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Measuring lichen specimen characteristics to reduce relative local uncertainties for trace element biomonitoring

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ABSTRACT

Local variation (within sampling sites) affects lichen air pollution biomonitoring of trace element deposition patterns. When comparing between sampling sites, global variation must be greater than local variation, thus reducing local variation is important in biomonitoring studies. To reduce local variability, sampling protocols are introduced, primarily minimum sampling height and less often sampling aspect. This study, introduces further protocols, which can help to reduce within site variation. First, the research design removed spatial variation by sampling a single tree. One–thousand and thirty–seven individual specimens of *Usnea subfloridana* were collected and aggregated into 97 samples based on similar collection height, aspect and mass. Samples were tested by inductively coupled plasma – atomic emission spectroscopy for total recoverable Al, As, Ba, Be, Cd, Co, Cr Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sr, Ti, Tl, V, and Zn. Fifteen of the elements tested were above minimum detection limits and their variation in concentrations were able to be partially explained with linear modeling. When explaining variation in concentrations with linear modeling, aspect was statistically significant for all of the 15 elements, height was statistically significant for 12 elements, and specimen mass was significant for 6 elements. We demonstrate that individually assessing and minimizing specimen collection aspect, height and mass prior to aggregating specimens into samples can reduce local variation, which will improve between site comparisons.

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1. Introduction

Plant biomonitoring of trace element air pollution, including the use of lichens, often is affected by local variation in specimen concentrations; this variation has been termed relative local uncertainty (RLU). These RLUs consist of variations that arise from biological and microclimatic variability, and from sample handling and analysis procedures (Wolterbeek and Bode, 1995). Relative local uncertainties affect biomontoring programs because RLUs must be accounted for when comparing results between sample sites. If the differences in the results of the monitoring between the sample sites are not greater than the RLU they should not be considered biologically significant and should not be used to support a conclusion of different levels of air pollution.

Relative local uncertainties determined in a study covering almost all of The Netherlands, using 10 x 10 km grid cells as sampling sites, range as high as 65% relative variance for Cs, 46% for As, and 48% for Fe. Twenty–eight sites were used to estimate local RLU values. The samples were individually collected from three to six trees of a single tree species. These individual samples were analyzed separately and were compared within–sites. RLU was independent of trace–element air pollution levels at the sampling sites. The RLU was an order of magnitude lower than the national variability, which is the variation necessary for a large–scale biomonitoring program to be appropriate (Sloof and Wolterbeek, 1991).

Another study with eighty–nine composite samples from 3–5 trees in 16 km^2 grids covering an area of about 20 200 km^2, tested 30% of these sites for within site variability (Jeran et al., 2007). The authors found that RLU was only 35% or lower for all elements tested, which was below the study–wide variation (Personal Communication).

Reductions in RLU at sampling sites allows for an increased sensitivity when determining significant differences between sites. The economics behind biomonitoring programs are always a concern; lichen biomonitoring has two main costs including the financial costs of analyses, and the cost of researchers to conduct the collection, preparation, and interpretation, among other functions, of the research. These costs limit the number of samples that can be tested in research projects; any method that will reduce RLU allows researchers to examine more sites as they require fewer samples at each site.

When researches sample large spatial extents, they combine many lichen specimens into composite samples to produce a sampling sites' mean value. Sensen and Richardson (2002) were able to determine that a chlor–alkali plant in New Brunswick, Canada, had a 2.4–3.4 km sphere of influence for elevated levels of mercury deposition using composite samples of *Hypogymnia physodes* with composites from at least three trees. Bennett and Wetmore (2003) analyzed data collected from four lichen species over a 15–year period; each sample was a composite of a single species. They found that Al, Cr, Fe, Na, Ni, and S had increased in thallus concentration during the study period; Cu, K, P, Pb, and Zn

had decreased; and Ca, Cd, Mg, and Mn were constant. This increase in S and Cr indicated that the sample site, Apostle Island, Wisconsin may not be as pristine as previously thought. *Xanthoria parietina* was used in Veneto (NE Italy) in a study that involved the collection of 200 composite samples over an 18 364 km^2 study area. This method was able to identify high–risk areas, based on concentrations of potentially harmful elements; the authors recommended these high–risk areas be monitored by instrumental methods (Nimis et al., 2000). Countless more studies have been conducted where general patterns of trace elemental thallus concentrations have been successfully determined both spatially and temporally using composite samples of lichen.

We test if lichen specimen mass can be used as a sampling control to reduce RLU for composite samples. Each individual specimen was individually collected and weighed prior to aggregation into composite samples. The common composite–sample controls are applied as well including: minimizing aspect and collection height range on the tree. It is well established that geographic separation will create variation in concentrations, the caveat for biomonitoring. In this initial study we limit our collection to a single tree to reduce concentration variability due to spatial variation, i.e. if mass does not significantly reduce variation at a single tree; it should not at larger scales.

2. Materials and Methods

2.1. Field collection

The sampling site was approximately 25 km northeast of the City of Thunder Bay, Ontario, Canada. Universal Transverse Mercator coordinates are Zone 16 N, 345805 m E and 5385358 m N. *Usnea subfloridana* typically grew to about 15 cm in un–stretched length; most specimens in the area were about 3–7 cm. *Usnea subfloridana* was chosen as the study species because: (1) in the study area it presented a large range in size, a characteristic we hypothesized to be explanatory to measured elemental concentrations; (2) it fits the suggested criteria of Bargagli and Nimis (2002) to use either fruticose or foliose species for biomonitoring projects; (3) the species was ubiquitous throughout the available study area; (4) it detaches from the substrate with relative ease; and (5) multiple species in the genus have been used previously in bioaccumulation studies (Poblet et al., 1997; Rossbach et al., 1999; Conti et al., 2009). It should also be included that species within the genus are known to be sensitive to pollution (Shrestha et al., 2012). We did not expect high levels of air pollution in this study area; otherwise, another species may have been chosen. The study area was dominated by *Abies balsamea* (L.) Mill. (balsam fir), which were approximately 60 years of age. All of the trees in the sampling site exhibited a similar growth pattern of lichen, with *U. subfloridana* being a dominant species. Other common lichen species were *Parmelia sulcata*, *Evernia mesomorpha*, and *Bryoria* spp.

Field collection was conducted on May 4, 2010. All specimens were collected within 12 hours. Specimens were collected from an *Abies balsamea* (L.) Mill. (balsam fir). Nomenclature for tree identification was Trees of Ontario (Kershaw, 2001). To safely fell the tree, a guide rope was necessary. The tree was guided to rest perpendicular to a previously felled tree; this was beneficial, as lichen specimens did not touch the ground. The tree was brought to the ground because we could not determine a suitable method to sample at heights much above our reach. The use of ladders was inappropriate because of the unstable ground, composed of loose soil and debris. Note: the land–owners use their local trees for fire wood, which this tree was used for after study. The study tree's height was about 17 m. North aspect was indicated on the tree prior to cutting it down at both at 1 m and 3 m above the ground. On the ground, four guidelines were run along the tree, representative of each cardinal direction. Lichens were collected from the tree trunk only; branch samples were not included in the collection. Sample collection began 1 m above the ground with an upper bound of 9 m; a total of 8 m of the tree trunk was sampled. Each collected specimen's height and aspect were recorded. Height was measured in 1 dm collars around the tree. Lichen aspect was recorded as one of 16 qualitative directions: 4 cardinal directions, 4 ordinal directions, and 8 further divisions. All specimens similar in appearance to the target species of *Usnea subfloridana* Stirt. (Brodo et al., 2001) were collected, and underwent microscopic species confirmation in the laboratory. Specimens were collected by inverting a polyethylene bag over the collector's hand to ensure that the inside of the bag was not touched. With the bag over top of the collector's hand, the specimen was grabbed firmly at the base and with a quick pull removed from the bark substrate. The bag was inverted to cover the lichen and sealed. Specimens were placed into a freezer for storage at the end of the field collection day until laboratory processing.

2.2. Laboratory processing

Lichen cleaning. During all laboratory procedures, FischerBrand™ Nitrile gloves were worn and samples were handled with plastic tweezers. In the laboratory, each sample was examined using a dissection microscope for confirmation as *U. subfloridana*. All work was done in a glass dish, which was cleaned between samples. Lichen specimens collected from the research tree, but above the collection area, were identified to species using all appropriate tests (chemical spot tests, etc) by the authors and also by Erika North, Curator of the Claude E. Garton Herbarium at Lakehead University. As well, some samples were sent to Irwin M. Brodo–author of Lichens of North America (Brodo et al., 2001)–for external identification, identified as *U. subfloridana* Stirt. These samples were all identified to be *U. subfloridana*. Specimens used in the analysis were only subject to visual examination.

Extraneous materials, which included parts of other lichens, seeds, small plant needles, and bark, were removed from each sample. Dead lichen material was removed from the specimens as well. During the cleaning procedure, it was common to find that multiple specimens had been collected simultaneously. These multiple–specimen samples were separated and treated as individual samples. Prepared specimens were frozen until weighing.

Lichen weighing. Each lichen specimen was air–dried for at least 24 hours. After the air–drying, the specimens were put in desiccators for 24 hours. Then, specimen mass was measured with an Ohaus Adventurer® Analytical Balance with a reliability of 0.1 mg. A Petri dish was placed on the scale to ensure the lichen did not touch the balance directly, and was wiped clean between uses.

Sample group delineation. Average specimen mass was well below the required analytical procedure material mass of 250 mg. The 1 037 individual specimens were combined into 97 composite–samples. Prior to group delineation, all specimens weighing over 250 mg were removed from the data set to be individually tested.

We grouped the lichen specimens into two aspect–groups based on the two dominate wind directions at the field site. The two groups are south–west, which included all lichens specimens with an aspect between 67.5° and 255°; and north–east, all lichens specimens with an aspect between 247.5°–45°. Within the wind groups, specimens were grouped into 5 dm height groups; a group that included all the lichens collected within 5 dm. The 5 dm groups included: Grouping 1 (1 to 5 dm collar groups), Grouping 2 (6 to 10 dm collar groups), Grouping 3 (11 to 15 dm collar groups)… Grouping 16 (76 to 80 dm collar groups). Within each height group, the lichens were sorted by mass. Starting with the heaviest specimens, the specimens were combined until a composite–sample's total mass reached the required analytical procedure material mass of 250 mg.

Sample Preparation. Lichens were cut into small pieces by hand with a ceramic knife, a process which took about 10–15 minutes per sample, for the 97 samples. Commonly, researchers use a Wiley mill (Richardson et al., 1995; France and Coquery, 1996; Chiarenzelli et al., 1997; Bennett and Wetmore, 2000; Bennett and Benson, 2005), but that was not possible because of the low initial lichen material mass and the loss of material during the milling process. The ceramic knife technique has been used previously (Chiarenzelli et al., 1997; Loppi et al., 1998) and ensures that there is no introduction of trace elements.

2.3. Analytical method for trace element analysis

Inductively–coupled plasma–atomic emission spectroscopy (ICP–AES) with a microwave–assisted digestion was used for trace element analysis of total recoverable Al, As, Ba, Be, Cd, Co, Cr Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sr, Ti, Tl, V, and Zn. Cut samples were dried at 100°C in an oven for at least 12 hours to assure dry mass. After drying, lichens were kept in a desiccator until just prior to weighing. Each sample was weighed to about 200 mg using a Denver P403 balance with a reliability of 0.1 mg, and the material was weighed onto FisherBrand™ Polystyrene Antistatic Weighing Dishes. Weighed samples were poured into CEM MARSXpress™ PFA Teflon® vessels, which are pressure vessels designed for temperatures up to 260°C. In each vessel, 3 mL of FischerBrand™ Trace Metal Grade concentrated HNO_3 was added, and the vessels were left to sit overnight for at least 18 hours. After at least 18 hours, 1 mL of FischerBrand™ Trace Metal Grade concentrated HCl was added to the vessels and left for an additional 3 hours.

Microwave digestions were done with the 3 mL HNO_3 and 1 mL HCl solution, and 200 mg lichen sample. A CEM MARSXpress, closed vessel Acid Digestion – MARS System was used. This instrument processes up to 40 samples simultaneously and rapidly monitors the temperature of each vessel using two highly sensitive IR internal temperature sensors (NIST traceable). The microwave program consisted of three steps: 25 minutes ramping to 180°C; holding at 180°C for 25 minutes; then a cooling cycle.

When the vessels cooled down below 40°C, 1.5 mL of H_2O_2 was added, causing a reaction lasting about 10 minutes. When the H_2O_2 reaction was completed, the samples were transferred to 50 mL Fischer Scientific Centrifuge Tubes and brought up to 25 mL with Type I distilled deionized water (Barnstead E–Pure Ultrapure Water Purification System with 18MΩ cm specific resistivity capability). Trace element analysis was then conducted on these solutions using a Varian Vista Pro CCD Simultaneous ICP–AES with a CETAC ASX–510 Auto Sampler. Final results are reported as µg/g dry mass.

Blanks and standards were run after every 11 lichen samples. Two standards were used to test for recovery, accuracy and precision: (1) Certified Reference Material BCR® –482, a lichen powder certified by the Community Bureau of Reference, and (2) Standard Reference Material 1570a, a spinach powder certified by the National Institute of Standards and Technology. Standards and blanks were processed in the same manner as all other samples. Accuracy, precision, and recovery were determined by following the methods: Accuracy (% Error) = [(Mean of the Standard)/(Expected Value)] x 100; Precision (Relative Deviation) = (Standard Deviation of the Standard)/(Mean of the Standard); Recovery (%)=[(Observed Standard Value)/(Expected Standard Value)] x 100.

2.4. Statistical analysis

Linear regression models determined the explanatory ability of our measured lichen characteristics, which included collection aspect, collection height, and specimen mass. We used dummy coding for aspect; a value of one was used when samples were in the north–east group. Our final models included all independent variables that were significant at α=0.05.

Partial correlation was used (Pearson's *r*) to determine the unique correlation of each independent variable – statistically significant in the linear regression models – to the dependent variable (elements). Partial correlation measures the correlation between two random variables, with the correlation of other random variables removed; i.e. What is the unique correlation between specimen mass and Al concentration, when controlling for correlation due to aspect and collection height to Al concentration. This value can be squared and treated similarly to the R^2 value from linear models. For both linear models and partial correlation, variables were transformed to approximate normal distributions.

3. Results

The lichen reference material had an average accuracy of 7.9%, precision of 4.79% and recovery of 92.07%. The spinach reference material had an average accuracy of 2.96%, precision of 4.27% and recovery of 96.59%.

Data for seven of the elements tested were below detection limits (DL) for most of the 97 composite–samples. The number of samples under the detection limits, of the 97 composite–samples tested, are in brackets beside each element: Ni (65), Co (92), Mo (96), As (97), Be (97), Tl (97), and V (97). None of these elements was included in the analysis. Pb was included in the analyses but 15 samples were below the detection limit. Cr and Ti both had 2 samples below DL, and all other elements were above DL for all samples (Al, Ba, Cd, Cu, Fe, K, Mg, Mn, Na, P, S, Sr, and Zn). Table 1 includes the means, medians (all samples and separated by aspect groupings), coefficients of variation, and standard deviations for the elements used in data analysis. Data were log–transformed to approximate normal distributions; all 0 values were set as ½ the minimum value for that chemical to allow for the log transformations. This was done for Cr (2 samples), Ti (2 samples), and Pb (15 samples).

Only one element modeled with linear regression, Mn, did not have any significant independent variables; all further discussion excludes Mn. Linear regression models were able to attribute between 7 and 57% of the variability in the trace element concentrations with the three independent variables measured: collection height, collection aspect, and specimen mass. The coefficients for each of the independent variables were both negative and positive depending on the specific element modeled. Table 2 includes the linear models' degrees of freedom (df), F statistic, adjusted R^2, significant independent variables, and coefficient sign for each independent.

Partial correlation R^2 while controlling for other significant predicators from the linear regression models ranged from 0.06 to 0.37 for height, 0.04 to 0.37 for aspect, and 0.04 to 0.15 for mass. Table 2 includes both the partial correlation Pearson's *r* and R^2.

4. Discussion

The independent variable, collection aspect, was significant in all linear regression models. For Al, Ba, Cd, Cr, Cu, Fe, Mg, Mn, Pb, S, Sr, Ti, and Zn the modeling indicates that a south–west aspect is associated with higher concentrations. These south–west samples' elevated concentrations are likely due to their exposure to the south–west winds; winds that would pass over the City of Thunder Bay. The elevated concentrations are likely due to emissions from the city, Thunder Bay's known main sources of on–site air releases for the following elements are: Al (fume or dust) from power generation; Cd from pulp and paper mills; Cr from industry; both Cu and Mn from industry and pulp and paper; and Pb from industry, pulp and paper, and power generation (NPRI, 2012).

Table 1. *Trace element thallus concentrations (μg/g) mean, standard deviation (SD), coefficient of variation (CV), and median (All samples, south-west samples only, and north-east samples only)*

Element	Mean	SD	CV (%)	Median		
				All	SW	NE
Al	197.3	45.3	22.87	184.2	224.4	175.8
Ba	31.8	4.2	13.32	31.6	34.26	30.6
Cd	0.4	0.1	22.73	0.42	0.46	0.4
Cr	0.3	0.2	51.52	0.3	0.46	0.23
Cu	3.0	1.5	49.17	2.67	3.08	2.44
Fe	241.5	67.0	27.73	224.6	282.0	205.4
K	2 521.9	340.0	13.48	2 453.9	2 335	2 608.4
Mg	933.0	129.5	13.88	906.6	1 001.9	878
Mn	365.8	45.8	12.51	365.5	377.6	355.5
Na	70.8	26.6	37.52	64.8	50.4	81.2
P	375.7	55.9	14.88	356.9	352.6	381.6
Pb	1.9	1.2	61.98	2.15	2.579	1.658
S	1 129.8	136.7	12.1	1 121.3	1 212.2	1 042
Sr	12.5	2.0	12.51	12.1	13.8	11.3
Ti	9.1	2.7	29.12	8.6	10.7	8.2
Zn	53.8	8.0	14.78	53.1	60.33	50.12

Linear regression models indicated increased concentrations of K, Na, and P in the samples from the north–east aspect grouping. The lichens in this group receive reduced solar radiation, which would result in longer moist periods following precipitation events. Lichen biological activity occurs when they are moist, a period when trace elements enter into their thallus. Longer periods of biological activity may be the cause for increased K, Na, and P concentrations. As well, lichen samples from the north–east aspect group may have reduced contact with rainfall and the associated leaching from rain water of trace elements; an analogous situation resulted in the reduced concentrations of K and Na–water soluble ions–in lichens washed prior to trace elemental analysis when compared to those which were not washed (Adamo et al., 2007). As well, K concentrations demonstrate a positive correlation with chlorophyll integrity (Kauppi, 1976; Garty et al., 1998); indication that lichens not exposed towards the primary pollution sources in Thunder Bay may be healthier.

An analysis of road lining and near road (250 m away from road) lichens, comparing both samples facing towards the road (street side) and those facing away (sheltered side) on trees, were tested for their bioaccumulation of traffic related elements. The results determined that samples facing towards and away from the road were similar in concentration of traffic related elements. Though, Al, Fe, and Pb were significantly different in concentrations between street side and sheltered side at road lining trees, and Cu, Sb, Al, and Pb were significantly different in concentrations between street side and sheltered side at the near road samples (250 m away) (Paoli et al. 2012). Paoli et al. (2012) suggest that turbulent diffusion is the cause for similarity between concentrations of street side and sheltered side lichens. This disparity to our results suggests that for longer pollutant transport, when diffusion should be less turbulent, that sampling aspect becomes more critical in the design of a sampling procedure.

The sample collection heights affected concentrations for 12 of the elements tested. The linear modeling indicated that for Al, Cr, Fe, K, Mg, Na, P, Pb, S, and Ti increased height resulted in increased elemental concentration in the lichen thallus. Direct rainfall is most likely decreasing these elements by leaching elements from within the thallus, and direct removal of particulate from the lichen upper surface. The particular tree studied grew with a dense canopy starting at about 18 m from the ground with few un–leafed branches along the stem, the dense canopy should protect lichen specimens from direct rainfall for specimens collected higher up on the tree. Ba and Cd concentrations decreased with increased specimen collection height on the tree, this decrease is likely attributed to the primary source of these two elements being soil particulate blown onto the lichens. Sample height is typically controlled as a minimum collection height in biomonitoring studies (Bargagli and Nimis, 2002). Our results suggest that sample collection should also have an upper bound to minimize within–site concentration variations. This type of control occurs in transplant studies where transplanted samples are located at a specified height above the ground (Conti et al., 2004).

Specimen mass was significant in the linear models for Al, Cu, K, Mg, P, and S. In the linear models, an increased mass resulted in decreased concentrations. Zonation of lichen trace elemental concentrations is understood to affect certain trace elements (Bargagli et al., 2002) – for example increased concentrations have been found in older thallus parts (Bargagli et al., 1987; Bargagli and Mikhailova, 2002) – this zonation may be responsible for the significant relationship to mass as lichens of greater mass would have greater amounts of older thallus. Our results are in disagreement to Senhou et al. (2002) who found a positive correlation between thallus trace element concentrations and lichen size, but for a different species than tested herein this research (*Evernia prunasti*), as well, their relationship was based on physical size, whereas ours is based on lichen mass.

Table 2. *Linear regression models for all elements, including the degrees of freedom (df), the F-statistic (F), the adjusted R^2 (R^2), the significant independent variables (Independent Variables), and coefficient sign (Sign). Pearson's r partial correlation coefficient (r) for each significant independent variable with controls for each of the other significant variables and the R^2*

	Linear Regression Model					Partial Correlation	
	df	F	R^2 (adj)	Independent Variables	Sign	r	R^2
Al	93	22.8	0.41 [c]	Height	+ [c]	0.47 [c]	0.22
				NE	- [c]	-0.48 [c]	0.23
				Mass	- [a]	-0.23 [a]	0.05
Ba	93	9.1	0.19 [c]	Height	- [b]	-0.26 [b]	0.07
				NE	- [c]	-0.42 [c]	0.18
Cd	93	12.1	0.23 [c]	Height	- [b]	-0.33 [c]	0.11
				NE	- [c]	-0.45 [c]	0.20
Cr	94	40.8	0.45 [c]	Height	+ [c]	0.36 [c]	0.13
				NE	- [c]	-0.61 [c]	0.37
Cu	94	4.3	0.07 [a]	NE	- [a]	-0.205 [a]	0.04
				Mass	- [a]	-0.206 [a]	0.04
Fe	92	29.0	0.37 [c]	Height	+ [c]	0.449 [c]	0.20
				NE	- [c]	-0.451 [c]	0.20
K	93	27.3	0.45 [c]	Height	+ [c]	0.554 [c]	0.31
				NE	+ [c]	0.596 [c]	0.36
				Mass	- [c]	-0.384 [c]	0.15
Mg	92	30.5	0.52 [c]	Height	+ [c]	0.575 [c]	0.33
				NE	- [c]	-0.534 [c]	0.29
				Mass	- [b]	-0.285 [c]	0.08
Na	92	19.7	0.28 [b]	Height	+ [c]	0.388 [c]	0.15
				NE	+ [c]	0.479 [c]	0.23
P	93	25.1	0.43 [c]	Height	+ [c]	0.606 [c]	0.37
				NE	+ [c]	0.511 [c]	0.26
				Mass	- [b]	-0.300 [b]	0.09
Pb	94	8.1	0.13 [c]	Height	+ [a]	0.251 [a]	0.06
				NE	- [b]	-0.265 [b]	0.07
S	93	41.3	0.56 [c]	Height	+ [c]	0.606 [c]	0.37
				NE	- [c]	-0.566 [c]	0.32
				Mass	- [c]	-0.384 [c]	0.15
Sr	94	23.5	0.30 [c]	NE	- [c]	NA	NA
Ti	93	23.1	0.39 [c]	Height	+ [c]	0.479 [c]	0.23
				NE	- [c]	-0.487 [c]	0.24
Zn	95	53.3	0.35 [c]	NE	- [c]	NA	NA

[a] *P < 0.05* , [b] *<0.01,* [c] *<0.001*

Linear models highlighted significant linear relationships between lichen specimen, aspect, collection height, and mass. Partial correlation, which reveals the amount of unique correlation between two variables while controlling for the correlation of other variables highlights that the independent variables were not equal in ability to explain variation in the models. The mean partial correlation Pearson's *r* values for collection height and aspect grouping were very similar at 0.21 and 0.23 respectively; the partial correlation Pearson's *r* values for mass was less than half of the first two at *r*=0.09. The lower *r* value associated to lichen mass is suggesting that key factors for local variability, which can be explained by a linear relationship to the lichen parameters measured, are more spatial characteristics (aspect and collection height) then the measured biological characteristic.

5. Conclusions

Lichen biomonitoring using *in situ* specimens for trace element analysis requires sampling controls to minimize within–site variability. Sampling methods should continue to use a minimum collection height, but should include a maximum collection height, e.g. samples collected between 1 m–1.5 m. Aspect of specimens collected needs to continue to be accounted for, either by selecting a particular range, appropriate to determine effects from nearby pollution sources, or collecting from all aspects to determine general conditions. When samples are to be collected from all aspects for general conditions, each site must be evaluated to ensure samples are collected from the entire aspect range. Lichen specimen mass should also be incorporated, we suggest using a bounding similar to both aspect and collection height, using an upper and lower bound for mass. These bounds would need to be determined empirically for each study. In conclusion, by limiting the specimen variation–prior to aggregation into composite samples to be analyzed by analytic techniques–the within site trace element concentration variability can be reduced resulting in stronger between site comparisons.

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