1999; Tumur et al., 2011). *Usnea* spp. is also commonly used for atmospheric deposition monitoring (Adams and Gottardo, 2012; Monaci et al., 2012; Wu et al., 2021). (3) They are all epiphytic lichens sharing the same substrate and microhabitats but differ in morphology, and therefore allowing us to highlight the influence of morphology on lichen element concentrations by minimizing the influence of habitat. All the three lichens attach to the bark of *Larix gmelinii* with a single holdfast (*Fig. 1c*). *RSI* has a large foliose thallus composed of fan–shaped and nonsorediate lobes with a width of 5-10 mm (*Fig. 1c, f, g*). *EVM* is a foliose–shrubby lichen (*Fig. 1c, d*) composed of many dorsoventral, strap–shaped, sorediate and narrower lobes (1-5 mm wide), its surface is the roughest with the densest soredia among the three lichens. *UAC* is a typical fruticose lichen, which is composed of sorediate main branches (0.4-1 mm in diameter) with a large number of side branches (*Fig. 1c, e*). The voucher specimens (L0079491, L0079492 and L0079493) of three lichens are kept in Kunming Institute of Botany, Chinese Academy of Sciences (KUN, CAS).

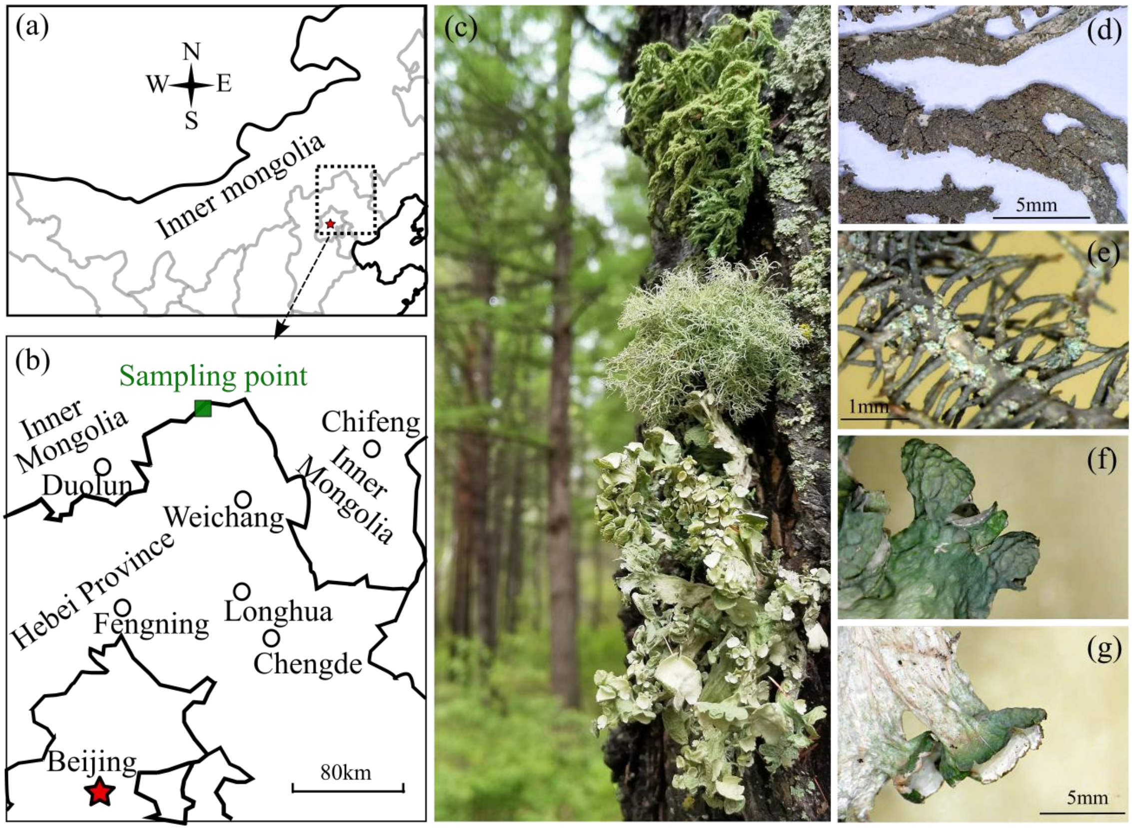

Figure 1. *Study area and three lichens. (a)-(b), Location of sampling point. (c), Evernia mesomorpha, Usnea aciculifera and Ramalina sinensis from top to bottom and the growing environments. (d), Evernia mesomorpha, showing densely sorediate lobes. (e), Usnea aciculifera, showing sorediate branches. (f), Ramalina sinensis, upper side of lobes. (g), Ramalina sinensis, lower side of lobes*

We collected 5 composite samples for each lichen. To test the CSRMC of the composite samples with a small sample size, we set the dry weight of each composite sample as 8-10 g, which is composed of 20-25 thalli with a diameter of 2-4 cm. To

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eliminate the element concentration bias caused by substrate (Adams and Gottardo, 2012), we followed the collection methods recommended by the most relevant studies (such as Agnan et al., 2014). Specifically, the lichen thalli were carefully collected together with the bark with a small blade from various aspects of *Larix gmelinii* trunk at the height of 1-2 m above the ground. The collected samples were placed in kraft paper bags, sealed and air–dried in the dark.

Pretreatment and element analysis

We removed the impurity from lichen surface under a stereomicroscope. The samples were not washed with water, because washing would cause element–specific loss of lichen elements (Boonpeng et al., 2021). All samples were dried in the dryer at 70 °C for 72 h to constant weight, and crushed and homogenized with a ball mill equipped with tungsten carbide canister (Retsch MM400; Retsch GmbH, Haan, Germany). HNO_3-H_2O_2 microwave digestion system was used to digest 200-300 mg of homogenized samples in PTFE tank.

Fifty-five elements (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Gd, Ge, Hg, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pb, Pr, Rb, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Tb, Th, Ti, Tl, Tm, U, V, Y, Yb, and Zn) were tested by inductively coupled plasma mass spectrometry (ICP-MS) in Hebei Research Center for Geoanalysis. The instrument used is Agilent 7700X (Agilent Technologies, Tokyo, Japan).

Four reference materials were used for quality control in the element analysis: IAEA-336 (a Portugal lichen) provided by International Atomic Energy Organization, GBW10014 (cabbage), GBW10015 (spinach) and GBW10052 (green tea) provided by the Institute of Geophysical and Geochemical Exploration, Chinese Academy of Geological Sciences. The quality control results show that the analytical accuracy and precision is generally less than 10%. The test results are consistent with the certified and/or recommended values.

Statistical analysis

Coefficient of variation (CV) is used to characterize the intra–site variability of each element, and CV = SD/mean×100%, where mean is the average value of element concentrations, and SD is standard deviation.

The element concentration of a composite sample can be regarded as a mean concentration averaged over several lichen individuals. According to the central limit theorem, the data of composite samples should obey the normal distribution. The CSRMC was estimated by calculating $P_{(\text{mean} \pm 0.25\times\text{mean})}$ with the normal distribution model. $P_{(\text{mean} \pm 0.25\times\text{mean})}$ is the cumulative probability within the confidence interval of mean ± 0.25×mean, and is calculated in Microsoft Excel Data Analysis package using the following function (*Eq.1*):

$$\text{NORMDIST}(\text{mean} \times 1.25, \text{mean}, \text{SD}, \text{TRUE}) - \text{NORMDIST}(\text{mean} \times 0.75, \text{mean}, \text{SD}, \text{TRUE}) \quad \text{(Eq.1)}$$

where normdist is a function, mean is the average concentration, SD is the standard deviation of the concentration, TRUE denote that the cumulative probability.

According to $P_{(\text{mean} \pm 0.25\times\text{mean})}$, CSRMC is divided into three ranks. Rank 1: $P_{(\text{mean} \pm 0.25\times\text{mean})} \geq 0.90$, indicating that the composite sample data can strongly represent the

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local mean element concentrations. Rank 2: 0.90 > $P_{(\text{mean} \pm 0.25\times\text{mean})} \geq 0.75$, indicating that the risk of the composite sample data deviating from the local mean element concentrations is acceptable. Rank 3: $P_{(\text{mean} \pm 0.25\times\text{mean})} < 0.75$, indicating that the composite sample method is not recommendable, because the risk of its data deviating from the local mean element concentrations is too high.

Independent Samples T test was used to analyze the significance of the concentration difference of each element among lichens, and Bonferroni corrections ($\alpha = 0.05/3 = 0.017$) were used in multiple comparisons. Paired experimental design was not considered, because there was no obvious altitude and habitat gradient on the selected transect line. The data analysis was completed in SPSS 25.0 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 shows the concentrations (mean and CV) of 55 elements and their differences among lichens (Independent samples T test with Bonferroni correction). For conciseness, CV, species difference in element concentrations and $P_{(\text{mean} \pm 0.25\times\text{mean})}$ (*Eq. 1*) are integrated into *Table 2*. *Table 3* summarizes the number and proportion of elements in different ranks of $P_{(\text{mean} \pm 0.25\times\text{mean})}$. These results show obvious species– and element–specificity of element concentrations and CSRMCs (*Tables 1, 2* and *3*).

Table 1. *Average content and variation coefficient of 55 elements in three lichens*

Element	***Evernia mesomorpha* (*EVM*)**		***Usnea aciculifera* (*UAC*)**		***Ramalina sinensis* (*RSI*)**	
	Mean (μg/g)	**CV (%)**	**Mean (μg/g)**	**CV (%)**	**Mean (μg/g)**	**CV (%)**
Ag	0.059A	21.94	0.040B	11.15	0.054A	9.76
Al	3576A	16.30	884.2B	20.26	1181B	22.48
As	2.011A	21.43	1.096B	12.26	1.404A	5.25
B	3.505A	13.03	1.528C	18.07	2.181B	6.20
Ba	27.08A	19.28	19.73A	8.66	9.178B	5.64
Be	0.089A	15.77	0.036B	10.07	0.034B	5.37
Bi	0.084A	6.09	0.090A	8.13	0.089A	5.76
Ca	2852AB	**41.66**	4682A	23.31	1050B	10.79
Cd	0.202B	9.41	0.330A	4.79	0.135C	6.05
Ce	4.062A	14.22	3.416A	13.17	1.803B	3.68
Co	0.728A	13.35	0.513B	11.48	0.414C	5.02
Cr	4.049A	14.55	1.673C	2.00	2.153B	7.94
Cs	0.464A	11.12	0.143C	4.34	0.191B	5.26
Cu	4.453A	13.47	2.960B	1.92	4.138A	3.71
Dy	0.212A	11.14	0.206A	6.58	0.105B	4.46
Er	0.107A	11.75	0.106A	14.08	0.055B	4.31
Eu	0.068A	13.01	0.064A	9.23	0.032B	4.65
Fe	1828A	16.75	506.2C	12.50	692.5B	4.82
Gd	0.277B	11.78	0.267A	5.83	0.134C	4.19
Ge	0.226A	12.41	0.149B	11.76	0.137B	4.91
Hg	0.135A	18.54	0.123A	11.15	0.226A	**30.36**
Ho	0.040A	11.90	0.039A	9.07	0.020B	3.80
K	4300A	14.72	3356A	8.98	2753B	4.98

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Element	*Evernia mesomorpha* (*EVM*)		*Usnea aciculifera* (*UAC*)		*Ramalina sinensis* (*RSI*)	
	Mean (µg/g)	**CV (%)**	**Mean (µg/g)**	**CV (%)**	**Mean (µg/g)**	**CV (%)**
La	2.078A	14.35	1.724A	11.81	0.940B	3.68
Li	1.642A	15.09	0.430C	12.60	0.625B	4.73
Lu	0.014A	14.22	0.012A	18.10	0.007B	4.82
Mg	895.3A	16.85	619.5B	4.16	406.8C	4.01
Mn	61.03B	11.65	83.19A	6.82	24.11C	5.48
Mo	0.194A	7.85	0.118C	3.48	0.143B	6.59
Na	437.8A	16.03	118.5C	6.86	154.0B	5.90
Nb	0.190A	10.42	0.067C	9.05	0.097B	5.60
Nd	1.734A	11.98	1.582A	11.93	0.807B	4.34
Ni	2.074A	14.88	1.016B	5.62	0.978B	4.02
P	1056A	**38.67**	613.5A	**26.29**	603.0A	5.10
Pb	4.388B	7.99	7.723A	5.93	2.393C	2.62
Pr	0.449A	12.58	0.400A	13.34	0.207B	4.19
Rb	8.214A	7.92	3.303B	10.50	3.731B	5.74
S	1246A	10.73	978.4B	4.18	1114A	5.37
Sb	0.120A	8.64	0.064C	8.13	0.086B	5.10
Sc	0.665A	17.57	0.213C	13.35	0.281B	4.32
Se	0.365A	7.05	0.408A	5.05	0.308B	4.33
Si	10648A	16.38	2621C	18.13	3658B	15.74
Sm	0.320A	12.08	0.301A	11.23	0.153B	4.08
Sn	0.260A	9.79	0.181B	6.22	0.233A	5.78
Sr	11.84A	24.03	13.17A	12.57	4.474B	5.29
Tb	0.040A	13.41	0.037A	6.65	0.019B	5.39
Th	0.497A	12.69	0.197B	11.45	0.223B	4.46
Ti	120.0A	10.24	42.41C	8.93	57.72B	6.27
Tl	0.073A	9.89	0.029C	9.43	0.033B	4.96
Tm	0.015A	12.12	0.014A	15.93	0.008B	4.33
U	0.152A	13.21	0.046C	16.34	0.067B	4.40
V	4.463A	14.93	1.424C	9.79	1.800B	5.05
Y	1.123A	13.50	1.246A	15.63	0.561B	4.06
Yb	0.096A	11.84	0.084A	16.78	0.049B	4.04
Zn	32.79A	7.17	31.34A	6.12	21.62B	2.69

Elements in bold face have CVs of > 30%. Different capital letters indicate significant differences between lichens. n = 5

In *Table 2*, 55 elements are divided into four groups according to the species difference pattern of element concentrations. Group I elements (Bi, Hg, and P) shows no significant difference in concentrations among lichens. Bi has a CV of less than 10% and a $P_{(mean \pm 0.25 \times mean)}$ of Rank 1. Hg in *RSI* and P in both *EVM* and *UAC* have CVs of larger than 25% and $P_{(mean \pm 0.25 \times mean)}$ of Rank 3.

Group II is composed of 26 elements, and has the lowest concentration in *RSI* among the three lichens. The group can be divided into three sub–groups: IIa (Ba, Ca, Ce, Dy, Er, EU, GD, Ho, K, La, Lu, Nd, PR, Se, SM, Sr, TB, TM, Y, Yb, and Zn), IIb (Co and Mg) and IIc (Cd, Mn, and Pb). The concentration of IIa elements shows no

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significant difference between *EVM* and *UAC*. The concentration of IIb elements is higher in *EVM* than in *UAC*, while the inverse occurs for the concentration of IIc elements. Except for the CV of Ca in *EVM* being 41.66%, the CVs of other elements in three lichens are all less than 25%. While the $P_{(mean \pm 0.25 \times mean)}$ of Ca in both *EVM* and *UAC* and Sr in *EVM* is Rank 3, the $P_{(mean \pm 0.25 \times mean)}$ of other elements in three lichens is mostly Rank 1.

Group III is composed of 20 elements, and has the lowest concentration in *UAC* among the three lichens. This group can be divided into two sub–groups: IIIa (Ag, As, Cu, S, and Sn) and IIIb (B, Cr, CS, Fe, Li, Mo, Na, Nb, Sb, SC, Si, Ti, TL, U, and V). The concentration of IIIa element shows no significant difference between *EVM* and *RSI*. The IIIb elements show a higher concentration in *EVM* than in *RSI*. The CVs of these elements is less than 22%. While the $P_{(mean \pm 0.25 \times mean)}$ of Ag in *EVM* is Rank 3, Si in three lichens is Rank 2, and other elements in three lichens is mostly Rank 1.

Group IV are composed of 6 elements (Al, Be, Ge, Ni, Rb, and Th), and has the highest concentration in *EVM* among the three lichens. But there is no significant difference between *UAC* and *RSI*. The CVs of these elements are less than 23%. Most elements in the three lichens have a $P_{(mean \pm 0.25 \times mean)}$ of Rank 1, with an exception of Al in *RSI* having a $P_{(mean \pm 0.25 \times mean)}$ of Rank 3.

Table 2. *Species difference and coefficient of variation in element concentrations, and* $P_{(mean \pm 0.25 \times mean)}$ *and its ranks for 55 elements in three lichens*

Group	Subgroup	Element	Species difference			CV%			$P_{(mean \pm 0.25mean)}$			Rank of $P_{(mean \pm 0.25mean)}$		
			EVM	***UAC***	***RSI***	***EVM***	***UAC***	***RSI***	***EVM***	***UAC***	***RSI***	***EVM***	***UAC***	***RSI***
I		Bi	A	A	A	6.09	8.13	5.76	1.00	1.00	1.00	1	1	1
		Hg	A	A	A	18.54	11.15	**30.36**	0.82	0.98	0.59	2	1	**3**
		P	A	A	A	**38.67**	**26.29**	5.10	0.48	0.66	1.00	**3**	**3**	1
II	IIa	Ba	A	A	B	19.28	8.66	5.63	0.81	1.00	1.00	2	1	1
		Ca	AB	A	B	**41.66**	23.31	10.79	0.45	0.72	0.98	**3**	**3**	1
		Ce	A	A	B	14.22	13.17	3.68	0.92	0.94	1.00	1	1	1
		Dy	A	A	B	11.14	6.58	4.46	0.98	1.00	1.00	1	1	1
		Er	A	A	B	11.75	14.08	4.31	0.97	0.92	1.00	1	1	1
		Eu	A	A	B	13.01	9.23	4.65	0.95	0.99	1.00	1	1	1
		Gd	A	A	B	11.78	5.83	4.19	0.97	1.00	1.00	1	1	1
		Ho	A	A	B	11.90	9.07	3.80	0.96	0.99	1.00	1	1	1
		K	A	A	B	14.72	8.98	4.98	0.91	0.99	1.00	1	1	1
		La	A	A	B	14.35	11.81	3.68	0.92	0.97	1.00	1	1	1
		Lu	A	A	B	14.22	18.10	4.82	0.92	0.83	1.00	1	2	1
		Nd	A	A	B	11.98	11.93	4.34	0.96	0.96	1.00	1	1	1
		Pr	A	A	B	12.58	13.34	4.19	0.95	0.94	1.00	1	1	1
		Se	A	A	B	7.05	5.05	4.33	1.00	1.00	1.00	1	1	1
		Sm	A	A	B	12.08	11.23	4.08	0.96	0.97	1.00	1	1	1
		Sr	A	A	B	24.03	12.57	5.29	0.70	0.95	1.00	**3**	1	1
		Tb	A	A	B	13.41	6.65	5.39	0.94	1.00	1.00	1	1	1
		Tm	A	A	B	12.12	15.93	4.33	0.96	0.88	1.00	1	2	1
		Y	A	A	B	13.50	15.63	4.06	0.94	0.89	1.00	1	2	1
		Yb	A	A	B	11.84	16.78	4.04	0.97	0.86	1.00	1	2	1
		Zn	A	A	B	7.17	6.12	2.69	1.00	1.00	1.00	1	1	1

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	IIb	Co	A	B	C	13.35	11.48	5.02	0.94	0.97	1.00	1	1	1
		Mg	A	B	C	16.85	4.16	4.01	0.86	1.00	1.00	2	1	1
	IIc	Cd	B	A	C	9.41	4.79	6.05	0.99	1.00	1.00	1	1	1
		Mn	B	A	C	11.65	6.82	5.48	0.97	1.00	1.00	1	1	1
		Pb	B	A	C	7.99	5.93	2.62	1.00	1.00	1.00	1	1	1
III	IIIa	Ag	A	B	A	21.94	11.15	9.76	0.75	0.98	0.99	**3**	1	1
		As	A	B	A	21.43	12.26	5.25	0.76	0.96	1.00	2	1	1
		Cu	A	B	A	13.47	1.92	3.71	0.94	1.00	1.00	1	1	1
		S	A	B	A	10.73	4.18	5.37	0.98	1.00	1.00	1	1	1
		Sn	A	B	A	9.79	6.22	5.78	0.99	1.00	1.00	1	1	1
	IIIb	B	A	C	B	13.03	18.07	6.20	0.94	0.83	1.00	1	2	1
		Cr	A	C	B	14.55	2.00	7.94	0.91	1.00	1.00	1	1	1
		Cs	A	C	B	11.12	4.34	5.26	0.98	1.00	1.00	1	1	1
		Fe	A	C	B	16.75	12.50	4.82	0.86	0.95	1.00	2	1	1
		Li	A	C	B	15.09	12.60	4.73	0.90	0.95	1.00	1	1	1
		Mo	A	C	B	7.85	3.48	6.59	1.00	1.00	1.00	1	1	1
		Na	A	C	B	16.03	6.86	5.90	0.88	1.00	1.00	2	1	1
		Nb	A	C	B	10.42	9.05	5.60	0.98	0.99	1.00	1	1	1
		Sb	A	C	B	8.64	8.13	5.10	1.00	1.00	1.00	1	1	1
		Sc	A	C	B	17.57	13.35	4.32	0.85	0.94	1.00	2	1	1
		Si	A	C	B	16.38	18.13	15.74	0.87	0.83	0.89	2	2	2
		Ti	A	C	B	10.24	8.93	6.27	0.99	0.99	1.00	1	1	1
		Tl	A	C	B	9.89	9.42	4.96	0.99	0.99	1.00	1	1	1
		U	A	C	B	13.21	16.34	4.39	0.91	0.99	1.00	1	1	1
		V	A	C	B	14.93	9.79	5.05	0.94	0.87	1.00	1	2	1
IV		Al	A	B	B	16.30	20.26	22.48	0.88	0.78	0.73	2	2	**3**
		Be	A	B	B	15.77	10.07	5.36	0.89	0.99	1.00	2	1	1
		Ge	A	B	B	12.41	11.76	4.91	0.96	0.97	1.00	1	1	1
		Ni	A	B	B	14.88	5.62	4.02	0.95	0.97	1.00	1	1	1
		Rb	A	B	B	7.92	10.50	5.74	0.91	1.00	1.00	1	1	1
		Th	A	B	B	12.69	11.45	4.46	1.00	0.98	1.00	1	1	1

$P_{(\text{mean} \pm 0.25\times\text{mean})}$ denotes cumulative probability of the concentration confidence interval ranging from 0.75×mean to 1.25×mean (*Eq. 1*). Different capital letters indicate significant differences between lichens

Rank 1 accounts for 83.6% of the total data points, followed by Rank 2 (11.5%) and Rank 3 (4.8%; *Table 3*). Amongst the three lichens, *RSI* has a highest proportion of Rank 1 and lowest proportion of Ranks 2 and 3, while *EVM* shows an inverse pattern (*Table 3*).

Table 3. *Element number and proportion (in parenthesis) in three ranks of* $P_{(mean \pm 0.25\times mean)}$ *in lichens*

Rank of $P_{(\text{mean} \pm 0.25\text{mean})}$	***EVM***	***UAC***	***RSI***	**Total**
Rank 1	41 (74.5%)	45 (81.8%)	52 (94.5%)	138 (83.6%)
Rank 2	10 (18.2%)	8 (14.5%)	1 (1.8%)	19 (11.5%)
Rank 3	4 (7.3%)	2 (3.6%)	2 (3.6%)	8 (4.8%)

$P_{(\text{mean} \pm 0.25\times\text{mean})}$ denotes cumulative probability of the concentration confidence interval ranging from 0.75×mean to 1.25×mean (*Eq. 1*). Rank 1: $P_{(\text{mean} \pm 0.25\times\text{mean})} \geq 0.90$. Rank 2: $0.90 > P_{(\text{mean} \pm 0.25\times\text{mean})} \geq 0.75$. Rank 3: $P_{(\text{mean} \pm 0.25\times\text{mean})} < 0.75$

Discussion

Species difference of element concentration

The element specificity of the species difference in element concentrations is so great that the 55 elements can be divided into four groups and seven sub–groups. Group I elements (Bi, Hg, and P) show not significant difference among three lichens (*Table 2*). The high CVs may be an explanation for the insignificant species difference for Hg (CV = 30% in *RSI*) and P (39% in *EVM*, 26% in *UAC*; *Tables 1* and *2*).

The species difference pattern of Group II-IV elements should not be attributed to experimental errors, because most of these elements has a CV of < 25% (*Table 2*), an acceptable variability reflecting natural fluctuation in element concentration of local lichens (Frati et al., 2005; Malaspina et al., 2014). Many important factors affecting the element concentration in local epiphytic lichens should not be the critical ones in the current study, such as the topography (Bajpai et al., 2022) and altitude (Bačkor and Loppi, 2009) of the study area, collection height and aspect on the tree, and lichen size (Varrica et al., 2000; Senhou et al., 2002). The reason is that a composite sample is composed of lichen thalli homogeneous in size, which were collected from all aspects at the same height of *Larix gmelinii* trees within a short transect line (50 × 1000 m).

In terms of the Group II element concentrations, *RSI* is lower than *EVM* and *UAC* (*Table 2*), possibly due to its smooth surface of broader and fewer lobes and the absence of soredia, and its erect/pendulous foliose thallus (*Fig. 1f, g*). It is reported that the fruticose lichens have higher element accumulation capacity than the foliose ones, and the presence of soredia and the narrower lobes increase the roughness of thallus surface (Di Lella et al., 2003). Similarly, *EVM* and *UAC* have rougher surface due to the shrubby thallus composed of narrower and denser lobes and the presence of soredia (*Fig. 1c, d, e*), which enable the two lichens to entrap and retain more amount of deposits.

The Group III element concentrations are the lowest in *UAC* among the three lichens (*Table 2*), which may be due to the fact that foliose lichens can capture more canopy leaching than fruticose lichens (Asplund et al., 2015). Wet deposition (Gallo et al., 2017) and canopy leaching (Asplund et al., 2015) can significantly change the elemental composition of epiphytic lichens by carrying essential nutrients including some elements in Group III (B, Cu, Fe, Mo, Na, S, and Si). Compared with the erect fruticose thallus of *UAC* (*Fig. 1e*), the dorsoventral foliose thallus enables *EVM* and *RSI* (*Fig. 1d, f, and g*) to capture higher amount of leachates, especially those in stem flow (Monaci et al., 2012).

EVM is the highest in concentration of Group IV elements, followed by *UAC* and *RSI* (*Table 2*). It should be noted that *EVM* is also high in concentration of Groups II and III. This may be attributed to the coarsest surface of *EVM* due to its densest soredia among lichens (*Fig. 1d, e, f, g*).

Species and element specificity of CSRMC

Generally, the composite sample does effectively represent the mean local element concentrations in lichens with a low cost, as seen from the high CSRMCs for most elements in three lichens (*Tables 2* and *3*). Geiser (2004) pointed out that the element content variability between two composite samples from the same location should be small. This is true in the current study, as only 4 (Ca in *EVM*, P in *EVM* and *UAC*, Hg in *RSI*) out of 165 CVs (3 lichens×55 elements) are more than 25% (*Tables 1* and *2*). The CSRMCs of Ranks 1, 2 and 3 account for 83.6%, 11.5% and 4.8% of the total, respectively (*Table 3*).

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The CSRMC shows obvious species differences just the same as the element content. *RSI* has the strongest CSRMC ($P_{(mean \pm 0.25 \times mean)}$ of Ranks 1, 2 and 3 accounting for 94.5%, 1.8% and 3.6% of the total elements, respectively), followed by *UAC* (81.8%, 14.5%, and 3.6%) and *EVM* (74.5%, 18.2%, and 7.3%). Interestingly, the surface of *RSI* is smoother than that of *EVM* and *UAC* (*Fig. 1d, e, f, g*). This association between morphology and CSRMC highlights the importance of using a single lichen species in the biomonitoring.

The CSRMC also shows obvious element specificity. Specifically, the CSRMC should be carefully considered for the three elements (Ca, Hg, and P) which sometimes have CVs of > 25% and $P_{(mean \pm 0.25 \times mean)}$ often falling into Ranks 2 and 3 (*Tables 1* and *2*). Therefore, when monitoring Ca, P and Hg using the composite samples, the size of a composite sample or number of replicates should be increased to reduce CVs, otherwise the three elements should be avoided in such studies. Compared with other elements, the higher local variability of Ca and P concentrations in lichens may be related to the greater uptake/release sensitivity of these elements to the environmental fluctuations, because calcium oxalate and P can be highly bioregulated in lichen thallus (Giordani et al., 2003). Hg is mainly transported and deposited in gaseous elementary substance (Selin et al., 2007). The high local variability of Hg concentration may be attributed to the fact that gaseous pollutant may be more unstable in lichens than particulate pollutants. In addition, we should also pay attention to the risk of CSRMC of Al and Si, which mostly falls into Rank 2 (*Table 2*). These two elements are crustal–derived elements, existing in large quantities in soil and rock debris particles. The other 50 elements have CVs of < 25% and $P_{(mean \pm 0.25 \times mean)}$ mostly falls into Rank 1 in each of the three lichens (*Tables 1* and *2*).

Our study is conducted in a background area with low heterogeneity of atmospheric element deposition. Further studies are needed to verify the suitability of this method in the polluted sites with potentially greater heterogeneity of atmospheric element depositions.

Conclusions

The element concentrations of *EVM*, *UAC* and *RSI* have strong element–specific species differences, which are related to the morphology and element source of lichens. Amongst the three lichens, *RSI* has the smoothest surface and lower concentration for most elements, while *EVM* has the roughest surface and the highest concentration for most elements. Foliose lichens accumulate higher amount of elements related to canopy leaching and wet deposition than fruticose lichens. The composite sample with a small sample size can effectively represent the local mean element concentration of lichens, but this representativeness has species difference and element specificity. CSRMC is the highest in *RSI*, but lower for Al, Ca, Hg, P and Si. When using the composite sample with a small sample size to monitor the atmospheric deposition, it is necessary to investigate the CSRMC of the elements of interest, and carefully select the lichen species and the number of composite samples to reduce the workload and financial expenditure with a satisfactory CSRMC.

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