Optimization of analytical methods for metal(loid)s in a lichen biomonitor

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by
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Declaration

I, Eve Mariel Kroukamp declare that the thesis/dissertation, which I hereby submit for the degree PhD Analytical Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

…………………………………..   ……………………..

Eve Mariel Kroukamp   **Date**

Dedication

This work is dedicated to my loving husband, Shaun Kroukamp, and our beautiful, happy child, Joshua Mark Kroukamp. My life would be incomplete without you in it. Joshua, you have inspired me to persevere, to do better and have brought so much joy to my life. Shaun, thank you for your love and patience, and for reading through drafts late at night. You are a partner for life, a very best friend and I look forward to our life together.

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Abstract

Although a number of studies have examined metals in lichens, these studies are often limited to total metal analyses of a few target analytes. Moreover, there has not been a strong focus on the speciation of metal(loid)s in lichens, where these types of studies are highly relevant in providing information about bioavailability, source apportionment and toxicity. Furthermore, little attention has been paid to sample preparation, storage and the optimization of extractions for species characterizations where these aspects are essential in such evaluations.

The use of the foliose lichen, *Parmotrema austrosinense* (Zahlbr.) Hale, as an appropriate biomonitor of air pollution in South Africa was investigated by evaluating the concentrations of various metal(loid)s in this lichen species growing at both an urban and mining impacted site. *P. austrosinense* was found to be reflective of the different sources of pollution at both of the sites chosen for this study, proving its fitness for use in future metal(loid) air pollution biomonitoring evaluations.

Thereafter, *P. austrosinense* was evaluated for metal and metalloid content at sites with varying sources and magnitudes of anthropogenic impacts. In this study the importance of combining multi-element studies with meteorological data was emphasized, as this allows for correlations between the observed concentration in the lichen thallus and those present as a result of air pollution to be drawn. The outcomes from this study not only serve as a baseline for future air biomonitoring studies in South Africa, but was also the first study to evaluate the concentrations of a suite of metals in lichens at the Cape Point Global Atmospheric Watch (GAW) baseline reference station, providing essential information for comparative assessments between other GAW sites around the globe.

After noting the combined use of multi-elemental and meteorological data as a useful tool for the assessment of lichen biomonitors, the use of a four-step sequential extraction scheme was employed for its novel application in the extraction of metals and metalloids from lichens. Here, greater attention was paid to sample preparation strategies and storage than had been done in studies by other researchers. Cryogenic freezing of samples using liquid nitrogen was found to be the most appropriate sample preparation strategy for total metal analysis, however this did not hold true for speciation analyses using sequential extractions, and a recommendation was made that the implementation thereof be revisited. A sample size of 10 g of lichen material was found to be adequate for providing a homogenous and representative sample of the lichen population for atmospheric pollution evaluations.

Thereafter the focus shifted toward the targeted analyses of arsenic species in lichens, and some of the lesser researched aspects, such as the optimization of extraction methods to improve the recoveries of inorganic arsenic species from the lichen matrix. An isocratic, High Pressure Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS) method, capable of baseline separating arsenobetaine (AsB) from arsenite (As III) using a Hamilton PRP X-100 anion exchange column was developed. The benefit of the developed chromatographic method is that five of the most toxicologically relevant arsenic species, namely As III, arsenate (As V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and AsB could all be quantified using a single analytical column with a single mobile phase, saving on the cost and time of analysis. Moreover, the extraction procedure used only deionized water as an extraction solvent, and employed a low concentration ammonium nitrate (17.5 mmol) in 1% MeOH solution as the mobile phase, making the routine application of

this method a more eco-friendly and "green" alternative to typical HPLC-ICP-MS methods for As speciation studies.

Having observed the benefits of both the sequential extraction, which was able to identify arsenic compounds that become available using solvents with different properties and ionic strengths, and the optimized chromatographic method which was able to further separate the soluble or exchangeable fraction into different species, the water extraction step of the sequential extraction was, for the first time, replaced with a chromatographic separation and applied to lichens from an urban impacted and rural site in South Africa. The findings from this study were significant, resulting in the highest mass-balance yields for arsenic than had been previously reported in literature for either chromatographic techniques or sequential extractions, being 104 % and 111% for the urban and rural sites respectively. The chromatographic method was thereafter applied to a bulk sample of the lichen, *P. austrosinense*, over a period of 1 month with assessments performed every week to determine the short-term stability of arsenic species in the water-extractable (bioavailable) fraction. It was found that the species of arsenic in the bioavailable fraction changed week by week. Here, the perception that the biotransformation capabilities of lichens makes them unsuitable biomonitors of air pollution was challenged. Instead, it was proposed that the predictable timing of the biotransformation of chemical species is an essential component of biomonitoring studies, and can assist in elucidating information about the timing of the exposure and the scale of the exposure event. Furthermore, the results were able to provide new insight into the current understanding of lichen metabolism, and a plausible explanation for a previously observed increase in As V postulated by other researchers.

This thesis contributes to the body of science related to the analytical practices employed in lichen biomonitoring of metal and metalloid air pollutants, with a strong focus on the carcinogenic species of the metalloid, arsenic. It is one of a handful of studies which evaluate As speciation in lichens, where the optimized extraction and novel combination of chromatographic and sequential extraction techniques are able to provide a substantial amount of information regarding source apportionment, and the timing and magnitude of pollution events. As such, this study provides additional insights into both the total analysis of a number of different metals and metalloids, and the optimized extraction and selective and sensitive separation of the various chemical forms of arsenic, making it of global interest. On a more local front, this is one of three published studies in South Africa which evaluated the concentration of heavy metals and metalloids in a lichen biomonitor, and is the first to address more than five elements, therefore the results will form a point of reference for future studies of this type.

Publications and Presentations

This thesis is based upon the following manuscripts:

Published papers

1. Kroukamp EM, Wondimu T, Forbes PBC. 2016. Metal and metalloid speciation in plants: Overview, Instrumentation, approaches and commonly assessed elements. *TrAC* 77:87-99. Impact Factor 7.034. DOI: https://doi.org/10.1016/j.trac.2015.10.007
2. Kroukamp EM, Godeto TW, Forbes PBC. 2017. Comparison of sample preparation procedures on metal(loid) fractionation patterns in lichens. *Environmental Monitoring and Assessment* 189:451. Impact Factor 1.804. DOI: https://doi.org/10.1007/s10661-017-6155-4

Submitted manuscripts

1. Kroukamp EM, Godeto TW, Forbes PBC. 2019. Spatial study of atmospheric metal concentrations in South Africa using the lichen biomonitor, *Parmotrema austrosinense*. Submitted to: *Environmental Monitoring and Assessment.* Impact Factor: 1.804
2. Kroukamp EM, Godeto TW, Forbes PBC. 2019. Optimized extraction of inorganic arsenic species from a foliose lichen biomonitor. Submitted to: *Environmental Science and Pollution Research.* Impact Factor: 2.8.
3. Kroukamp EM, Godeto TW, Forbes PBC. 2019. Species distribution patterns of arsenic in a lichen biomonitor. Prepared for submission to: *Chemosphere.* Impact factor: 4.427.

Book Chapter

Patricia B.C. Forbes, Leandri van der Wat and Eve M. Kroukamp, Chapter 3: Biomonitors, in Comprehensive Analytical Chemistry vol. 70: Monitoring of Air Pollutants: Sampling, Sample Preparation and Analytical Techniques, Patricia Forbes (Ed.), (2015), 53-108, Elsevier, Netherlands. ISBN: 978-0-444-63553-2 http://dx.doi.org/10.1016/bs.coac.2015.09.003

Conference Presentations

Oral presentations

1. Kroukamp EM, Godeto TG, Forbes PBC. 2017. Optimized speciation of 5 arsenic species using anion exchange HPLC-ICP-MS and its real life application in a lichen biomonitor of air pollution. *European Winter Plasma 2017 Conference*, 19-24 February 2017, St Anton Am Arlberg, Austria.

Poster presentation

1. Kroukamp EM*, Wondimu TG, Forbes PBC. 2014. Preliminary investigation into the use of lichens as biomonitors of air pollution in South Africa. *Analitika 2014 Conference*, 7-11 September 2014, Parys, South Africa.
   * Award for second best mass spectrometry student poster.
2. Kroukamp EM, Godeto TW, Forbes PBC. 2017. Sample preparation strategies for the determination of metal(loid) fractionation patterns in lichen air biomonitors. *Canadian Society for Chemistry 2017 Conference*, 28 May - 1 June 2017, Toronto, Canada.

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List of Abbreviations

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As III	Arsenite
As V	Arsenate
AsB	Arsenobetaine
DMA	Dimethylarsenic acid
GAW	Global Atmospheric Watch
HPLC-ICP-MS	High Pressure Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry
MAP	Mean Annual Precipitation
MMA	Monomethylarsonic acid
SEM	Scanning Electron Microscope
TETRA	Tetramethylarsonium ion
TMAO	Trimethylarsine oxide

Chapter 1 Background and Motivation

In this chapter an overview of the problem which motivated this study is given. In addition, aims and objectives with the context of the thesis are presented.

1.1. Problem Statement and rationale

Metal air pollution from increasing infrastructure, industrial development and motor usage has become of global concern within the last few decades, where the presence and high abundance of certain metals in air have been directly linked to increased incidences of cancer and respiratory disease (Pope 1989, Cohen 2000, Nyberg *et al.* 2000, Järup 2003, Reynolds *et al.* 2003). Since these particles are absorbed by lung tissue during inhalation, they are considered to be one of the most important, potentially harmful agents on human health (Fernández *et al.* 2000, Fernández Espinosa *et al.* 2002, Ayrault *et al.* 2013). As such, the monitoring of metal and metalloid air pollutants is of increasing importance.

Metal air pollution is often evaluated by trapping dust and airborne particulate matter onto filters through high-volume air-sampling apparatus (Fernández *et al.* 2000, Fernández Espinosa *et al.* 2002, Feng *et al.* 2009), however this type of sampling only provides an assessment of air quality on the day of sampling, forgoing long-term temporal information about the abundance and bioavailability of these contaminants. These types of assessments are also expensive to conduct both from an instrumentation and consumables perspective.

Epiphytic lichens, in contrast, are reflective of environmental concentrations of airborne metals over long periods of time (Walther *et al.* 1990) and are widely accepted biomonitors of air pollution (Branquinho *et al.* 1999). Lichens lack a cuticle and as a result may assimilate metal ions, among other pollutants, from the atmosphere (Branquinho *et al.* 1999; Adamo *et al.* 2003). Thallus concentrations of metals then reach an equilibrium with the surrounding air (Sloof 1995, Kularatne and De Freitas 2013), and consequently concentrations within and upon the thallus are deemed to be reflective of atmospheric metal concentrations.

In South Africa, the mining and processing of high-grade geological deposits of chromium, platinum, vanadium, vermiculite, manganese, palladium, rutile, gold, zirconium, ilmenite and coal (Gerber *et al.* 2002) results in a number of harmful metals and metalloids entering the air. Studies involving the evaluation of trace metals in the South African atmosphere are limited to just a few, most of which employ air filters for the monitoring of atmospheric pollution, and tend to only address a few target analytes (Okonkwo *et al.* 2009, Van Wyngaardt *et al.* 2011, Beukes *et al.* 2013, Van Zyl *et al.* 2014). Studies involving the use of lichens as biomonitors of metal air pollution in South Africa are even less common, with only a few reported studies to date (Panichev and McCrindle 2004, Forbes *et al.* 2009).

In the application of lichens to monitor trace metal(loid) air pollution, there is no global consensus about which sample preparation strategies should be employed and little investigation has been done into how these strategies affect the overall concentration obtained. The speciation of metal(loid)s of toxicological interest is also fairly under-researched, limited to just a handful of lichen-based studies globally (Koch *et al.* 2000, Farinha *et al.* 2004). This study addresses this paucity of information related to sample preparation and metal(loid) speciation strategies.

1.2. Aims and Objectives of the thesis

1.2.1. General aims of the thesis

The aims of this study were to:

- investigate method development, optimization and speciation of key metal(loid)s in a lichen biomonitor of air pollution.
- evaluate different sample preparation strategies for total and speciated metal(loid) concentrations and provide a recommendation based upon the results
- explore new protocols and provide information about trace metal(loid) concentrations in air in regions which have not previously been evaluated.

1.2.2. Objectives

The objectives of this study were to:

- determine suitable sampling sites in South Africa with variable impacts.
- investigate the total metal(loid) content for a variety of different metals at three different, previously uncharacterized sites with varying degrees of impacts.
- relate the total metal(loid) concentrations to environmental conditions, the surrounding geology and potential sources in the area.
- assess the role of seasonal variations on bioavailability and bioaccumulation of certain metal(loid)s on lichens.
- compare different sample preparation strategies for the analysis of total metal(loid)s and partitioned metal(loid)s from a sequential extraction on the bioavailability and extractability from the lichen matrix for the first time.
- develop a method for the enhanced extraction and chromatographic separation of five species of arsenic in a lichen biomonitor using anion exchange chromatography.
- combine, for the first time, a chromatographic separation with a sequential extraction for the target analyte arsenic, to provide an in-depth overview of bioavailability of this metalloid.

1.3. Context of the thesis

Paper 1 provides a review of the speciation of metals in plants and the importance of such studies. Some of the sampling considerations for such types of analyses and the techniques employed to evaluate metal species in plant matrices are also discussed. Finally, the key target elements which have been evaluated in speciation assessments are examined.

Conference Paper 1 is a short chapter focused on the evaluation of the foliose lichen, *Parmotrema austrosinense*, as an appropriate biomonitor of metal air pollutants in South Africa

Paper 2 involves the comparative assessment of metal concentrations in lichens from three areas in South Africa with distinctly different climates and anthropogenic impacts. The concentration of a number of different metals

and metalloids in lichens from these sites were evaluated and were related to meteorological parameters and potential pollutant sources.

Paper 3 evaluates various sample preparation strategies, short term stability and the mass balances of metals and metalloids from the employment of a sequential extraction protocol developed for soils and air filters to a lichen matrix for the first time.

Paper 4 focuses on the optimized extraction of arsenic from a lichen matrix and the development of a cost-effective and relatively ecofriendly chromatographic method for the baseline separation of five of the most toxicologically relevant arsenic species. The chromatographic technique was thereafter applied to lichens from two sites in South Africa with varying degrees of impacts.

Paper 5 substitutes the total water extraction step from the sequential extraction of lichens as discussed in Paper 3 with the optimized extraction and chromatographic separation of five arsenic species in lichens as developed in Paper 4. This step was thereafter followed by the other sequential extraction steps as discussed in Paper 3 to improve mass-balance recoveries and provide information about source apportionment. The short-term stability of arsenic in the lichen *Parmotrema austrosinense* was assessed in the water extractable fraction, shedding some light on the detoxification mechanisms of the lichen and how this can assist in pinpointing the timing of pollution events.

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Chapter 2 Introduction

This chapter provides a brief overview of the factors contributing to air pollution and application of biomonitors in evaluations of atmospheric pollution. It will also provide an introduction into biomonitoring and the use of lichens as biomonitors of metal(loid) pollutants in air.

Since the mid-1800's scientists have observed a correlation between certain chemicals and altered states of health in plants, animals and humans (Nylander 1866, Wiłkomirski 2013). This began an age of bioindicator studies, where various lifeforms were assessed for changes in behaviour, physiology and abundance as a result of pollution (de Bruin 1990). Plants were used as some of the earliest monitors of pollution, and through the monitoring of atmospheric deposition on plants, the elucidation of information pertaining to pollutant source, quantity and toxicological effects has been possible. Over time, an increasing number of studies were also able to link environmental concentrations of metals to those in biological populations, and from these findings, the field of biomonitoring became established (de Bruin 1990). Biomonitors are living entities which are able to assimilate pollutants over an extended period of time and thus reflect environmental conditions (de Bruin 1990). Of paramount importance in biomonitoring studies is the selection of an appropriate biomonitor, where species presence, prevalence, ability to accumulate toxicants, ease of sampling and ecological relevance all help to assist biomonitor choice (Jeran *et al.* 1996).

Considering that pollutants in the air are often dispersed by wind, the use of biomonitors in such evaluations can be especially useful. This is because traditional sampling techniques, such as the use of air filters, are difficult to implement over large areas due to the infrastructure requirements and associated costs of such approaches. These types of techniques are also biased in favour of prevailing winds and only represent pollution at the time of sampling. Biomonitors, in contrast, allow for ease of sampling throughout and beyond the exposure area, and offer an added benefit of being reflective of pollution over a longer period of time (Walther *et al.* 1990). Large volumes of the chosen biomonitor species can often be collected, making the collected material less likely to be biased in favour of dominating winds, and sampling is also cost effective.

Although a number of different biomonitors such as fungi, mosses, lichens, pine needles, bark and tree leaves have been used in the assessment of airborne pollutants (Kansanen and Venetvaara 1991, de Nicola *et al.* 2013), it is the use of lichens which has drawn a lot of attention in national air biomonitoring programs. This is because lichens are widely available throughout the world, and are found at extremes of temperature and altitude (Forbes *et al.* 2015). Moreover, through Scanning Electron Microscopy (SEM), it is clear to see differences in the amount of material deposited on the surface of lichens at sites with different sources and magnitudes of pollution (Fig. 1 and Fig. 2), where this translates into differences in the concentration of the various pollutants.

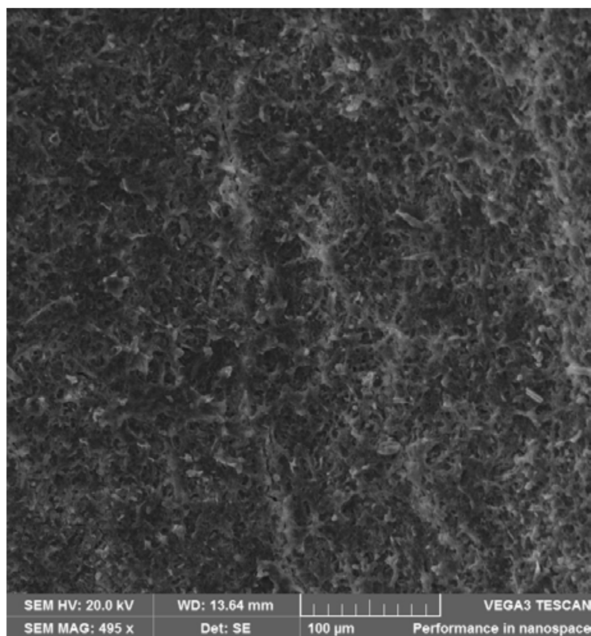

Fig. 1 Secondary electron micrograph of the foliose lichen, *P. austrosinense*, from a Global Atmospheric Watch (GAW) reference site showing almost no visible debris on the lichen thallus. Micrograph was captured using a Tescan Vega™ SEM, accelerating voltage: 20 keV, working distance: 13.64 mm, magnification of 495 x

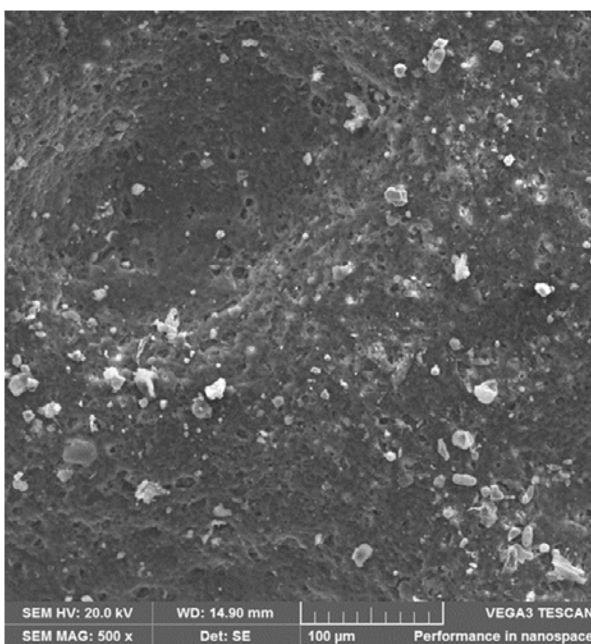

Fig. 2 Secondary electron micrograph of *P. austrosinense*, from a mining-impacted site showing ample visible debris on the lichen thallus. Image taken using SEM, accelerating voltage: 20 keV, working distance: 14.90 mm, magnification of 500 x

To understand why lichens are so effective as biomonitors of air pollution, it is first necessary to understand their physiology. Lichens are the product of a mutual relationship between a fungus and an algae (Forbes *et al.* 2009, Forbes *et al.* 2015). They are slow growing, perennial and have a uniform morphology throughout their

lives (Loppi and Pirintsos 2003, Forbes *et al.* 2015). The absence of the cuticle in lichens allows for the uninhibited movement of nutrients from the air over the whole thallus, allowing for the uptake of metals and other pollutants to levels which exceed ambient concentrations (Loppi and Pirintsos 2003, Forbes *et al.* 2015).

There are a number of different growth forms of lichens, namely fruticose, crustose, squamulose and foliose (Fig. 3). Foliose lichens have been shown to accumulate higher concentrations of metals than fruticose lichens in some studies (Glenn *et al.* 1991), where additional benefits of this growth form are the large surface area, ease of extraction from the substrate, and their presence in a variety of different habitats. Epiphytic lichens do not acquire any nutrients from the bark or tree, only using the tree as a substrate (Bargagli *et al.* 2002, Forbes *et al.* 2015).

Fig. 3 Foliose epiphytic lichen, *P. austrosinense*

The accumulation of metals by lichens can take place through a number of different processes, including the diffusion of gases across the cell membrane, entrapment of particles on the lichen surface, or ion-exchange (Forbes *et al.* 2015), where ion-exchange requires a moist surface for the exchange to take place and is a swift, passive process, obeying the principles of mass and charge balance (Nieboer *et al.* 1976). Some of the major factors effecting the accumulation of toxicants from the environment in lichen thalli are the prevailing wind direction (de Bruin 1990), rain, stemflow and lichen species, where the secretion of calcium oxalate (Fig. 4) may affect the distribution of metal(loid)s between the inter-,intra- and extra-cellular components of the lichen thallus. It is therefore essential that all of these aspects are considered during lichen choice and sampling, where strategies such as sampling from all around the tree and taking into account meteorological factors can assist in ensuring that unbiased sampling takes place.

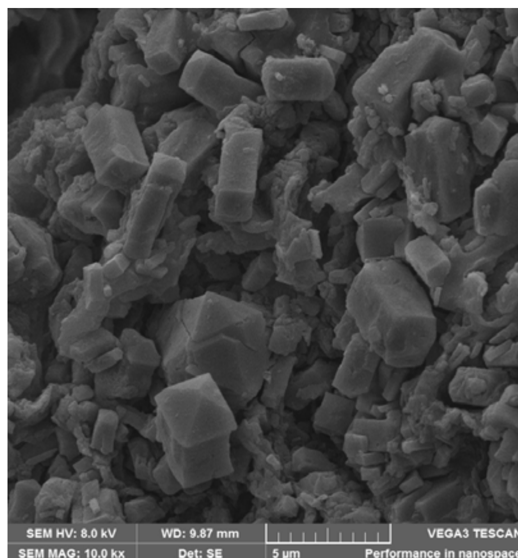

Fig. 4 Secondary electron micrograph of crystals on the surface of the foliose lichen, *P. austrosinense*, from a mining-impacted site. These were determined to be calcium oxalate crystals using Scanning Electron Microscopy Energy Dispersive Spectroscopy (SEM-EDS). Micrographs were taken using SEM, accelerating voltage: 8 keV, working distance: 9.87 mm, magnification:10 000 x

To date, most atmospheric particulate analyses using lichens as biomonitors in South Africa have involved the quantification of only one or a few target metals and do not tend to be extensive in terms of the number of elements which are assessed (Forbes *et al.* 2009, Panichev and McCrindle 2004). Sample collection and sample preparation studies vary greatly and there is little standardization of techniques (Kroukamp et al. 2017), where the effect that these have on the metal concentration in lichens has not been extensively evaluated and so there is a paucity in lichen biomonitoring studies in this respect.

In order to gain a holistic understanding of air pollution it is also essential to identify and quantify the different chemical species of key elements since this relates to the availability, mobility, uptake mechanisms and toxicity of that element (Ressler *et al.* 2000, Fernández Espinosa *et al.* 2002, Stanislawska *et al.* 2013). However, these studies are uncommon in lichen analyses (Kuehnelt *et al.* 2000, Farinha *et al.* 2004, Machado *et al.* 2006), and are plagued by the same issues as observed by total metal analysis in terms of sample collection, preparation and storage, where this is an area requiring further investigation.

One of the key elements which is of toxicological interest is arsenic. Arsenic is a metalloid which exists in a number of different chemical forms, some of which are carcinogenic. These can be inorganic; such as arsenite (III) and arsenate (V) or organic species; such as dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), arsenobetaine (AsB), trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA) and different arseno sugars (Guerin *et al.* 2000, Machado *et al.* 2006). The most toxic forms of arsenic are inorganic (Guerin *et al.* 2000, Machado *et al.* 2006) and it is these forms which are most commonly found in the terrestrial environment and attached to particulate matter in air (Chung *et al.* 2014). Anthropogenic sources of arsenic in the atmospheric environment include cigarette smoke, medical waste incineration, crematoriums, copper smelters, glass manufacturing plants and gold mining activities, among others (USEPA 1998, Chung *et al.* 2014). Methods evaluating As species in lichens are few in number and tend to have poor recoveries (Koch et al. 1999, Kuehnelt et al 2000), making conclusions between source apportionment and the timing of pollution events difficult to

characterize. The optimization of techniques for the extraction of arsenic species from lichens, along with the development of a method which can appropriately distinguish between the various chemical forms of arsenic within a lichen matrix would greatly benefit air pollution biomonitoring studies and assist with drawing conclusions between source and atmospheric concentrations.

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Chapter 3 Literature Review

This chapter presents an overview of the importance of speciation in plant studies and discusses the instrumentation employed, important analytical considerations, and commonly assessed elements in speciation studies.

3.1. Paper 1

This paper was formatted in accordance with the journal *TrAC*, *Trends in Analytical Chemistry* an Elsevier journal in which it has been published.

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Metal and metalloid speciation in plants: Overview, instrumentation, approaches and commonly assessed elements

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ABSTRACT

The ability of plants to hyper-accumulate metals and metalloids from the surrounding environment may pose a significant health risk to both humans and animals since plants form a substantial component of diet. This attribute, however, has also been identified as a useful tool in bioremediation and biomonitoring studies; where assimilated metals and metalloids in plants often correlate to environmental exposure. Since the bioavailability and toxicity of these elements depend upon their chemical form, speciation studies are essential in determining their mobility and metabolic pathways. This can be done in a number of ways where sampling, pre-treatment and storage are all important factors affecting speciation. Appropriate analytical techniques for speciation studies can either be direct methods such as XAS, or indirect methods which require species separation prior to analysis. Separation techniques can either be in the form of sequential extractions or column separation and analyte detection often utilises instrumentation such as ESI-MS, ICP-MS and ICP-OES.

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Abbreviations: µXRF, Micro-X-ray Fluorescence; AFS, Atomic Fluorescence Spectroscopy; API, Atmospheric Pressure Ionization; CEC, Capillary Electrochromatography; CID, Collision Induced Dissociation; CRI, Collision Reaction Interface; CRM, Certified Reference Material; CZE, Capillary Zone Electrophoresis; CZE-ICP-MS, Capillary Zone Electrophoresis Inductively Coupled Plasma Mass Spectrometry; ESI-MS, Electrospray Ionization Mass Spectrometry; EXAFS, Extended X-ray Absorption Fine Structure; FAAS, Flame Atomic Absorption Spectroscopy; FTIR, Fourier Transform Infrared Spectroscopy; GC-MS, Gas Chromatography Mass Spectrometry; GC-MS-HGAAS, Gas Chromatography Mass Spectrometry Hydride Generation Atomic Absorption Spectroscopy; GFAAS, Graphite Furnace Atomic Absorption Spectrometry; HEN, High Efficiency Nebulizer; HFBA, Heptafluorobutanoic acid; HG-GC-QFAAS, Hydride Generation Gas Chromatography Quartz tube Flame Atomic Absorption Spectrometry; HPLC, High Pressure Liquid Chromatography; HPLC-HG-AFS, High Pressure Liquid Chromatography Mass Spectrometry Atomic Fluorescence Spectroscopy; HPLC-ICP-MS, High Pressure Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry; HPLC-UV-HG-AFS, High Pressure Liquid Chromatography Ultra Violet Hydride Generation Atomic Fluorescence Spectroscopy; IC, Ion Chromatography; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry; IEC, Ion Exchange Chromatography; IEF, Isoelectric focussing; IPC, Ion Pairing Chromatography; IP-RPLC-ICP-MS, Ion Pairing Reversed Phase Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry; IS, Ion Spray; ITP, Isotachophoresis; LC-VG-ICP-MS, Liquid Chromatography Vapour Generation Inductively Coupled Plasma Mass Spectrometry; MAE, Microwave Assisted Extraction; MECC, Micellar Electrokinetic Capillary Chromatography; NMR, Nuclear Magnetic Resonance; PCA, Principal Component Analysis; PM, Particulate Matter; QTOF-MS, Quadrupole Time of Flight Mass Spectrometry; RPLC, Reversed Phase Liquid Chromatography; RTILS, Room Temperature Ionic Liquids; SEC, Size Exclusion Chromatography; SE-HPLC-ESI-MS-MS, Size Exclusion High Pressure Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry; SeMC, Methyl-L-selenocysteine; SEM-EDX, Scanning Electron Microscope Energy Dispersive X-ray Microanalysis; SeMet, Selenomethionine; SWV, Square Wave Voltammetry; TA, Target Analysis; TDS, Total Dissolved Solids; TFA, Trifluoroacetic acid; TOF-SIMS, Time of Flight Secondary Ion Mass Spectrometry; UAE, Ultrasonic Assisted Solvent Extraction; XANES, X-ray Absorption Near Edge Structure; XAS, X-ray Absorption Spectrometry.

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1. Introduction

The importance of speciation studies is evident from the increasing number of publications focussing on this type of analysis. This is because total elemental concentrations do not provide sufficient information about the bioavailability and fate of metals and metalloids in the environment [1], whereas speciation studies are able to [1–7]. Such analyses can be described as the identification and measurement of specific forms of an element according to its molecular or complex structure, electronic or oxidation state or its isotopic composition [8]. The interest in metal and metalloid species in plants (which are generally collectively referred to as "metals" in this paper) is well established, as plants are natural, dietary sources for major, minor and trace essential minerals [9]. Since metals are non-biodegradable [10,11], metal loading in plants is therefore an important consideration when assessing potential health impacts [12,13], where metals can be assimilated either as elements or as metabolites [14].

The uptake of metal(loid)s by plants is either a passive or active process. Vascular plants take up metals from their roots, transpiration through stomata on the leaf surface, and deposition on the surface of leaves and bark. Non-vascular plants, however, often lack cuticle and stomata [15], and factors such as surface water, rain and passive diffusion from substrates facilitate the movement of metals into these plants [16]. The uptake, translocation, transformation and accumulation of metallic species in crop plants is extremely important from an animal and human health perspective [17,18]. Since a number of plants and their produce are distributed globally, their composition needs to be determined to meet import and export criteria [19]. The testing procedures employed can prove to be challenging, as the toxicity or beneficial nature of metals is not necessarily determined solely by its organic or inorganic form [6,20] or the oxidative species present [6,21]. Therefore toxicity is often linked to the solubility of the chemical form and the ligand species. Knowledge of potential sources of pollutants within a particular area is advantageous as it can provide information about the compounds present and their physical and chemical properties.

Metal(loid)s in the environment usually occur as a mixture of inorganic and organic compounds with varying degrees of toxicity. Natural sources of these elements in plants include that arising from soil microbial activity or the weathering of geological formations, as was investigated by Eiche et al. [18], who evaluated the speciation of Se in edible crops growing in seleniferous soils in India (Table 1). Such studies of metal speciation and metal homeostasis in plant crops can also be used in the biological engineering, otherwise known as biofortification, of food crops to increase the uptake of beneficial metals by plants into key plant components [41]. Metals can also occur in the environment as a result of anthropogenic activities, and biomonitoring studies are often undertaken to assess the risks associated with such exposures.

Biomonitoring studies make use of direct monitoring of biological organisms, which are able to assimilate contaminants over a long period of time and remain in equilibrium with the system [42–45]. These types of studies are therefore not as subjective as those involving ecosystems which are only sampled at a specific point in time [46,47]. By using plants as biomonitors, information on metabolic pathways, detoxification mechanisms and source apportionment can be obtained. A study by Bergqvist et al. [28] showed that higher concentrations of inorganic As in edible crops correlated with the concentrations of As in the soils from an abandoned glassworks in which they were grown. Thus a clear link between cause and effect could be observed. These concentrations were higher than those in plants grown in less contaminated soils; namely greenhouse and agricultural alum shales (Table 1). Plants are not only used to assess soil contamination in biomonitoring studies, but can also be used in the biomontoring of atmospheric pollution. Botanical matrices such as moss, pine needles, tree bark, leaves, grass, ferns and lichens have been employed in this regard [48,49]. Metal speciation studies in such cases can be either intra-, inter- or extracellular. An example of the latter is a study by Schreck et al. [50], who determined the speciation of Pb in atmospheric fallout dust on the surface of edible plants.

Biomonitoring can also be employed in aquatic ecosystems such as estuarine, salt water and freshwater systems through the use of aquatic plants such as the water hyacinth, *Eichhornia crassipes* [51]. Here, *in situ* water hyacinth was used to evaluate and remediate mercury contaminated waters from a chloralkali plant. Since certain species of plant have been shown to accumulate higher concentrations of metals than others, plants have also played a large role in bioremediation efforts [52]. Bioremediation-phytoremediation projects exploit the ability of plants to assimilate metals in concentrations higher than their metabolic requirements. Hyperaccumulating, metal tolerant plants are commonly used in the phytoextraction of heavy metals from metal-loaded soils, such as those found in mining [53] and sawdust heaps [54] Certain plant species are known to possess higher bioconcentration potentials than others, such as was found in the fern, *Pteris vittata*; where these plants are commonly used in the removal of As from contaminated soils and groundwater [23]. Livestock and agricultural farmers often populate catchment areas with riparian vegetation such as bulrushes and reeds to form man-made wetlands; reducing water flow, downstream silt build-up and the eutrophication of rivers [55–57].

To determine a suitable plant species to be used for the mentioned applications, familiarity with the elements of interest, as well as an understanding of their speciation will greatly assist with the selective extraction of elements [33]. Moreover, hyper-accumulation

Table 1
Summary of published studies dealing with the separation of species of metals in plants

Metals speciated	Plant material	Plant species	Location	Extraction method	Sample mass (g)	Column	Mobile Phase	Analytical technique	Reference
As	Lichens and green plants	*Alectoria ochroleuca, Usnea articulate, Achillea millefolium, Alnus incana, Asplenium viride, Dryopteris dilate, Equisetum pratense, Fragaria vesca, Rubus idaeus, Vaccinium myrtilis, Vaccinium vitis idaea, Picea abies, Larix decidua, Deschampsia cespitosa.*	Austria	MeOH: H_2O (9:1)	0.5, 0.8, 1	Anionic species: Hamilton PRP-X-100 (250 × 4.1 mm; 10 µm) Cationic species: Zorbax 300-SCX cation-exchange column (150 × 4.6 mm)	Anionic: pyridine (20 mM, pH 2.6, CHOOH) Cationic: $NH_4H_2PO_4$ (20 mM, pH 5.6, 25% NH_4	HPLC-ICPMS	[22]
As	Brake Fern	*Pteris vittata*	Greenhouse study using soils from Florida, USA	MeOH:H_2O (1:1)	0.010	Anionic species: Hamilton PRP-X-100 (250 × 4.6 mm, 10 µm particle size)	K_2HPO_4 & KH_2PO_4 (0.015 M, pH 5.9)	HPLC-HG-AFS and HPLC-ICP-MS	[23]
As	Lichens	*Parmelia sulcate*	Portugal	Sequential	0.2–0.5	Separation carried out on pre-column. Anionic species: Hamilton PRP-X-100 (250 × 4.1 mm) Cationic species: Altech Adsorbosphere SCX 5U (250 × 4.6 mm)	Anionic: KH_2PO_4 (15 mmol/L, pH 6.1, NH_4OH) Cationic: pyridine (2.5 mmol/L, pH 2.65, HCL)	HPLC-UV-HG-AFS	[24]
As	Lichens and tree bark	*Parmelia caperata, Platanus hybrida*	Portugal	MeOH:H_2O (1:1)	0.2–0.5	Anionic species: Hamilton PRP-X-100 (240 × 4.1 mm) Cationic species: Altech Adsorbosphere SCX 5U (250 × 4.6 mm)	Anionic: KH_2PO_4, (20 mmol/L, pH 6.0) Cationic: pyridine (2.5 mmol/L, pH 2.65)	HPLC-HGAFS	[21]
As	Flowering plant	*Calluna vulgaris,*	Spain	MAE and DB[a] Orthophosphoric acid (0.3 M)	0.2	Anionic species: Hamilton PRP-X-100 (250 × 4.1 mm; 10 µm)	TRIS(hydroxymethyl amionoethane) pH 7, adjusted with glacial acetic acid.	HPLC-ICP-MS	[25]
As	Rice, wheat	*Rice Flour CRM, NIST SRM 1568a Rice flour, commercial rice, Wheat*	USA, Czech Rebublic (commercial rice), wheat (Italy)	MAE and Water bath extraction	0.25	Anionic species: Hamilton PRP-X-100	Malonic acid (2, 5 or 10 mm at pH 5.6 adjusted with aqueous ammonia)	HPLC-ICP-MS	[26]
As	Signalgrass	*Brachiaria brizantha*	Brazil	HNO_3 (2% v/v)	0.2	Anionic species: IonoSpher A (250 × 4.6 mm)	Phosphate buffer 12.5 mmol/L, pH 5.4	HPLC-ICP-MS	[27]
As	Carrot, lettuce, spinach	*Daucus carota L, Lactuca sativa L., Spinacia oleracea*	Sweden	MeOH:H_2O (1:1)	0.1- 5	Anionic species: Hamilton PRP-X-100 (250 × 4.6 mm)	$H(NH_4)_2PO_4$ (pH 5.8)	XANES and Sequential extraction with HPLC-AAS	[28]
As	Rice	SRM 1568b and CRM 7503-a and rice from local market	USA	Enzyme-assisted water phase microwave extraction (0.5% amylase solution)	0.2	7100 CE system. 60 cm coated fused silica capillary with i.d. 100 µm	Na_2CO_3 (8 mm, pH 11) buffer	CE-ICP-MS	[29]
As	Rice	*Yerua, Camba, ZHE733, Puita and El Paso 144*	Argentina	Heat assisted extraction (sand bath)	~1.0	Heated PRP-X-100 column	NaH_2PO_4/Na_2HPO_4 buffer	HPLC-HG-AFS	[30]
Cd	Flowering plants, grasses, maize	*Silene cucubalis, Agrostis tenuis, Rauwolfia serpentina, Zea mays L.*	France	Sequential and online	0.010	SEC column: Superdex Peptide HR 10/30 (300 × 10 mm). Superdex 75 HR 10/30 and Superdex 200. Guard column: TSK PWXL (40 × 3 mm)	TRIS-HCL buffer (30 mm, pH 8.5) ES-MS-MS: Orifice potential 40 V, Ionspray voltage 4100 V.	SE HPLC-ICP-MS, SE HPLC-ES-MS-MS	[9]
Cd	Edible plant	*Arabidopsis thaliana*	Poland	Sequential extraction	0.040	Superdex 75 HR 10/30 (300 × 10 mm)	30 mM Tris buffer with 10 mM of NaCl 7.4	SEC-RPLC-ESI-MS	[31]

(*continued on next page*)

Table 1 (*continued*)

Metals speciated	Plant material	Plant species	Location	Extraction method	Sample mass (g)	Column	Mobile Phase	Analytical technique	Reference
Cd	Flowering plant	*Arabidopsis halleri*	Japan	Extraction				^{113}Cd NMR	[32]
Mn	Hazelnuts		Turkey	Sequential and online	0.1	Heated column (26°C) 200 column oven and a vacuum degasser. Brownlee DB Aq. C-18 (150 × 4.6 mm i.d. and 5 µm film thickness). Guard column (40 mm) with the same stationary phase material.	MeOH/H_2O (gradient mobile phase 100:0 (A%/B%, v/v) for 0–1 min, 95:5 (A%/B%, v/v for 1 min, 97:3 (A%/B%, v/v) for 2 min and 98:3 (A%/B%, v/v) for 2 min)	ICP-MS and HPLC-ICP-MS	[17]
Ni	Green plant	*Thlaspi arvense L. Thlaspi goesingense*	Germany:					XAS	[33]
Ni	Tree	*Sebertia acuminata*	New Caledonia	Sequential and on-line	0.127	SEC column: Superdex Peptide HR 10/30 (300 × 10 mm). Preparative SEC on Superdex 30 preparative grade column (600 × 26 mm, Mx< 10000Da) lypholized at −50°C	SEC: $NH_4C_2H_3O_2$ (5 mmol/L, pH 6.8) and degassed ultrasonically.	SE HPLC-ES MS-MS, CZE-ICP-MS	[34]
Ni	Green plant	*T. caerulescens*	France	Sequential and on-line	1	SEC column: Superdex Peptide HR 10/30 (300x 10 mm).	SEC: $NH_4C_2H_3O_2$ (5 mmol/L, pH 7) and degassed ultrasonically.	SE HPLC-ICP-MS,	[4]
Ni	Flowering plant	*Alyssum serpyllifolium ssp. Lusitanicum*	Portugal					SWV	[35]
Sb	Flowering plant, moss, vascular plants, lichens, fungi	*Mimulus sp., Funaria hygrometrica, Drepanocladus sp. Typha latifolia, Bidens cernua,Cladonia sp, Lycoperdon s. Coprinus comatus.*	Canada	MeOH: H_2O (1:1)	0.5 ± 0.005, 1–2 for moss	GC-MS: PTETM (5.30 m × 0.32 mm, 0.25 µm Supelco)	Buffer: $NaBH_4$ (2% w/v) and $NaBH_4$ (6%)	HG-GC-AAS, Headspace HG-GC-MS	[36]
Se	Garlic and mustard	*Allium sativum and Brassica juncea*	Spain	UAE	0.2	Ion pairing reverse phase column C8 (250 × 2.0 mm, 5 µm) and SEC Shodex Asahipak GS-220 HQ (300 × 7.6 mm, >3000 Da)	IPRLC: 0.2% (v/v) heptafluorobutyric acid, 10% (v/v) MeOH (pH 2.5) SEC: 10 mm ammonium acetate buffer (pH 6.5)	HPLC-ICP-MS	[37]
Se	Yeast and Clover	*Not mentioned*	China	Water extraction and enzymatic extraction	Not mentioned	C18 column (Shim-pack CLC, 5µm, 4.6 × 150 mm, Japan)	0.4% (v/v) [BMIM]Cl, 0.4% (v/v) [BMMIM]BF_4, and 99.2% high purity deionized water (pH = 6) was used for isocratic elution at a flow rate of 1.0 mLmin^{-1}	RTILs improved RPLC-ICP-MS	[38]
Se	Kale	*Brassica oleracea var. alboglabra L.*	Thailand	0.1 M HCl in 10% v/v MeOH	0.1	Reverse phase column Inertsil® C18 (250 × 4.6 mm, 5 µm)	8 mm BSA, 4 mm TFA pH 4.5	HPLC-ICPMS	[39]
Se	Wheat and mustard	*Triticum aestivum and Brassica juncea*	India					SE HPLC-ES MS-MS, CZE-ICP-MS	[18]
U	Sunflower and oilseed rape	*Helianthus annuus and Brassica napus.*	France					HG-GC-AAS and HG-GC-MS	[40]

[a] DB: Digestion block.

of metals in different parts of the plant may vary quite considerably, and knowledge of these properties can be beneficial. There are concerns, however, that the process of assimilation of metals by plants may result in the biotransformation of certain metallic and metalloid species [9,58], as was found in the case of As in the lichen species *Parmelia caperata* (Table 1) [21]. This biotransformation is a potential issue for other elements as well [21] and as a result, non-living biomass has also been assessed for its suitability in bioaccumulation and bioremediation studies.

Inactivated mosses and lichens have been employed in bioremediation studies as biosorptive agents; absorbing metal ions from industrial waste waters to reduce toxicity [59,60]. A study assessing copper (as Cu II) in lichen biomass, *Cladonia rangiformis hoffm.*, was used to illustrate this, as copper is a common by-product of industry, processing and mining wastes [60]. The effect of pH was found to be a defining parameter in the absorption of Cu [60,61] and is also likely to have an influence on the chemical species present. The absorption of Cu at low pHs was found to be inhibited, assumedly due to a competition between H_3O^+ and Cu (II) at the lichen surface, whilst at pH > 5, bioabsorption was also found to be inhibited [60]. Similar effects were observed in studies by Uluozlu et al. [61] who monitored the bioabsorption of Pb (II) and Cr (III) into the lichen *P. tiliaceae* using Fourier Transform Infrared Spectroscopy (FTIR). Since a number of different factors can disturb the equilibrium in the chemical species, appropriate means of sampling and sample pre-treatment are important in any chemical speciation analysis.

2. Sampling and sample pre-treatment

A number of sample pre-treatment techniques have been established for the speciation of simple matrices; however, complex matrices still present a significant challenge. This is partly due to shifts in chemical equilibria which may occur during this step, affecting the quality of the results, the extent of which are often difficult to quantify [1,62,63]. Consequently, sampling and sample stabilization in complex matrices are considered to be the most important steps in speciation analyses [1,63]. To prevent alterations, the pre-treatment procedures are often not as aggressive as one would use for total element determinations [25,27].

In biological materials, contamination during sampling and from sampling apparatus may result in altered species distributions [1,63,64]. The following recommendations in terms of sample collection have been made:

- Samples should be collected using acid-washed plastic [65], nylon, ceramic or teflon tweezers [66].
- Sampling time should be kept to a minimum [1].
- Powder free latex, nitrile or vinyl gloves should be worn.
- Samples such as roots and stems, which are in close contact with the ground, should be gently washed of adhering materials so as to limit contamination [48].
- Samples such as leaves, bark, epiphytic mosses and lichens, should be collected 1–2 m above the ground to limit contamination by "back-splash", and are generally not washed [48,49].
- Samples of similar sizes should be chosen to reduce variations which can be introduced from longer growth periods [15].
- Storage should take place in acid washed polyethylene or polypropylene bottles or paper bags [1,67].
- Samples should be stabilized as soon as possible which can involve steps such as drying, placement in an inert environment or freezing.

Sample pre-treatment should involve as few steps as possible; thereby limiting the chances of sample loss and species alteration [68]. There are conflicting approaches to the washing of samples, where some authors prefer to remove extraneous materials in this manner [48], while others are concerned that washing steps may result in the dissolution of ionic compounds, altering their concentrations [67]. Inevitably, it comes down to the desired information. If the assessment of the adhering particles, isotopic ratios, or the speciation of metals within the sample are of interest, then it has been recommended that the samples are not washed [69]. If the bioconcentrated fraction is of interest then it is recommended that the samples are washed [70]. To assess the distributions of metallic species within the bioconcentrated fraction, conditions which maintain the original species, but avoid contamination by soil, bark or other particles need to be employed. For this reason other cleaning procedures, such as nitrogen air jets and ultrasonic baths or probes may be recommended.

Nitrogen air jets are especially useful in speciation studies as they are thought to provide an inert and clean environment, limiting contamination and changes in the species pattern distributions. Some samples, such as mosses, are especially porous and as a result are difficult to clean effectively [71], and so a number of techniques have been employed to remedy this. Spagnuolo et al. [71] found that nitrogen air jets were too aggressive for the cleaning of mosses at higher pressures (4–8 bars) and were unable to remove adhering particles at lower pressures [71] and results were found to be irreproducible and unpredictable. In comparison, the use of ultrasonic probes for cleaning procedures was found to produce reproducible data and ensured the integrity of the cell membrane [71]. The procedural requirements for immersing the sample in a liquid phase however, was found to encourage the leaching of metals from the sample, compromising the quality of the results [71]. Ultrasonic baths have also been used as a gentler alternative to ultrasonic probes, and have been used to clean foliose lichens such as *Parmelia sulcata* [72]. Consequently they are often the preferred cleaning procedure for speciation analysis [66].

The choice of preparation techniques also depends upon the type of analytical instrumentation which will be used for a particular chemical species. Laurette et al. [40], for example, recommended that root samples be washed with distilled water and soaked in 10 mM Na_2CO_3 to remove adsorbed uranium from the root surfaces of oilseed rape and sunflower plants prior to analysis with X-ray absorption spectrometry (XAS) with X-ray Absorption Near Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS) (Table 1).

A degree of heterogeneity in chemical species may be expected between samples within a population [7,59]. To account for this, it is recommended that a large, representative set of samples be collected, stabilized, pulverized and homogenised [73]. However these steps require the manipulation of the sample, which can lead to contamination [65]. Techniques commonly used in retaining species integrity and preventing analyte losses include enzymatic hydrolysis, volatilization with and without pre-concentration, as well as different leaching and extraction methods [1,26,63,74]. Some of the challenges surrounding homogenization can be addressed by drying or freezing samples prior to this step; increasing the ease by which homogenization can take place, and ending microbial activity which may otherwise disrupt the chemical equilibrium between species. Commonly employed techniques are air drying [15,66], oven-drying [75], freezing to between −20°C to −80°C [76], freeze-drying [22] and cryogenic freezing with liquid nitrogen [40,77].

It has been recommended that biological tissue samples be freeze dried and stored under cold conditions until processing [1,68]. The freeze drying process removes water molecules by freezing the water within the sample. By keeping the pressure below the triple point and gradually increasing the temperature, the ice crystals are sublimated [78], which limits water-based reactions [68]. Freeze drying, however, may remove certain volatile species from the sample [79],

and in some cases has been found to alter the major chemical speciation patterns [68]. This observation was confirmed by Eiche et al. [18], who noted that the drying step (air drying and subsequent freeze drying) resulted in poor recoveries of some species of Se in wheat and Indian mustard plants (Table 1). For this reason, techniques involving the formation of water on the surface of the sample are often avoided and some researchers have chosen to rather freeze their biological materials in liquid nitrogen [33,40], and store under cold conditions until processing. Presumably this is based on the assumption that the species distributions will experience little alteration during cryofixation and thus the original species integrity can be ensured. Sample stabilization steps for transition elements require further consideration as they are especially susceptible to oxidation, which can compromise the species integrity. Therefore, studies which focus on evaluating the species distribution patterns for these elements should be conducted in an inert environment [78].

Quality control procedures in the analysis of chemical species should ensure that the data captured is an accurate and true reflection of the species concentrations. Mass balances should be determined throughout the procedure and species spikes are avoided as spikes may affect the species equilibrium [1,68]. If the study is to involve the validation of a new technique, cross correlation of results with an existing technique is highly recommended [62] and certified reference materials should be analysed where possible.

There are a variety of analytical techniques which may be employed in the determination of appropriately collected and pretreated samples, and these are discussed in Section 3.

3. Direct analytical techniques

3.1. XAS

Synchrotron X-ray Absorption Spectroscopy is a powerful, element-specific speciation tool [7], and is commonly used in the assessment of metal-uptake, toxicity and tolerance in plants, particularly in hyper-accumulation studies [7,80]. Measurements using XAS provide a plethora of information about the target analyte in the sample, such as ligand identification in terms of oxidation state and symmetry, as well as information about neighbouring atoms [80]. XAS follows the Beer-Lambert Law, where signals from XAS are deconvoluted using chemometrics [7].

XAS is able to provide quantitative metal or metalloid speciation data for environmental samples, irrespective of their physical state and provided that the original species integrity is maintained throughout the entire analytical procedure [2,7]. XANES or EXAFS is typically used after principal component analysis (PCA) and target analysis (TA) to determine the analyte species identity. XANES is the analysis which occurs within the energy region of 50 eV, below the elements absorption edge to 200 eV above this [81].

Although this technique has shown to be a valuable tool in speciation analysis, poor detection limits, maintenance of the species integrity and limitations on the number and variety of species within a sample which are quantifiable make calibration a challenge and reduce its applicability. The quantification of transition elements has been especially problematic, although new instrumentation may address this [7]. Where these elements are of interest, sample and species integrity is maintained by cryofixation of specimens and sample runs taking place at low temperatures [7]. The limited availability of certified reference materials (CRMs) for the analytes and species of interest for this technique presents an additional challenge [7]. Consequently, laboratories need to carefully synthesize in-house standards with low errors and appropriate matrix matching.

4. Separation techniques

Two separation techniques which are commonly employed in speciation analysis are extractions and chemical separation using chromatography [82]. Sequential extractions characterize molecular groups according to similar chemical traits; where the fractions are linked to bioavailability [1,3,82,83]. These techniques, however, are not fully selective, since two or more species are often extracted together, and thus do not allow for the identification of any single chemical species [1]. For this reason, extractions are not regarded as true chemical speciation analyses [1] whereas separations using chromatography are [82,84]. Moreover, sequential extraction procedures are not as suitable for routine analysis as chromatographic techniques because they are often involved and time consuming processes [85].

The measurement of analytes in extracts from sequential extraction procedures have mostly used Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) [86], Graphite Furnace Atomic Absorption Spectrometry (GFAAS) [82] and Flame Atomic Absorption Spectroscopy (FAAS) [87] for quantification, whilst a number of hyphenated techniques such as High Pressure Liquid Chromatography – Inductively Coupled Plasma – Mass Spectrometry (HPLC-ICP-MS) [21,84] and Hydride Generation – Gas Chromatography – Quartz Tube Flame Atomic Absorption Spectrometry (HG-GC-QFAAS) [84] have been widely used for the detection and characterization of individual chemical species in extracts. The extraction and separation techniques are described further in Sections 4.1 and 4.2, whilst the detectors employed are discussed in Section 4.3.

4.1. Extraction techniques

Extractions can be either single step or sequential; where the latter treats the sample with leaching agents of increasing strength of interaction [1,82,83]. The sequential extraction method optimized by Tessier et al. [83] is most commonly used, however some issues were the lack of selectivity with respect to individual species and the pH dependency of the method [1,87]. Several adaptations have thus been suggested for different matrices including plants such as lichens [24]. Improvements have included the use of less aggressive reactants and extractants not being replaced in each step but rather added to each step, with small aliquots set aside for analysis [24].

Ultrasonic-Assisted Solvent Extraction (UAE) has gained popularity in the last few years as an attractive alternative to Microwave Assisted Extraction (MAE) techniques as it does not require high temperatures, pressures, or harmful chemicals [71,88,89]. A solid sample is placed into a solution and is exposed to ultrasonic energy, which results in the formation of imploding bubbles, leading to high localized temperatures and pressures [89]. In this way compounds or elements can be extracted from the sample matrix [71]. Ultrasonic methods also increase the rate of reaction in sequential extractions [88] and assist with the homogenous suspension and distribution of slurries [90]. The application of UAE in the extraction of weakly bound organic compounds from their respective matrices is also possible [88], provided that the temperature does not exceed the tolerances of the analyte [90]. UAE has been used in the extraction of selenium species from *Allium sativum* and *Brassica juncea* prior to chromatographic separation using HPLC-ICP-MS [91] (Table 1).

Factors such as particle size, extractant concentration, sonication time, sample mass to extractant ratio [72,91,92], and the use and choice of surfactants [91] all impact on the extraction efficiency. Extractions using ultrasonic probes are more efficient, reproducible and repeatable than the use of ultrasonic baths [72] which do not have a uniform distribution of ultrasound energy and

also lose power over time [93]. An additional benefit of using this technique is that it involves fewer analytical steps than many other extraction techniques, making it less susceptible to contamination [72].

4.2. Hyphenated techniques

Reactions of metals and metalloids are seldom related to their elemental properties, but rather to their oxidation state or their role as a component of larger macromolecules [1]. Therefore coupling of different separation techniques with either element or molecule-selective analytical techniques, or both, form the basis for most speciation analyses [1,62]. Methods of separation commonly include Liquid Chromatography (LC), Capillary Electrophoresis (CE) and Gas Chromatography (GC), where detection methods can either be elemental or molecular [1]. Coupling methods require ease of interfacing between the separation and detection system, and adequately low detection limits [29]. Some of these techniques which are commonly employed are briefly discussed in this section.

4.2.1. Liquid Chromatography (LC)

Liquid chromatography is an extremely useful technique because both the mobile and stationary phases used in the separation can be altered depending on the analyte; improving species stability and analyte separation [1]. It can be used to separate thermally labile and non-volatile compounds and is easily interfaced with ICP-MS making it suitable for speciation studies [94]. Unlike GC, derivatization to produce volatile compounds is not required. It is also more reproducible than CE [38]. Species alterations may still occur, however. Contamination of stationary phases or denaturing of biomolecules brings with it the risk that the species of interest may not be quantitatively recovered [1,7,62]. It is therefore necessary to ensure that the metal-ligand bond is stronger than any bonds which can form between these components and the stationary phase [62]. LC columns commonly used to separate chemical species are based upon factors such as retention time, size and mobility [1,5,62]. Different columns, their benefits and disadvantages will be discussed in the following sections.

4.2.1.1. Size Exclusion Chromatography (SEC). Size Exclusion Chromatography (SEC) is a technique which often precedes detection, differentiating between free metal ions and elements bound within a macromolecular structure [62]. In this way, separation is dependent upon the molecular size of the analyte [95,96]. Its ease of coupling to on-line detection systems makes this type of separation extremely efficient [62]. Speciation is gentle, and does not result in high element species losses, or alterations to the species type on the column [1]. The mobile phase is determined by assessing sample-solvent solubility and its chemical compatibility with the stationary phase [95]. As shown in Table 1, mobile phases commonly employed in SEC techniques are Tris-HCL buffer [9], Tris buffer with NaCl [31], ammonium acetate [4,34,37] and sodium borohydride [36].

Concerns surrounding SEC, however, are that electrostatic effects may be observed in a non-neutral column, giving an uncontrolled recovery of metallo-complexes [1,9]. Furthermore, artefacts may be formed due to interactions between the packing material of the column and the metal or ligand [9]. The chemical conditions of the mobile phase also affect the composition of the complex and may further impact the quality of the results [9]. Other issues with SEC are the lack of standards for analysis, causing the resulting information to be based upon the volume eluted, and is therefore regarded as being strictly qualitative [9,96].

4.2.1.2. Ion Exchange Chromatography (IEC). In Ion Exchange Chromatography (IEC), cations or anions are exchanged between the ionic sites on the stationary phase and the mobile phase [1,5]. This technique is widely used, and can be applied to both ionic and non-ionic compounds, although ligand exchange reactions are needed to resolve non-polar compounds [95]. IEC generally has high separation efficiency, where the retention of the ionic species is determined by pH, ionic strength of the mobile phase and the nature of the ion exchanger [1,95,97]. Properties such as cross-linkage, particle size, and functional groups in the stationary phase resins determine the distribution coefficient, and thus the efficiency of the chromatographic separation [98]. Salt-buffered solutions containing methanol or acetonitrile are commonly used as mobile phases [95]. Separations carried out using higher cross-linked resins possess greater mechanical strength, and experience less swelling in solution than other resin grades, although it should be noted that the ion exchange is not as efficient in these resins [98].

Disadvantages of using this technique are the possible retention of some organic ions due to hydrophobic interactions between the sample and the stationary phase [1,98] and poorer selectivity in comparison to Ion Pairing Chromatography (IPC) [97], which is discussed in Section 4.2.1.3.

4.2.1.3. Reversed Phase Liquid Chromatography (RPLC). Reversed Phase Liquid Chromatography (RPLC) can be used to separate both ionic and non-ionic compounds [1,94]. Separations are quick, efficient and highly reproducible [1]. In this technique the mobile phase commonly consists of aqueous mixtures of methanol, acetonitrile, tetrahydrofuran or heptafluorobutyric acid (HFBA) [1,37,95]. The stationary phase is generally a carbon chain bound to the stationary phase support material and is less polar than the mobile phase solvents [5]. The ion pairing reagent in the mobile phase promotes ionic interaction, which is achieved through both the ionic and organic regions of the analyte [5]. Ionizable analytes interact with the ionic end, and the organic end of the mobile phase interacts with the stationary phase [5]. This allows the analysis to resemble an ion exchange column with the added benefit of being cheaper, having higher resolving power and being more robust than IC columns [5,94]. Columns typically used in this technique are C_8 [37] and C_{18} [38,39] (Table 1).

An adaptation of this technique, Ion Pairing Reversed Phase Liquid Chromatography ICP-MS (IP-RPLC-ICP-MS), can be used for the concurrent separation of anionic, cationic and neutral molecules provided that an appropriate ion-pair reagent is added to the mobile phase [5,38]. Trifluoroacetic acid (TFA) is a useful ion pairing reagent for IP-RPLC; achieving good separations, with the drawback of having long retention times and consequently, poor peak resolution [38]. HFBA, in comparison, is a stronger ion-pairing reagent with better chromatographic resolution than TFA [38].

Poor tolerances to high concentration matrices, and less effective separation than that achieved for IC [5] are disadvantages of this technique. Organic solvents and acids may alter the chemical species of the analyte [1] and the variation of buffer solutions, pH control and the addition of salt to the mobile phase is often required [1,5,95]. Consequently, this technique is limited to strongly bound metals, where covalent element-ligand bonds are preferred [1]. In terms of detection, coupling this technique with ICP-MS may cause carbon loading on the cones and cooling of the plasma, resulting in ionization effects [1,5]. This poses a challenge in analysis both in terms of increased interferences and degraded detection limits [1,5] and will be discussed further in Section 4.2.1.4.

4.2.1.4. Addressing analytical challenges in LC. There are a number of ways to improve the detection limits and reduce artefact formation in liquid chromatographic techniques. These include, but are not limited to, the preparation of the samples in the LC buffer which allows the eluent and organic modifier to be in a diluted state [1,99]. Peltier-cooled spray chambers are also recommended in ICP-MS systems when dealing with organic matrices, as well as the

entraining of small quantities of oxygen into the plasma to burn off excess carbon from the cones [1,5]. The introduction of oxygen into the plasma is not expected to alter the chemical species since the introduction of the chemical species is dependent upon the elution time of the column. However, additional oxygen in the plasma may result in additional polyatomic formation and can also alter the ionization conditions of the plasma which may impact quantification. Lastly, for volatile elements such as Hg, better LODs can be achieved by using alternative techniques such as Liquid Chromatography Vapour Generation ICP-MS (LC-VG-ICP-MS), allowing for direct plasma introduction [99].

4.2.2. Capillary Electrophoresis (CE)

CE is a powerful separation technique with a high resolving power and is commonly employed in the speciation of macromolecules, although the separation of ions possessing different charges is also possible [29,99]. In this technique, different modes of separation such as Capillary Zone Electrophoresis (CZE), Micellar Electrokinetic Capillary Chromatography (MECC), Isoelectric Focussing (IEF), Isotachophoresis (ITP) and Capillary Electrochromatography (CEC) are possible on a single instrument [1,100]. CE techniques only slightly disturb the equilibrium between different elemental species, and the absence of a stationary phase eliminates possible interactions between the stationary phase and the analyte [1]. The coupling of CE to MS is determined by the degree to which the analyte is electrophoretically mobile and the differences in the structure of macromolecules [100]. Dead-volumes on ICP-MS are low; with flow rates of less than 0.5 μL/min [99]. However, techniques such as Electrospray Ionisation (ESI), Ion Spray (IS) and Atmospheric Pressure Ionization (API) are often employed to change the phase to a gas prior to mass spectrometric detection [100]. Mobile phases can be in the form of sodium carbonate and columns such as 60 cm coated fused silica capillary have been reported [29].

Limitations of this technique relate to small sample volume allowances, and ensuring that the sample is adequately homogenous [99]. Detection limits and peak resolution are inferior to those in LC, and alterations to the chemical species may occur due to the application of high voltages [1,29]. The choice of nebulizer is important as those used in coupling CE to ICP-MS often introduce backpressure which may compromise electrophoretic separation by producing laminar flow in the capillary [99]. Since the analyte mobility is dependent upon pH and the strength of the electric field, migration time shifts may also occur with CE analysis, although this is commonly dealt with by introducing internal standards and using the method of standard additions for additional quality control [1].

4.2.3. Gas Chromatography (GC)

Hyphenation of GC to ICP-MS has increased sensitivity and allowed for higher matrix tolerances than that found in GC-MS [101]. Although GC is used to separate volatile species, the species of interest in these applications are commonly not volatile [1,101]. To achieve a gaseous sample, complex sample preparation is required, where this may put the sample at risk of contamination and species loss or alteration [1]. The separation of the analyte species often takes place at elevated temperatures which, when coupling to detectors such as ICP-MS, requires that the transfer line be heated [1]. As an alternative, some studies have coupled GC to other detectors such as AAS or AFS, and this has been found to exhibit promising results [101].

4.3. Detection techniques

4.3.1. Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray Ionization is a sensitive, soft ionization technique for ionisable analytes, which is easily coupled to HPLC [1,102]. Extremely advantageous is the ability of this technique to allow for the conversion of a liquid sample into its gaseous form, thereby ensuring that little of the original species information is lost [103]. It is especially useful for organic solvents which often pose problems in ICP-MS, and is used in the identification of unfragmented molecules, where further fragmentation can be induced and controlled through collisions (Collision Induced Dissociation; CID) [1]. Lead, for instance, can be directly speciated without any previous analyte separation [104]. ESI-MS is often used as a complementary detection method for species evaluation by HPLC-ICP-MS [104–107]. Information obtained by ESI-MS is highly dependent upon the species of the analyte and therefore requires a substantial amount of off-line investigations for quality assurance [100].

In ESI there are a number of important factors affecting ionization and the formation of an appropriate spray. These include the solvent type, flow, composition and type of the capillary, applied potential, distance to counter-electrode, surface tension, analyte and electrolytes in the sample, as well as electrochemical processes at the probe tip [102]. The positioning of the CE capillary also needs to be optimized, where studies by Schramel et al. [100] have shown that the ideal position for the analysis of Se, Cu and Sb species was 0.5–0.7 mm outside the ESI stainless steel tip.

An undesirable effect in this type of analysis is the formation of ion-solvent clusters, where a single species may be split into multiple signals due to the solvent forming a gas and expanding in the vacuum [103]. This, along with electrolytic processes occurring at the tip of the ESI needle, may result in the transformation of chemical species [1]. In comparison to ICP-MS, ESI-MS also has poorer detection limits [106]. Ionization in the presence of salts is inefficient and further degrades these limits [62,106]. For this reason it is often recommended that either SEC or RPLC is run with salt-free buffers, and that the analyte is pre-concentrated through freeze-drying prior to analysis [62].

4.3.2. Atomic Absorption Spectrometry (AAS)

Although Flame Atomic Absorption Spectroscopy (FAAS) offers a cheap alternative to a number of other techniques, detection limits are generally insufficient for the determination of environmentally relevant concentrations of elements [1,28,94]. The addition of matrix modifiers in the analysis of some elements may compromise species integrity and the high flow requirements of such systems also pose a significant problem when coupled with other techniques such as HPLC [1]. For this reason, hydride Generation Gas Chromatography Atomic Absorption (HG-GC-AAS) techniques are often preferred, and are considered to be a valuable method for detecting hydride forming elements such as As [108] and Hg [109].

Graphite Furnace Atomic Absorption Spectrometry (GFAAS), on the other hand, requires only a few microliters of sample, offers low detection limits, is capable of analysing slurries and solids and requires little sample pre-treatment [1,91,94]. Caution in the use of this technique should be exercised to ensure that the samples are representative of the sample population and are homogenous [91]. It is important to note that GFAAS cannot be coupled to HPLC because the data points are generated too infrequently for peak identification [1,94]. For these reasons, the use of AAS systems is uncommon in species characterization studies.

4.3.3. Inductively Coupled Plasma (ICP)

ICP technologies have the benefit of being capable of high sample throughput, multi-element detection, possess a wide linear dynamic range, exhibit long term stability and have greater sensitivity in comparison to many other techniques [110,111]. In comparison to flame techniques, argon plasma techniques (such as ICP) are relatively inert with plasma temperatures reaching 10 000 K; breaking any molecular bonds; including those of refractory compounds which is typically not possible with techniques such as FAAS [1,110].

4.3.3.1. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). ICP-OES is one of the most common techniques used in the analysis of macroelements in plant biomonitoring studies. It is a multi-element technique where most modern instruments allow for simultaneous measurements of emission spectra, and background corrections can be easily applied through the software [1,110]. It is a relatively robust technique and is utilised for a wide range of analytes from numerous applications [112] and is sometimes a preferred technique to ICP-MS in situations where monoisotopic elements are of interest. Most modern ICP-OES systems allow for the optimization of parameters such as plasma, auxiliary and nebulizer gas flows; sampling height; peristaltic pump flows; and RF power, all of which can significantly improve the detection limits of specific elements [110,112].

In terms of environmental analytes, the detection limits of this technique are poor when compared with ICP-MS, although markedly superior to AAS. Interferences arising from many wavelengths of light being emitted simultaneously may occur [112]. Rare earth elements, for example have numerous emission wavelengths and are often poorly quantified at sub-ppm levels unless complex background corrections are applied [112]. The nebulizer and spray chamber needs to be carefully selected for each specific matrix, and argon humidifiers may be used in samples where salt concentrations are high. It is important to ensure that standards and samples are closely matrix-matched in both ICP-OES and ICP-MS analyses, where any deviations may result in signal drift [113]. Poor transport efficiency and tolerance of organic solvents can be dealt with through the use of ultrasonic or glass frit nebulizers [110] and cooled spray-chambers. Solvent loads on the plasma from organic matrices can be further reduced through aerosol thermostating, introduction of a condenser, use of a micro-HPLC column and increased RF power [110].

4.3.3.2. Inductively Coupled Plasma Mass Spectrometry (ICP-MS). ICP-MS is often the favoured analytical technique for speciation studies due to its wide availability, low detection limits, wide dynamic range, versatility and ability to determine isotope ratios, which allow it to be used in source apportionment studies [76]. It is also easily interfaced with LC columns [29]. It is a highly sensitive, multi-element analytical technique, where analyte concentrations can be determined either sequentially (quadrupole/hexapole MS) or simultaneously (double-focussing or magnetic sector MS) [1,5,94].

As a stand-alone technique, ICP-MS can only provide information about the total metal content and not the oxidation state of the metal or its interactions in biological systems including the metal binding sites in biomolecules or its alkalyted form [5,106]. Coupling to HPLC, however, allows for the identification of individual metallic species, which is achieved through the determination of the retention time of species [1]. Further advantages are the allowance of ICP-MS to remove difficult matrices on-line [94]. HPLC-ICP-MS requires samples to be in the liquid form and thus an extraction step is required for non-aqueous samples [7], and so the species of interest should be sufficiently stable so as to avoid species alterations [7,62], as is the case in all speciation analyses. On-line detection systems reduce the chance of species alterations resulting from interaction time of the matrix with the analyte, and contamination in terms of storage and losses [1]. Off-line systems (fraction collection and subsequent delivery to detection), however, allow quality control checks to be maintained throughout the entire separation procedure [1]. To benefit from both of these aspects it is recommended that methods are developed off-line for overall quality control, and then adjusted for the on-line mode [1].

Disadvantages of ICP-MS are that it is not as robust as ICP-OES and has poor tolerance for total dissolved solids (TDS); typically in the range of 0.1–0.2% [113]. High concentrations of acids in samples may also have a negative effect on the lifetime of the sampling cones [1]. When using HF in sample digestion, for example, it is recommended that platinum cones be used [1]. Chloride ions which are present in HCl matrices are not only extremely corrosive but also pose a problem in the analysis of low concentrations of ^{75}As which has a polyatomic overlap of $^{40}Ar^{35}Cl$. Unfortunately, As is monoisotopic and so the use of an alternative isotope is not an option. Correction equations, collision or reaction gases may therefore be applied to reduce or remove this interference [29]. Some instruments have a Collision-Reaction Interface (CRI) which uses modified sampler and skimmer cones to eliminate these interferences prior to mass separation [27] whereas others use a collision or reaction cell prior to the quadrupole/hexapole.

Salt and carbon loading in the plasma from organic extraction media, as one often finds in HPLC techniques, may result in changes in the plasma temperature and consequently affect ionization. Resulting deposits on the cones from these matrices distort the ion beam and degrade the mass resolution of the instrument [105]. Several techniques are employed to reduce this, such as sample dilution, the use of ion-exchange columns, and the introduction of oxygen into the plasma to burn off excess carbon [1]. Physical changes, such as viscosity and surface tension may also affect sample transfer and analysis [1,5].

In the development of an HPLC-ICP-MS method there are a number of parameters which require optimization. The column flow of the HPLC needs to be matched to the sample uptake rate of the ICP-MS, therefore requiring careful selection of the nebulizer and optimization of instrumental parameters [5,105]. Nebulizers in this application are usually concentric, and in the form of high efficiency nebulizers (HEN) as these types have good reproducibility due to improved aspiration in comparison to the crossflow types [1,5,105]. Other types of couplings to ICP-MS are also possible such as CE-ICP-MS. In such cases, the electrical circuit at the end of the capillary and flow rates between the column and the ICP-MS need to be optimized.

5. Commonly assessed metals and metalloids

5.1. Arsenic

Arsenic is a carcinogenic metalloid [64] which occurs in the environment as a result of human activities and naturally through the weathering of arsenic based geological structures [63]. Prolonged exposure to low concentrations of bioavailable arsenic species has been linked to a number of toxic effects, the extent of which is dependent upon the speciation [63,64].

In terrestrial environments, inorganic forms of As are dominant [64], toxic [21,64,84], and competitively inhibit phosphate metabolism [114]. As (V) is most commonly found in soils under oxidising conditions [64], making it the predominant form to be taken up by plants, however it may be reduced to lesser toxic arsenite via plant metabolic processes [25]. As (III), on the other hand is found under reducing conditions [64], has a high affinity for the thiol groups in proteins and consequently inactivates a number of enzymes [114].

Instrumental techniques for the separation of arsenic species have been primarily in the form of HPLC- or Gas Chromatography Hydride Generation Atomic Absorption Spectroscopy (GC-MS-HGAAS), HPLC or GC -ICP-MS, HPLC or GC -ICP-OES [114] or High Performance Liquid Chromatography Hydride Generation Atomic Fluorescence Spectroscopy (HPLC-HG-AFS) [30]. HPLC is the most common of these techniques in the separation of As species, where Ion Chromatography (IC) is mostly used for plant extracts [64]. By coupling these techniques with ICP-MS, detection is completed on-line and is highly sensitive [63,64]. Other separation techniques used in the

determination of non-volatile species are ion pairing liquid chromatography (IPLC) and SEC [63,115]. Due to the variety of As compounds available in nature, adequate separation is sometimes difficult to achieve, where co-eluted peaks may require further separation and identification [64,114].

An example of the use of HPLC-ICP-MS, shown in Table 1 is in the determination of arsenic species in lichens and plants near a decomissioned arsenic smelter site in Austria [22], whilst sequential extractions were used to separate As species in lichens to assess As air pollution in another study [24] (Table 1). Extractions were performed on powdered lichens using water, $CaCl_2$ and H_3PO_4 respectively. It was concluded that biotransformation of As may have occured, where the lichens were thought to methylate the As species as a protective measure.

XAS has also gained popularity in the analysis of As species as it allows for the direct analysis of As species in plant material with little sample preparation [64]. It is also able to provide an indication of the locality of As species within the complex plant matrix [64]. Detection limits with this method, however, are rather poor and are limited to the dominant As species [64]. Furthermore, this technique is not as widely available as ICP-MS and standards are difficult to come by. As shown in Table 1, Bergqvist et al. [28]. investigated the presence of As in carrots, lettuce and spinach using XANES and HPLC-AAS. Organic forms of As were not found in any of the plant species and only inorganic forms were present. The concentrations of As were found to be higher in impacted sites and was linked to higher extractable As in the soils. However, the results from HPLC-AAS and XANES spectrometry did not correlate. Analysis via HPLC-AAS indicated that As (V) was the major form of As in the samples, whereas XANES readings found As (V) and As (III) to be more or less equally represented in the sample, which may be indicative of poor detection limits of HPLC-AAS for this species. Alternatively the extraction procedure used for HPLC-AAS itself may have altered the oxidative species of As in the sample.

Although to a much lesser extent, Capillary Electrophoresis (CE) [29] and on-line speciation using HPLC-HG-AFS [30] have also been explored as alternative methods for As speciation.

5.2. Selenium

Selenium is an essential micronutrient which is toxic at elevated concentrations [18,63] and its uptake from soil is dependent on a number of parameters, including speciation [18]. As shown in Table 1, a number of different methods have been used in the determination of selenium species. A common method of detection is XAS, where short scan times reduce the likelihood of species alteration from redox reactions, and also prevents the volatilization of Se [18]. IPRP-HPLC-ICPMS is also commonly used [39]. In a study by Maneetong et al. [39], the most abundant form of Se in hydrophonically grown kale seedlings was selenomethionine (SeMet) which correlated with the findings by Eiche et al. [18]. In contrast to the latter study, however, Maneetong et al. [39] did not find any major inorganic species of Se. Other identified species were methyl L-selenocysteine (SeMC) and two unknown species. It was also found that these species of Se decreased after 15 days, which was attributed to biotransformation of the selenium species. The study by Eiche et al. [18] further proved that Se in these soils is highly bioavailable and that hyper-accumulation of the metal will occur. It was hypothesized that Se is taken up in the form of selenite and is stored and biotransformed in the root into the organic forms of Se. The two most mobile forms of Se; namely selenite and SeMet; were found to be in highest concentrations in the leaves, thus confirming their mobility. In comparison to concentrations found in the roots and leaves, Se in the stems were low; implying that this part of the plant is merely a passage between the roots and leaves.

Room temperature ionic liquids (RTILs) have been found to be an attractive additive in HPLC columns in the analysis of selenium species, as found in studies such those conducted by Chen et al. [38] which analysed Se species in yeast and clover, where good resolution of Se species could be achieved in 8 min. The RTILs did not alter the pH of the mobile phase and therefore ensured species integrity. No memory effects were observed and an injection volume of only 10 μL was required [96].

5.3. Antimony

The speciation of antimony in plants has also received some attention. Industrial sources of Sb are from mining and smelting where Sb is applied in the production of flame retardants, plastics, textiles, semiconductors and parasite drug treatments [36]. As shown in Table 1, a study by Koch et al. [36] found Sb (V) to be the dominant form of Sb in biota using HG-GC-AAS and headspace HG-GC-MS for quantification. This finding was unexpected as this is the most stable oxidation state of Sb under normal environmental conditions, which should limit its mobility in biological systems therefore there may be some level of biotransformation of the oxidative species by the biomonitor organisms [36]. Antimony (III) and dimethylantimony were also found in moss from this study [36], however, some of the recoveries of Sb using this method were very poor; and varied from 0.7% in *Funaria hygrometrica* to 95% in *Typha latifolia*. This could point to some issues with sampling or extraction; for example, it was thought that the extraction method using methanol and water (1:1) may be inadequate to extract the antimony species. Furthermore, the use of sonication for 20 minutes could result in heat build-up and cause species alterations or losses. Although not considered in this study, the addition of the matrix modifier $NaBH_4$ in the confirmation of methylantimony species using HG-GC-AAS may also have introduced undesired effects on the antimony species present.

5.4. Cadmium

Cadmium commonly enters the environment from the burning of fossil fuels and municipal wastes [116] and is carcinogenic, teratogenic and mutagenic in high concentrations [31]. Its speciation in plants has been investigated both in terms of analytical method development [9] and translocation mechanisms in the plant itself [32]. Due to the biological and biochemical transformations of Cd in plant roots, the bioavailability and toxicity trends in roots are different to those in soil [117]. A study by Ueno et al. [32] identified Cd species using ^{113}Cd Nuclear Magnetic Resonance (NMR). In this study, the inorganic form of free Cd ions was found to be dominant, where 85.7% was present as the free metal, 7.7% was complexed with sulfate, 3.2% was present as a Cd-citrate complex and 0.1% was present as a Cd-Histidine (Cd-His) complex. This implied that the mobility of Cd is not dependent on the ability of the ion to complex with organic ligands, and rather suggests that the transfer of Cd to the xylem is an energy dependant process. It was also surmised that Cd uptake is competitively inhibited by Zn, which is likely due to Cd and Zn following similar metabolic pathways. There is also a close relationship between Fe and Cd homeostasis in plants, where Fe deficiency caused a significant increase in the amount of Cd present in the xylem sap. In this study, the main organic anion present in the xylem sap was citrate, followed by His. Cysteine and gluthathione metal complexes were below detection limits. A study by Połeć-Pawlak et al. [31] found that 89% of cadmium accumulated in the roots and inhibited growth of root hairs. Furthermore, this study found that the majority of the accumulated cadmium was found within the water-soluble peptide fraction [31].

5.5. Lead

Lead enters the environment primarily through the production of lead batteries, paints and gasoline [118]. The toxicity of Pb is well documented and can affect every organ; although the toxic effects are primarily neurological [118]. A study by Schreck et al. [50] investigated smelter activities in battery recycling operations as potential sources of Pb air pollution. Lead containing particulate matter (PM) within and on the surface of leaves were determined using Micro-X-ray Fluorescence (μXRF), Scanning Electron Microscopy coupled with Energy Dispersive X-ray Microanalysis (SEM-EDX) and Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). The species identified within this study were $PbSO^{2+}$, PbO^{+} and $PbCO^{+}$ and Pb^{+} [50].

5.6. Manganese

Manganese is ubiquitous in the environment and is commonly used in the production of steel and aluminium alloys, and has also been introduced into gasoline as a lead replacement [119]. Fractionation of Mn species is used in bioavailability and mobility studies in food crops, such as hazelnuts, as shown in Table 1 [17], where Mn species were determined using HPLC-ICP-MS. Mn was extracted using fractionation methods such as water, diethyl ether, n-hexane and methanol. In this study, most of the Mn present was found to be associated with proteins (54–87%) with a small component associated with oils.

5.7. Nickel

Nickel is commonly assessed in metal speciation studies. It is released into the environment through activities such as battery production, electroplating, development of alloys and colouring of ceramics [120]. A study by Alves et al. [35] determined the speciation of Ni in xylem sap using Square Wave Voltammetry (SWV), where citric, oxalic, malic and malonic acids were found to have the greatest effect on species distributions. Nickel has also been assessed in hyper-accumulating plant species, *T. goesingense* and *T. arvense*, using XANES [33]. It was found that 87% of the Ni which was not bound to the cell wall was chelated by citrate, and thus confirmed that Ni-organic complexes play an important role in Ni tolerance mechanisms. This study also found that 25% of intracellular Ni was bound to histidine, and this bond was thought to assist the movement of Ni from the cytoplasm into the vacuole. At low pHs it was found that Ni-His complexes were destabilized and resulted in Ni ions favouring complex formation with organic acids such as citrate. It was noted that the accumulation of Ni by a non-accumulator takes place in the cytoplasm, and often causes the death of the plant [33].

Nickel speciation has also been assessed in a metal hyper-accumulating tree species, *Sebertia acuminate*, which is endemic to New Caledonia [34]. This study by Schaumöffel et al. [34] used complementary techniques such as SE-HPLC-ESI-MS-MS and CZE-ICP-MS to accurately quantify various Ni species at both the molecular and elemental level. Stable nickel compounds such as nicotianamine and nickel citrate complexes were identified along with 5 other stable complexes, implying that this technique cannot be used for the identification of non-stable complexes. It was also found that a combination of these methods was required for adequate separation and identification of nickel species. This study recommended that future studies involve a Quadrupole Time of Flight Mass Spectrometer (QTOF-MS) or ESI-MS for more sensitive and accurate measurements of less concentrated Ni complexes. Similar experiments were conducted by Vacchina et al. [4], regarding a different plant species, *T. caerulescens* [4] using SE-HPLC-ICP-MS and SE-HPLC-ESI-MS. It was found that metal complexes could be identified through the complementary use of HPLC-ICP-MS, CZE-ICPMS and ESI-MS-MS. Results from CZE-ICP-MS showed that the nickel species present was Ni (II) and a second unidentified nickel peak was thought to be a stable nickel complex. This complex was further characterized using ESI-MS-MS and was found to be a previously unreported nickel compound which is produced by this plant assumedly in response to nickel stress.

6. Conclusions and future outlook

It is clear that the application of plant speciation studies is extremely important in a number of fields, however the accurate quantification of metallic species is a complex and challenging task. Quality control is critical in speciation analyses, which highlights the use of certified reference materials (CRMs), whenever possible, to improve reliability of analytical results. In the absence of a suitable CRM, physically different methods should be employed for comparison.

Future directions in metal speciation studies are likely to focus on the development of improved sample preparation and analytical techniques so as to ensure that the original species profile is not altered. Literature reports on speciation of metals in plant materials have focused on a limited number of elements, primarily As, Cd, Mn, Ni, Pb, Se and Sb. In view of the importance of speciation studies highlighted here, future studies are likely to focus on additional elements which are of toxiciological and environmental relevance. Moreover, speciation studies are also likely to become widely applied in the evaluation of food commodities with plant origins; the biofortification of desired chemical species in food crops; and is also likely to find a place in the development of pharmaceuticals since bioavailability and metabolic pathways are dependent upon chemical speciation.

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Chapter 4 Experimental Methods, Results and Discussion

This chapter outlines the materials and experimental methods for the determination of metal(loid)s in a lichen matrix, first through an initial assessment of the choice of biomonitor organism as a reflection of atmospheric metal(loid) concentrations, followed by site comparisons of total metal content in relation to meteorological parameters and then speciation studies and method development, with a focus on arsenic. In each case information is reported in accordance with the format of the journal to which it was submitted.

In terms of sampling sites in the first study (Section 4.1), two accessible sites were chosen with sufficiently different impacts. The sampling at the University of Johannesburg (UJ) took place in a parking lot and the sampling at Waterval Boven (WB) took place on private land with the owners' permission. The aim of this study was to determine whether or not the lichen, Parmotrema austrosinense was a suitable lichen species for being able to demonstrate differences in metal(loid) contents between sites with different sources and magnitudes of impacts. Due to the bias which could be introduced in providing an assessment of air quality from the direct impact of car traffic in the parking lot at UJ and the lower abundance of lichens at the WB site, as well as the inability to gain further permission from the land owner to sample in this area, other sites were investigated for subsequent studies..

Three sites with sufficiently different impacts, namely Cape Point as a relatively unimpacted reference site, Johannesburg (JHB) Botanical Gardens near the JHB central business district (CBD) as an urban site, and a private farm in the Waterberg Mountain range which was adjacent to a number of cattle and agricultural farms as well as a conservation area (Marakele National Park) were identified for further evaluation. The Cape Point site was only used for a total metal comparison study (Section 4.2) since it is a conservation area and the sampling permit limited sample quantities. The urban site was used for method development for the sequential extraction study (Section 4.3). The urban and rural sites were used for method development for As speciation in lichens (Section 4.4) and the development of a combined chromatographic separation and sequential extraction method (Section 4.5). The key focus of Sections 4.3-4.5 was method development, where the developed method was then applied to sites with different impacts to determine whether or not differences could be observed and could be ascribed to differences in sources between sites. This will serve as a basis for future studies regarding metal fractionation patterns in air with the use of the lichen biomonitor, Parmotrema austrosinense.

The certified reference materials (CRMs) used in the various studies in this chapter are provided in Appendix A.

4.1. Conference Paper 1

This work is a short study which evaluated the suitability of the foliose lichen, *Parmotrema austrosinense* (Zahlbr) Hale, as a biomonitor of air pollution and is written in the style of the conference it was presented at as a poster (Appendix C) where it received second prize for the student poster in the category “Mass Spectrometry”.

Kroukamp E, Wondimu T. Forbes P. 2014. Preliminary investigation into the use of lichens as biomonitors of air pollution in South Africa. *Analitika 2014 Conference*, 7-11 September 2014, Parys, South Africa.

Preliminary investigation into the use of lichens as biomonitors of air pollution in South Africa.

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Introduction

Air pollution is known to adversely affect human health. It is therefore imperative to monitor the presence of pollutants, including heavy metals, in air resulting from motor emissions (Giordano *et al.* 2010), consumption of fossil fuels, industrial processes and mining, among other anthropogenic activities. The determination of metals in air pollution is commonly done through the use of theoretical dispersion models and *in situ* measurements of air pollutants (Sloof 1995), the latter often requiring the use of expensive equipment for collection.

Economical options have been explored for this type of monitoring, where biomonitors, such as lichens, are deemed as valuable alternatives to traditional air filtering techniques and have been shown to accurately reflect environmental concentrations of a number of pollutants including heavy metals (Branquinho *et al.* 1999, Jeran *et al.* 2007, Forbes *et al.* 2009, Koz *et al.* 2010, Pawlik-Skowronska and Bačkor 2011, Demiray *et al.* 2012, Kularatne and de Freitas 2013). Since lichens lack a cuticle, they allow uninhibited movement of metals from the external atmosphere into the intracellular spaces within the plant structure (Branquinho *et al.* 1999). In the case of particulate contaminants, metals do not accumulate in the cell wall and are instead accumulated in the thallus (Brown 1987, Koz *et al.* 2010, Kularatne and de Freitas 2013) which reaches equilibrium with concentrations in the air (Sloof 1995, Kularatne and de Freitas 2013). There are a number of different factors which may influence the accumulation of heavy metals by lichens, where these include the lichen species, sampling height, tree species, climate and wind, and need to be factored in when making comparative assessments.

In this study we aim to investigate the accumulation of metals in lichens at two sites in South Africa with varying degrees of potential environmental impacts, which were chosen due to ease of accessibility and the high level of impacts in each area which were of differing types. In doing so we aim to determine if lichens can be used as biomonitors of air pollution by metals in South Africa. Methodological considerations will also be assessed.

Methodology

Site Description

Waterval Boven (WB, also known as Emgwenya, GPS co-ordinates: 25.674057 S, 30.364525 E, Fig. 1) is situated in Mpumalanga on the edge of the escarpment in South Africa. Surrounding areas between Machadodorp and Carolina contain a quarry and three mines comprised of a coal, nickel and chrome mine (Cockburn 2013, McCarthy and Humphries 2013). The town of Waterval Boven is 1423 m above sea level and experiences high rainfall from late spring to late summer (November-March) with a mean annual precipitation (MAP) of 851 mm rainfall (Climate

2019a). Maximum temperatures for summer and winter are 27 °C and 18 °C respectively, and minimum temperatures are around 14 °C and 3°C respectively.

The University of Johannesburg (UJ, GPS co-ordinates: 26.186530 S, 27.996949 E) is in the City of Johannesburg (Fig. 1) in Gauteng, South Africa. As with many large cities it has high population and traffic densities, but also has a number of industries including smelting, coal mining, scrap production, mine dumps, coal fly ash, iron and steel production, gold mining activities, crematories, medical waste incineration, combustion of fossil fuels and gas works in close proximity to the site. Johannesburg is 1757 m above sea level and experiences high rainfall from late spring to mid-summer (November-January) with a mean annual precipitation (MAP) of 790 mm rainfall (Climate 2019b). Maximum temperatures for summer and winter are 27 °C and 17 °C respectively, and minimum temperatures are around 13 °C and 3°C respectively

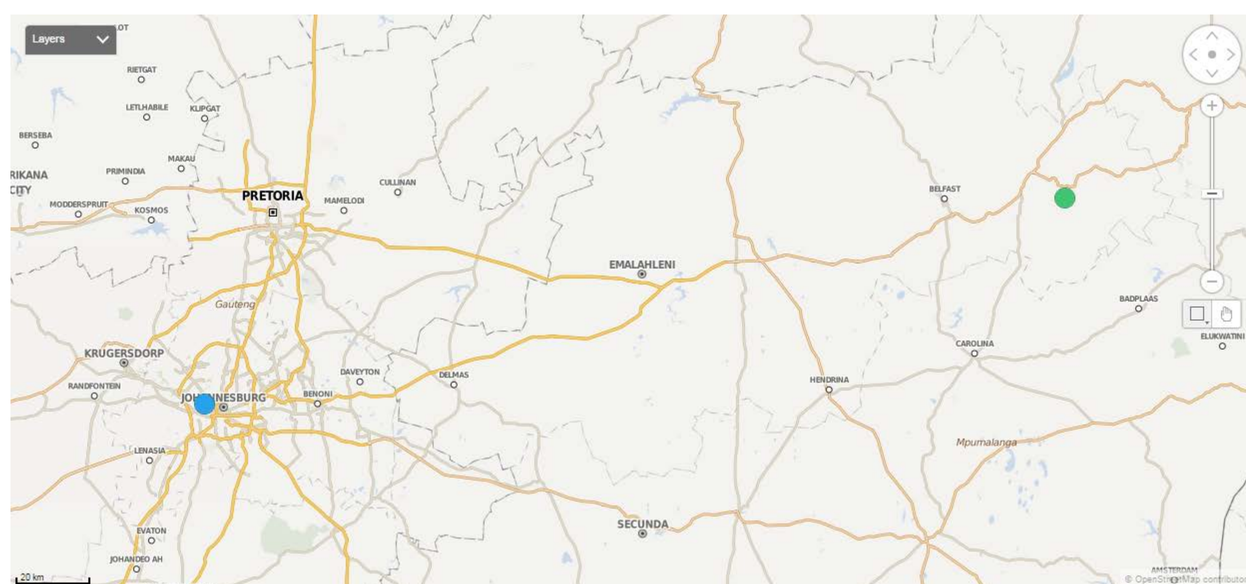

Fig. 1 Map showing sampling sites at the University of Johannesburg (blue dot) and Waterval Boven (green dot) graphed with Spotfire™ Software (TIBCO).

Sample collection

Healthy lichen samples, *Parmotrema austrosinense* (Zahlbr) were collected from trees of the UJ (n=21) and Waterval Boven (n=13) sites, carefully cleaned by removing any attached substratum while ensuring that the whole lichen remained intact. Afterwards, each individual lichen thallus was rinsed using deionized water, and dried in an oven at 50 °C for 8 hours (Pawlik-Skowronska and Backor 2011). For each lichen sample the entire thallus was then accurately weighed using an analytical balance (Mettler Toledo, XP 205) and individually digested using a method adapted from the Milestone Application Note for lichen digestions (HPR-FO-55, Milestone 2014), where the HNO_3 volume was increased to accommodate requirements of the microwave digestion system being used (CEM Mars 6). The digestion took place using 10 mL of HNO_3 (Suprapur®, 65 %, Merck) and 1 mL of H_2O_2 (Suprapur, 30%, Merck) (Pawlik-Skowronska and Backor 2011, Mendil *et al.* 2009), whereafter the samples were gravimetrically diluted (50 x) using deionized water. Calibration standards (n=9) for each element were prepared from individual stock solutions of 1000 mg/L (Merck), matrix-matched to the samples (using the same type and

concentration of acid) and gravimetrically diluted. Gallium, Ru and Re were chosen as internal standards for the analysis in order to appropriately represent the mass range and ionization efficiencies of the analytes being studied, and was added to all blanks, standards and samples prior to dilution. The certified reference material (CRM) DOLT-4 (Dogfish liver, NRC-CNRC) was evaluated for quality control to ensure the accuracy of the method of analysis and validity of the calibration standards, where another CRM was not available at the time. The CRM digest and blanks were spiked with a low concentration (10 µg/L) and high concentration (50 µg/L) multi-element standard to verify the analytical results obtained. The samples were analysed first using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Spectro Arcos, Spectro Analytical Instruments). Elements which were below the detection limits of the ICP-OES were analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NexION 300X, PerkinElmer Inc.) using the conditions outlined in Table 1.

Statistical analyses

Statistical analyses took place using one-way ANOVA and appropriate *post hoc* tests. All statistical analyses took place using the statistical program SPSS 22.0 with significance set at the 95% confidence interval ($p < 0.05$).

Table 1: Experimental conditions for the ICP-MS

Condition	Name/value
Nebulizer	Meinhard® glass microconcentric
Spray Chamber	Quartz baffle cyclonic
Injector	Quartz 2.0 mm
Triple Cone Interface	Nickel/Aluminum
Plasma Gas Flow	18 L/min
Auxiliary Gas Flow	1.4 L/min
RF Power	1500 W
Replicates per Sample	3
Dwell Time	20-200 ms
Mode of Operation	Standard & Collision (using He gas)

Results and Discussion

As shown in Fig. 2a and Fig. 2b, Cu, Zn, As, Pb, Mo and B were found to be significantly ($p < 0.05$) higher in the University of Johannesburg site in comparison to the Waterval Boven site and could originate from the numerous anthropogenic activities in the area, where some point sources could include scrap production (source of Cu; USEPA 1985), coal fly ash, iron and steel production (sources of Zn; ATSDR 2011), gold mining activities,

crematories, medical waste incineration (sources of As; USEPA 1998), combustion of fossil fuels (source of Mo; TDSHS 2012) and gas works in close proximity to the site.

Since mining stock piles are often susceptible to drying, metals present in the fine, mined dust near the Waterval Boven site can be dispersed by wind (Branquinho *et al.* 1999, Sloof 1995) and taken up by lichens. The presence of these dusts may explain the significantly higher ($p < 0.05$) concentrations of Al, V, Cr, Fe, Co and Se found in lichens from this site. High standard deviations for the samples can be explained by analysis taking place on individual lichen sample, where depending upon orientation to prevailing winds the concentrations could vary greatly. Future studies should rather pool samples per site to ensure that more representative data is acquired.

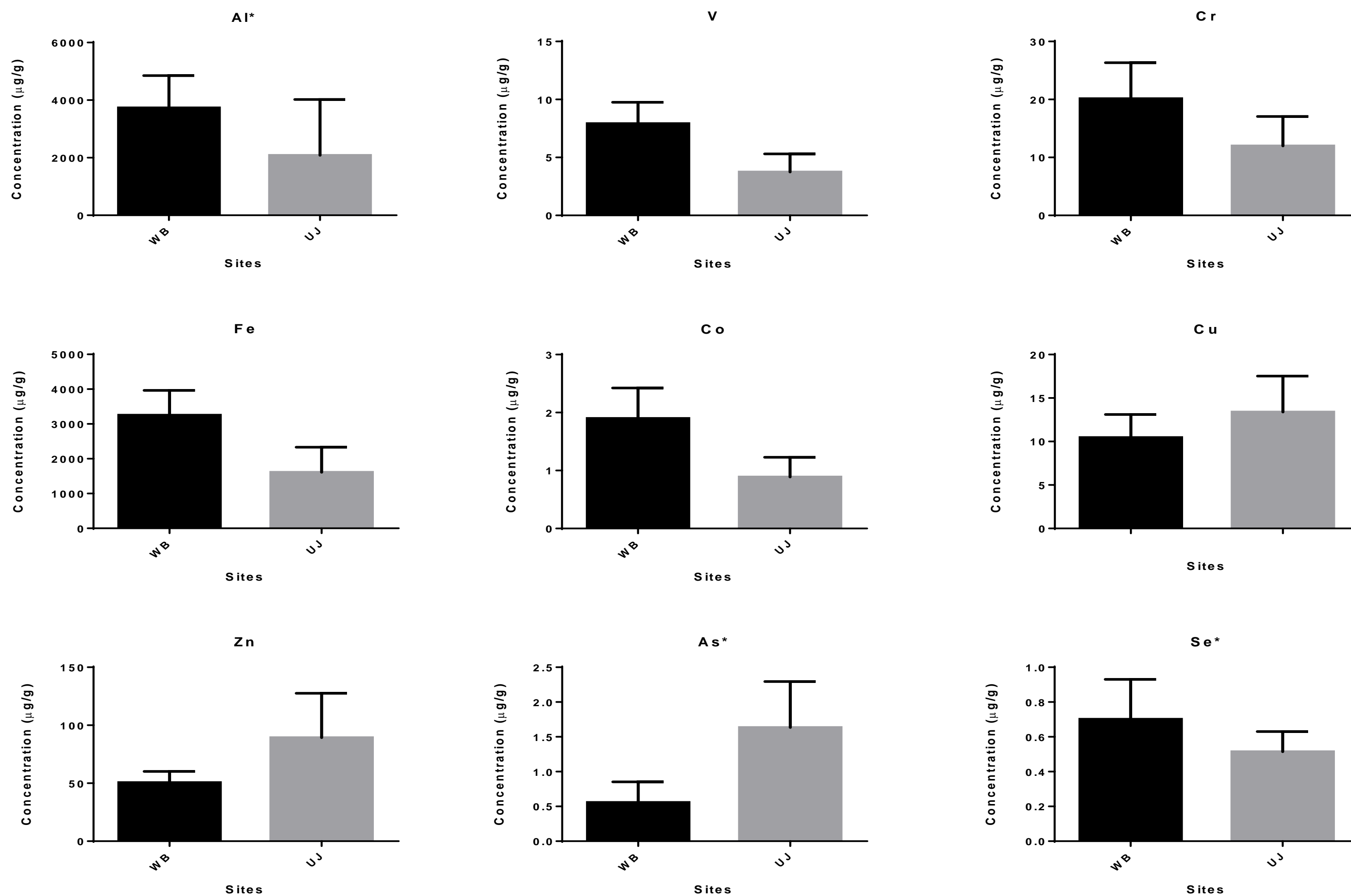

Fig. 2a Concentrations of metals which showed statistically significant differences (*p<0.05*) between the University of Johannesburg site (UJ) and the Waterval Boven Site (WB) where * denotes sites from which datapoints which were 20% out of the calibration range were removed

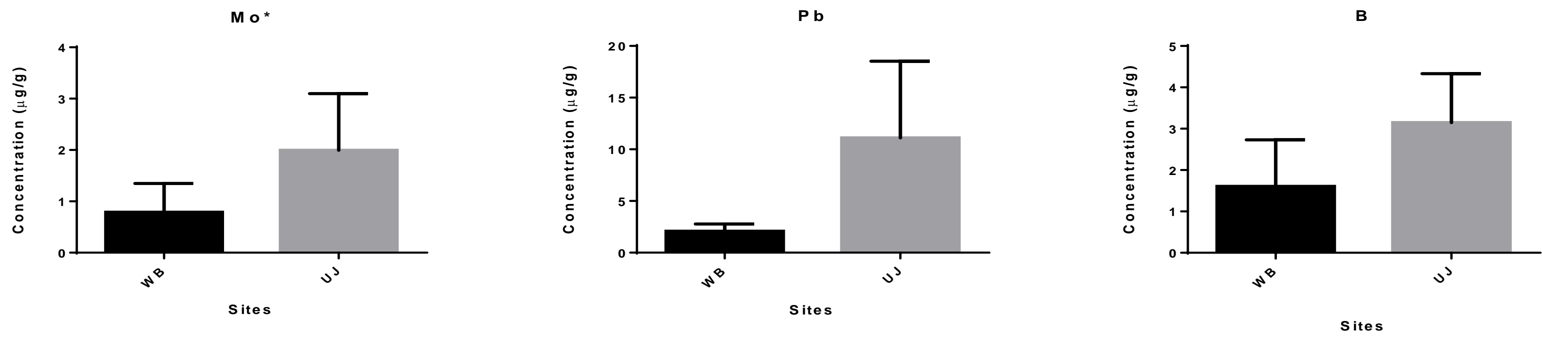

Fig. 2b Concentrations of metals which showed statistically significant differences (*p<0.05*) between the University of Johannesburg site (UJ) and Waterval Boven site (WB) . where * denotes sites from which datapoints which were 20% out of the calibration range were removed

Spike recoveries were within an acceptable range (USEPA 1987) for most elements (Fig. 3), although higher than expected recoveries were found for As, Se, Rb, Sr and Mo in the 50 µg/L spike of the DOLT-4 CRM, presumably due to carbon enhancement effects of this matrix (Larsen and Stürup 1994). As and Se spike recoveries were low in the 10 µg/L spikes of the CRM matrix, where this is likely to be due to a top-weighed calibration, causing inaccurate results at lower concentrations. These results show that the lichen matrix does not have a significant effect on the accuracy of the analytical results. All CRM recoveries were between 90-110% except for Ni which was recovered at 79% and Se which was recovered at 117%, where the latter is likely due to carbon enhancement effects of the DOLT-4 matrix. The good CRM recoveries of this analysis prove the analytical accuracy of the method (Fig. 4). All internal standard recoveries were within an acceptable range of between 60-125% (Fig. 5) of the original blank response reading (USEPA 1992) although long term drift was observed over the course of the sample run.

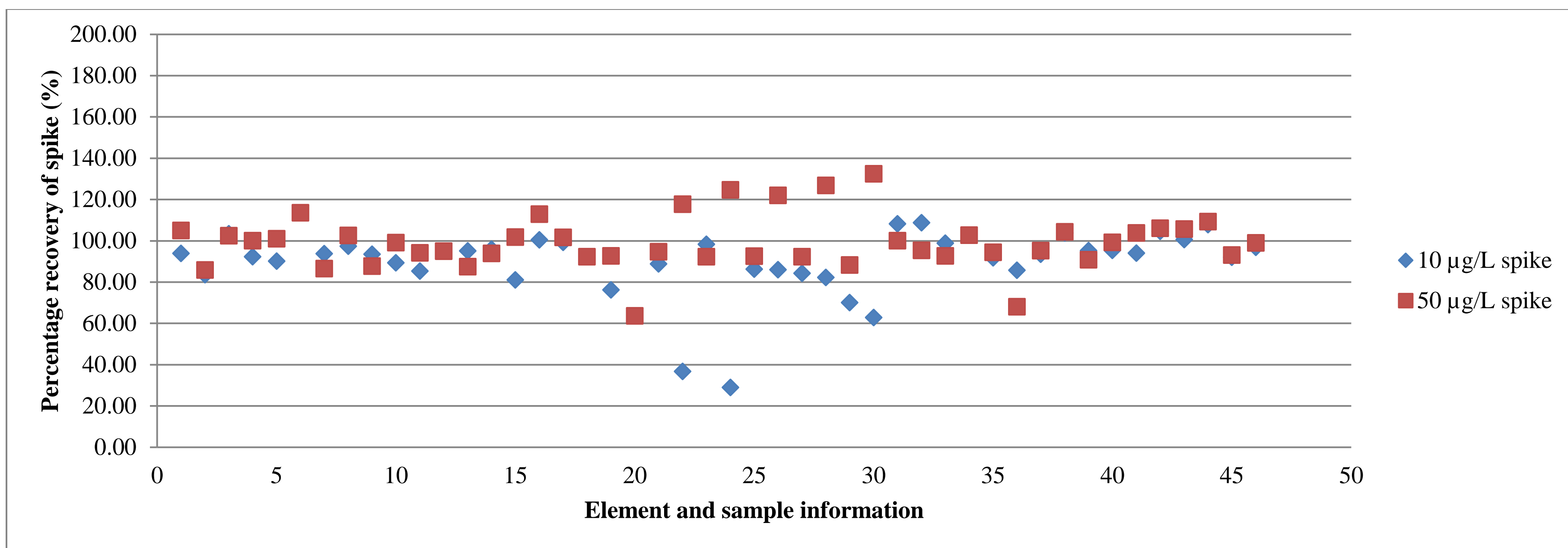

Fig. 3 Spike recoveries (%) of simple (spiked blank) and complex matrices (CRM DOLT-4)

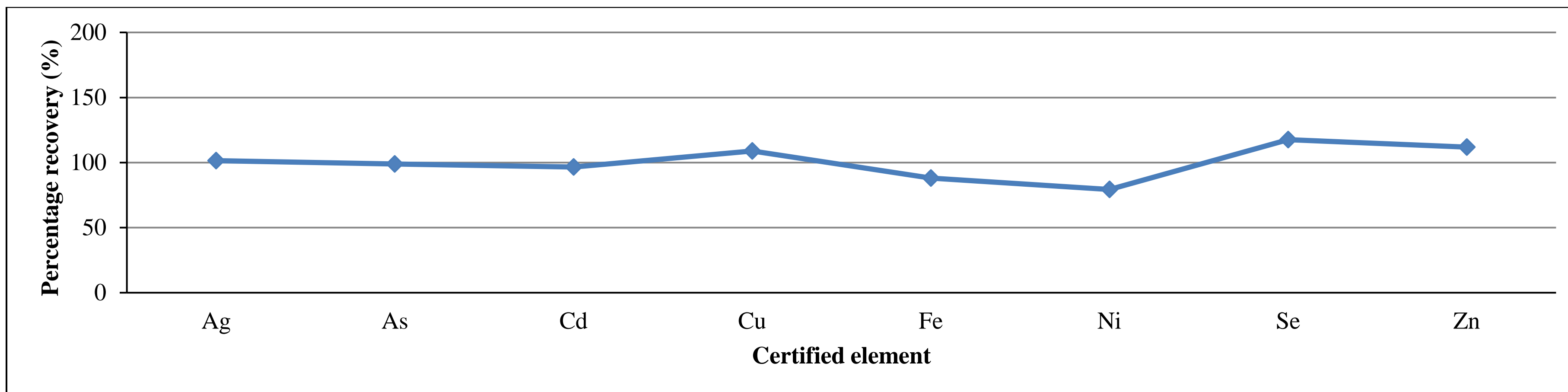

Fig. 4 Percentage recoveries of the certified reference material DOLT-4

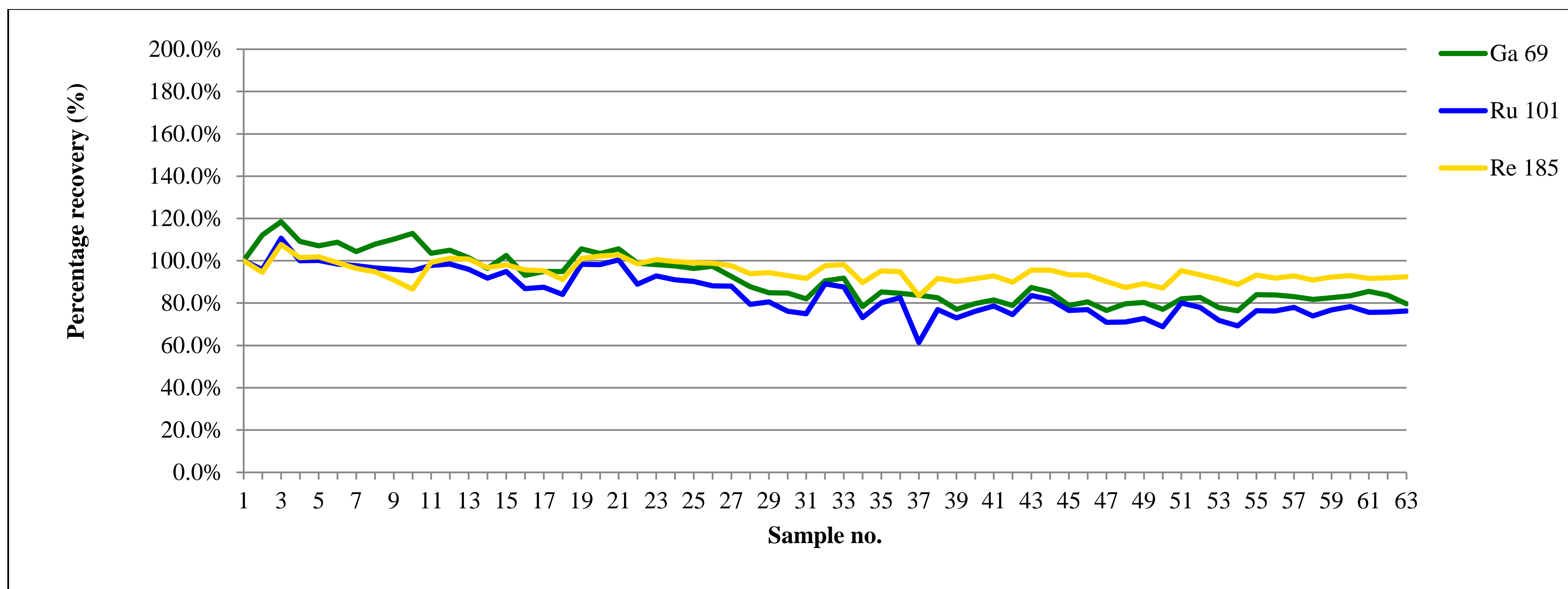

Fig. 5 Internal standard recoveries (%) over the sample run

Conclusion

The findings from this study support the use of lichens as sensitive monitors of air pollution by heavy metals in South Africa, where levels of metal(loid)s in lichens appeared to correlate with those mined or emitted near the chosen sites. Lichens were found to be an affordable alternative to conventional air-filtration methods and were widely dispersed at both sites, indicating its fitness for purpose in South African air biomonitoring studies. Recommendations for future work are to pool the sample per site together to ensure a homogenous and representative sample is provided.

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4.2. Paper 2

This paper was formatted in accordance with the journal *Environmental Monitoring and Assessment,* a Springer journal. At the time when this PhD was submitted for examination, this paper was under review.

Kroukamp EM, Godeto TW, Forbes PBC. Spatial study of atmospheric metal concentrations in South Africa using the lichen biomonitor, *Parmotrema austrosinense*. *Environmental Monitoring and Assessment*. *Under review.* Impact Factor 1.804. *DOI pending*.

Spatial study of atmospheric metal concentrations in South Africa using the lichen biomonitor, *Parmotrema austrosinense*

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Key words:
Air pollution
Biomonitoring
Cape Point air quality
Johannesburg air quality
Lichen
Waterberg air quality

Abbreviations:

GAW	Global Atmosphere Watch
JHB	Johannesburg
KNP	Kruger National Park
LOD	Limit of Detection
LOQ	Limit of Quantification
MMT	Methylcyclopentadienyl manganese tricarbonyl
PGMs	Platinum Group Metals
SD	Standard deviation
WMO	World Meteorological Organization

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Abstract

Lichens are widely accepted biomonitors of air pollution. In this study, lichens *Parmotrema austrosinense* (Zahlbr.) Hale, were collected from three different sites within South Africa which had different anthropogenic impacts and climatic conditions, namely Cape Point Nature Reserve, Johannesburg Botanical Gardens and a farm in the Waterberg Mountains. Bulk lichen material was air dried, powdered, digested and analysed using ICP-MS for Al, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Se, Sn, Th, Tl, U and Zn. Of these analytes, statistically lower concentrations of V, Cr, Mn, Cu, Cd and Pb were found in lichens from the Cape Point site than in lichens from the Johannesburg and Waterberg sites, whereas concentrations of Al and Zn were only significantly lower than the Johannesburg site. These findings confirm the appropriateness of Cape Point as an atmospheric reference site. Concentrations of Ni were significantly higher in lichens from the Cape Point site in comparison to both other sites, and this was thought to be from both natural and anthropogenic sources. As can be expected, the urban-impacted Johannesburg site had significantly higher concentrations of Al, V, Cr, Cu, Zn, Cd and Pb than both other sites. Manganese concentrations, however, were statistically higher in lichens from the Waterberg site than in lichens from both the Cape Point and Johannesburg sites, in agreement with geological information. The findings from this study show, for the first time, that lichens are useful biomonitors for elucidating differences in atmospheric metal contamination between different regions of South Africa.

Introduction

Air pollution is a growing concern throughout the world. Unlike other forms of pollution, which tend to be more localized, air pollution expands far beyond the source boundaries (Adamo et al. 2003; Fernández-Espinosa and Ternero-Rodríguez 2004). Particulate matter is often small enough to be carried by the wind and can travel long distances, being deposited into waterways, onto land and the surfaces of plants, and can even enter the respiratory passages of terrestrial mammals (Patrick and Farmer 2007). Human populations in particular have been shown to experience acute and chronic toxic effects as a direct consequence of air pollution, where these effects are more pronounced in people living in industrial and densely populated urban areas in comparison to their rural counterparts (Brugha et al. 2018; Shahadin et al. 2018). Therefore, the monitoring of air pollution in relation to potential sources is extremely important.

Biomonitoring is a powerful form of environmental monitoring, which utilizes animals or plants to provide an indication of surrounding pollutant concentrations (Forbes et al. 2015). Epiphytic lichens are widely used biomonitoring organisms, forming the backbone of a number of national air-quality biomonitoring initiatives (USDA 2004; SOER 2009). Some of the toxicants commonly monitored in such initiatives are heavy metals, where concentrations within the lichen thallus have been directly linked to environmental concentrations (Bari et al. 2001). Lichens have also been shown to retain a number of elements for 2-5 years (Walther et al. 1990) and, provided *in situ* lichens are used, allow for a comprehensive evaluation of atmospheric conditions over extended periods of time.

Some additional characteristics making lichens suitable air-biomonitoring organisms, are the absence of a root system and cuticle (Branquinho et al. 1999; Adamo et al. 2003). Since there is no cuticle, metals deposited on the lichen surface easily cross the 'atmosphere-lichen boundary', and hyperaccumulate at concentrations higher than their surroundings (Loppi et al. 2003). Lichens also rely solely upon the air as a source of nutrients, and therefore any concentrations of metals found within the lichen thallus are a direct consequence of their presence in air (Bargagli et al. 2002; Bačkor and Loppi 2009). Moreover, lichens are easy to collect, sentinel, slow growing and found in a variety of different habitats, all of which are important features of an appropriate biomonitor (Jeran et al. 2007; Forbes et al. 2009; Demiray et al. 2012). Foliose lichens, in particular, are often chosen for metal biomonitoring assessments due to their propensity to assimilate higher concentrations of metals than some of the other thallus forms (Glenn et al. 1991), and the ease of their removal from the substrate.

Factors affecting the uptake and detoxification of heavy metals by lichens are humidity, precipitation, temperature, proximity to the source, predominant wind direction in relation to the source, pH and season (Bačkor and Loppi 2009). Metal metabolism can also play a significant role in detoxification mechanisms, and may account for the high metal concentration tolerances which some lichen species have been found to exhibit (Rola et al. 2016). Metabolic processes are thought to deal with metals in lichens through the sequestration of heavy metals into oxalate crystal deposits on the lichen surface (Sarret et al. 1998) and/or complexation of metal(loid)s with lichen acids (Branquinho et al. 1999). Comparative assessments should therefore aim to use the same species of lichen, thereby reducing bias which may occur due to the use of different lichen species with differing metabolic processes.

The use of lichens in the monitoring of atmospheric metal concentrations in South Africa is poorly researched, with only a few reported studies to date which have covered a few metals at individual sampling locations (Panichev and McCrindle 2004; Forbes et al. 2009). This study aims to address this paucity by providing a wide range of atmospheric metal concentrations present in the foliose lichen species, *Parmotrema austrosinense,* Hale (*P. austrosinense*), which is a foliose lichen species native to Southern Africa and is widely distributed throughout the area. It has been used as a biomonitor of air pollution in two previous studies within South Africa (Forbes et al. 2009; Van der Wat and Forbes 2015) and in an investigative study on sample preparation (Kroukamp et al. 2017). Moreover, it has been shown to be reflective of environmental concentrations of metals (Kroukamp et al. 2014), making its use appropriate in this study. Three different sites with varying climates and different sources of metals in air were evaluated and compared. Seasonal wind and climate data was also provided, thereby giving supporting information to the conclusions drawn.

Materials and methods

Site description

All sampling sites were located in South Africa (Fig. 1) and are discussed in detail in the sections below.

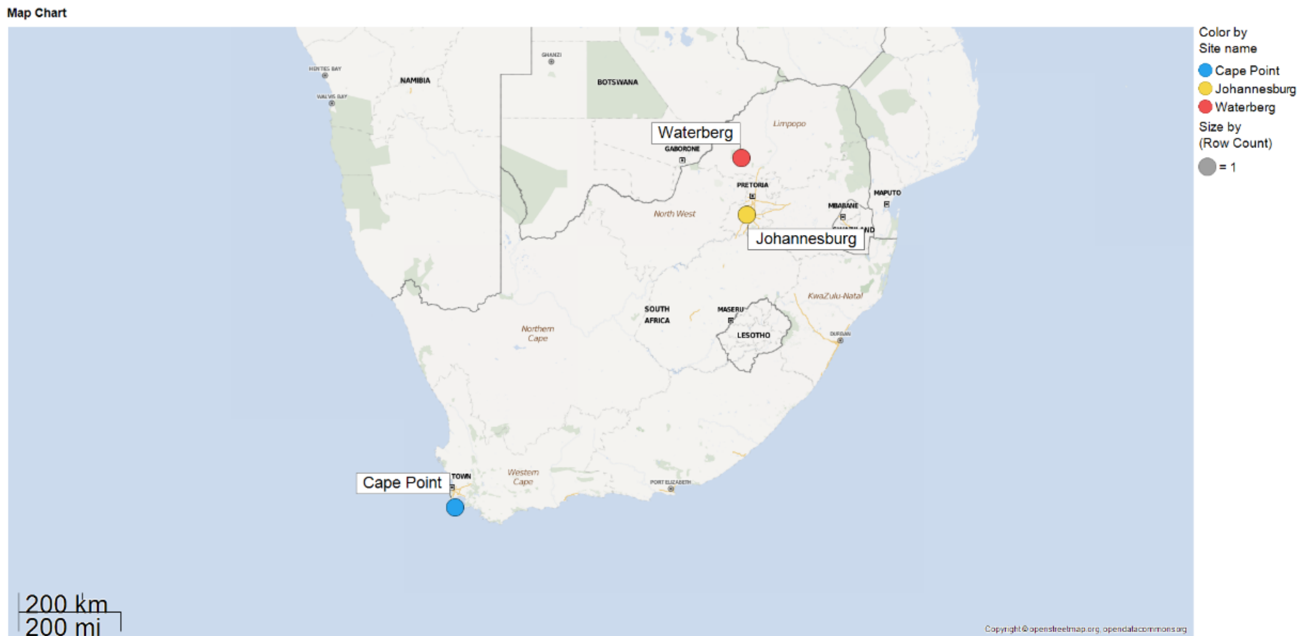


Fig.1 Map of lichen sampling sites: Cape Point, Johannesburg and Waterberg mapped using Spotfire^{TM} software

Cape Point

Cape Point (GPS co-ordinates 34.35558 S, 18.49468 E) lies on the outskirts of Cape Town city at the southern tip of South Africa. It has a unique microclimate due to the warm Agulhas current of the Indian Ocean to the East, and the cold Benguela current of the Atlantic Ocean to the West. Cape Town typically has hot and dry summers and wet and cool winters, with the rainy season starting in April and running until September (Davidge 1978). Lichens within this area grow predominantly on Fynbos, which refers to a group of evergreen shrubs unique to South Africa (WWF 2017). Winds within this area are often high, and facilitate the distribution and deposition of sea-spray onto surrounding vegetation. Lichens were collected on 27 October 2016 from five locations along the point which were positioned according to which ocean it faced, and the degree of wind protection. The locations were as follows: Location 1 (GPS co-ordinates 34.35338 S, 18.48963 E), Location 2 and 3 (GPS co-ordinates 34.35326 S, 18.49006 E, close together, GPS co-ordinates did not differ), Location 4 (GPS co-ordinates 34.354936 S, 18.49352 E) and Location 5 (34.35558 S, 18.49468 E). There are few anthropogenic impacts on Cape Point air quality due to its distance from urban and industrial activities. It is also not close to any other land masses and has been identified by the World Meteorological Organization (WMO) as a suitable Global Atmosphere Watch (GAW) baseline station (Brunke et al. 2016).

Johannesburg

Johannesburg (JHB) is the largest city in South Africa, having the most expansive man-made metropolitan forest in the world, and a population of over 4.4 million residents who are distributed over an area of 1645 km^2 (WPR 2018). Johannesburg is located on the escarpment, experiencing rainfall in the summer months between December and February, and has cold, dry winters. This site was located close to a main arterial road which links the suburbs to the city centre (6 km away) and consequently has high traffic volumes throughout the year. Impoverished communities within the city and surrounding areas use wood, petroleum, oil, coal and, in some cases, car tyres to fuel fires for cooking and heating households. The outer edges of the city lie along a gold belt known as the 'Witwatersrand', and is home to a number of industries and mines including steel, brass, aluminium and gas works, and gold and coal mining. All of the aforementioned factors contribute towards the air pollution in Johannesburg and the surrounding areas. Samples to represent this urban setting were collected on 1 December 2015 from a variety of different trees in the Johannesburg Botanical Gardens (GPS co-ordinates 26.159211 S, 28.001003 E).

Waterberg

The Waterberg Mountain range is located in the Limpopo Province where the predominant vegetation is mountainous savanna (Codron et al. 2005). The annual rainfall within this area is usually > 600 mm, with the rainy season occurring during the summer months between October and March (Codron et al. 2005). Samples were taken from a variety of different

trees within a game farm in the area on 7 March 2016 (GPS co-ordinates 24.488 S, 27.8377 E), at 50-100 m away from a dirt road within the farm. Direct anthropogenic impacts in this area are predominantly in the form of agriculture, cattle and game farming. Further afield is Thabazimbi, a mining town (50 km away), where mining activities in areas surrounding the sampling site include platinum, haematite and clay mining.

Meteorological data collection

Comprehensive meteorological data such as rainfall, temperatures, humidity and wind direction were obtained from the South African Weather Service for the nearest weather stations to the sample sites, namely Cape Point Weather station (GPS co-ordinates 34.3530 S, 18.4890 E, elevation 228 m), Johannesburg Botanical Gardens Weather Station (GPS co-ordinates 26.1560 S, 27.9990 E, elevation 1624 m) and Lephalale Weather Station (GPS coordinates 23.6760 S, 27.7050 E, elevation 839 m). Seasonal results were averaged for the 3 years prior to sampling for rainfall, temperature and humidity. Seasons were defined as stated by the South African Weather Service (SAWS 2018), with Spring being 1 September – 30 November, Summer being 1 December- 28/29 February, Autumn being 1 March-31 May and Winter being 1 June-31 August. Wind directions were summarized into number of occurrences per season for the 3 years prior to sampling based upon 3 readings per day (morning, noon and evening), although it should be noted that not all sites had data available for every day.

Sample collection, preparation and analysis

Lichens were sampled between late-spring and early autumn at the different sites where sampling took place just after or during the rainy season at each site (2015-2016). Sampling took place at a distance of >100 m away from tar roads, from all around the tree and between 2-4 m above ground level to prevent bias in the results due to automobile pollution, prevailing winds, and to avoid the stemflow and "soil backsplash" zones respectively (Monaci et al. 2012; Kroukamp et al. 2017). Collection took place at least 5 days after a major rain event so that the loading of metals onto the lichen surface had been regained (Gailey et al. 1985; Brown and Brown 1991; Kularatne and de Freitas 2013). Lichens were collected between 11:00 and 14:00 each day when lichens are less metabolically active (Hutchinson et al. 1996), and to prevent any bias which may occur due to dilution from morning dew on the lichen surface. Lichens were removed from the substrate using plastic tweezers and nitrile-gloved hands. Whole thalli samples were collected throughout each location to ensure that the population was adequately represented (Bargagli et al. 1987; Madrid and Cámara 1997; Gräfe et al. 2014). Unrelated materials, such as bark, were removed prior to storage, thereby preventing contamination, and samples were stored in acid-washed polypropylene bottles until processing.

Samples were cleaned using plastic forceps under a magnifying lamp at the laboratory and patted to remove any remaining dust. Washing was avoided since this was shown to alter the chemical composition of lichens (Frati et al. 2005; Cansaran-Duman et al. 2009; Kroukamp et al. 2016). Ten grams of cleaned lichen material for both the Johannesburg and Waterberg sites were collected, whereas much less sample was available for processing from the Cape Point Site due to its nature conservation status as will be discussed in more detail. Samples were then shredded and passed through an Endecott mesh sieve. Fractions > 1 mm but < 4.699 mm were collected and ground under liquid nitrogen in a porcelain pestle and mortar, as this approach has been shown to be the most appropriate form of sample preparation for lichen digestions (Kroukamp et al. 2017). Air-dried, cleaned, pulverized samples were then stored in acid-washed polypropylene bottles in a cool, dark place until digestion.

As a consequence of the nature conservation status of the Cape Point site, there were restrictions on the overall lichen mass which could be removed from this site. Samples were taken from five locations (i.e. Location 1, 2, 3, 4 & 5), and were not pooled into a single bulk material. Instead, samples from each individual location were pooled and homogenized independently. Subsets from each respective bulk material were then taken for analysis. This was done to provide supplementary information to Cape Point Nature Conservation about metal distributions within the site but are not to be covered here due to the small population sizes per location which prevented statistical analyses (available in Online Resource 1), therefore averaged values across the Cape Point sites are presented. The total number of samples for the Cape Point locations was twenty-one (n=21). Ten replicate samples (n=10) from Johannesburg and 20 replicate samples from Waterberg (n=20), were used for comparison, although it should be reiterated that these were obtained from pooled, homogenized, bulk-lichen material from each respective site. Consequently, the standard deviations (SD) between replicates for these sites are expected to be much smaller than those from the Cape Point site. Since samples were pooled, the tree species was not noted as this should play no role in the overall metal concentration profiles.

Powdered lichen material (0.1 g per replicate) was digested using a Mars 6 microwave digestion system (CEM) with 10 mL HNO_3 (65%, Merck, Suprapur®) and 1 mL H_2O_2 (30%, Merck, Suprapur) in accordance with the Milestone application note HPR-FO-55 (Milestone 2014), where the chosen mass was known to be sufficiently homogenous due to prior studies (Kroukamp et al. 2017). The digests were filtered through pre-conditioned (using 0.5 mL MeOH) PTFE syringe filters (0.45 µm, Membrane Solutions) and diluted to 50 mL using deionized water (18 $M\Omega.cm^{-1}$, MilliQ Millipore). An aliquot (1 mL) of this was diluted to 10 mL with deionized water as this dilution had been found to be most appropriate in past studies (Kroukamp et al. 2017). Samples were then analysed using a NexION® 300X Inductively Coupled Plasma Mass Spectrometer (ICP-MS, PerkinElmer Inc.) using a range of six freshly-prepared, matrix-matched standards.

Isotopes which did not have any isobaric interferences were chosen for each polyisotopic analyte of interest. Transition elements and metalloids were analysed using a collision gas (He ≥ 99.999%, Afrox) to reduce polyatomic interferences on these elements. Samples were analysed for Al, As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Se, Sn, Th, Tl, U, V and Zn. Rhodium/ Ru, In and Lu were used as internal standards depending upon their absence from the site. A tea certified reference material (CRM, INCT-TL-1) was used for quality assurance and was digested in the same manner as the lichen samples.

Statistical analysis

Statistical analyses were performed using the statistical analysis program, SPSS 22.0 and one-way ANOVA. Appropriate *post hoc* tests; Dunnets T-3 test, Scheffes Analysis, Homogeneity of Variance and Brown-Forsythe were used, and statistically significant differences were determined with a 95% confidence interval ($p < 0.05$). Only results which are statistically significant and above the detection limits of the instrument are discussed.

Results and discussion

Analytical data validation

Based on their suitability relative to mass and ionization potentials, Rhodium/ Ru, In and Lu were used for correcting instrument drift and matrix effects. The recovery for these internal standards was within the range of 70-130%, indicating absence of significant matrix effects (Keith 1996). The accuracy of analytical results was also checked by analysing a tea certified reference material (CRM, INCT-TL-1). All analytes were within the 95% confidence interval as indicated on the CRM certificate except for Ni and Cd which had 87% and 83% recoveries respectively. This was likely due to very low concentrations of these elements in the CRM material as well as inaccuracies and a 'top-weighed' calibration curve for these elements. Limits of detection (LOD) were determined by $3S_{y/x}/b$ and limits of quantification (LOQ) were determined by $10S_{y/x}/b$ where "$S_{y/x}$" is the standard deviation of the calibration curve and "b" is the slope of the curve.

Sources of metals

In this study, only Al, Cd, Cr, Cu, Mn, Ni, Pb, Zn and V were found to exhibit statistically significant differences in concentration between sites, and therefore only these elements will be discussed in further detail. The potential natural and anthropogenic sources of these metals are first elaborated upon and are followed by site comparisons. It should be noted that the larger degree of variation of metal concentrations in samples from the Cape Point site are expected due to the manner in which sampling took place at this site, as explained previously.

Aluminium

Aluminium is ubiquitous in the environment and comprises a third of the earth's crust (Gourier-Fréry and Fréry 2004). It is found predominantly in wind-blown soil particles from shales and rocks. Despite its abundance, it is listed as a potential neurotoxicant in humans (Domingo 1995) and can exist in high concentrations in polluted environments. Anthropogenic contributors to Al in the atmosphere are the combustion of fuels, and the metals industry (Hashimoto et al. 1992). It is also used in explosives, fireworks, the manufacture of cans, foil, roofing, water-treatment, the production of pharmaceuticals and cosmetic products, and is released into the environment as a consequence of the mining and processing of Al ores, coal-fired power plants and incinerator emissions (ATSDR 2008).

Cadmium

Natural concentrations of Cd in the atmosphere result from windblown particles, volcanic activity, biogenic emissions and forest fires, although these concentrations are generally low. Cadmium in the atmosphere typically comes from non-ferrous metal industry emissions, and is commonly found in association with Zn and Pb metal processing. It is also released into the atmosphere through the incineration of waste, iron and steel production, Cd-Ni battery production, cigarette smoke, and fertilizer and cement production (WBG 1998a; WHO 2007). The resuspension of dusts containing Cd can greatly contribute to the long-range transport of this metal (WBG 1998a; WHO 2007). Cadmium toxicity can cause decalcification of bone, renal damage, reproductive system defects, damage to the pulmonary and gastric systems and is a known carcinogen (Rahimzadeh et al. 2017).

Chromium

Chromium can occur naturally in volcanic dust and gases, rocks and soils. However, high environmental levels are typically due to anthropogenic activities such as the ferrochrome industry, manufacture of dyes and pigments, leather and wood preservation (Lushchak et al. 2009), and the treatment of cooling tower water. It is also released from ore refining, automobile brake linings, catalytic converters and cement producing plants. The respiratory tract is the main organ for Cr toxicity, resulting in acute and chronically toxic effects through its inhalation. In cases of acute exposures, symptoms such as shortness of breath, coughing and wheezing may be experienced whereas chronic exposures have been shown to lead to bronchitis or pneumonia, reduced pulmonary function and in some cases, cancer (Sullivan 1969; USEPA 2000).

Copper

Copper is an essential micronutrient, where excess concentrations of inhaled copper can cause nose, eye, throat and mouth irritation, adverse gastric effects such as nausea, as well as headaches and dizziness. Moreover, some diseases which are associated with age, such as Alzheimer’s, neurodegenerative diseases, arteriosclerosis, diabetes mellitus and others have thought to be related to excessive copper intake (Brewer 2010). Natural copper concentrations in air are low and are due to windblown dusts and soils, volcanic activity, suspended decaying vegetation, forest fires and sea spray. High levels of atmospheric copper are typically due to anthropogenic activities such as copper mining, smelting and processing, the combustion of fossil fuels, and the production of phosphate fertilizers and fungicides for plants (ATSDR 2004).

Lead

Lead is a metal which has a neurotoxic effect. Only a minute fraction of the Pb found in environmental samples has a natural source, with the majority being anthropogenic in origin, resulting from impacts such as ore and metal processing, its use in leaded aviation fuel, lead batteries and paint production. Additional sources are the production of ceramics, cosmetics, ammunition and in the past, plumbing materials (USEPA 1996; USEPA 1998; USEPA 2013; WBG 1998b).

Manganese

Although manganese is omnipresent in the environment and forms a major constituent of the earth’s crust, it can still be a health concern. Fine particulate matter can penetrate deep into the lung tissue, and for manganese, the main path of chronic exposure is through inhalation (Michalke et al. 2007; Hoet et al. 2012). High concentrations of Mn can result in a condition called manganism which has similar symptoms to Parkinson’s disease, and causes the neurodegeneration of the central nervous system (Ellingsen et al. 2003; Nádaská et al. 2012). Anthropogenic contributions to Mn in air are steel production, Mn ore mining, production of dry battery cells, welding activities, and more recently, the use of Mn as an anti-knock agent in fuels (Ellingsen et al. 2003).

Nickel

Nickel may lead to toxic effects in the respiratory tract and can influence the immune system (Cempel and Nikel 2006). Nickel concentrations in air are often higher in industrialized areas, being used in a number of different industries. These industries include the production of stainless steel and alloys, and the combustion and incineration of coal, diesel, fuel, waste and sewage (Cempel and Nikel 2006). It can also be released during copper and platinum refining, petroleum refining, electroplating, aircraft manufacture, motor vehicle production, ship building, and the production of catalysts, batteries, dyes, pigments and ceramics (USEPA 1984). Natural sources of nickel in the atmosphere tend to be low, and include dust from weathered rocks and soils where Ni is the 24th most abundant element in the earth’s crust (USEPA 1984;

Cempel and Nikel 2006). Other natural atmospheric sources of Ni are forest and vegetation fires, volcanic emissions and sea salt (USEPA 1984).

Vanadium

Vanadium is the 22nd most abundant element in the earth's crust and is found naturally in the atmosphere as a result of sea spray, windblown continental dusts and through volcanic emissions (Vučković et al. 2013). High concentrations, however, may result in lung damage (ATSDR 2012a,b). It is widely used in the steel industry as an anticorrosive and in the production of ceramics, catalysts and super-magnets. It is also released in the burning of fuel oil and coal, and occurs in small quantities in cigarette smoke (ATSDR 2012a,b).

Zinc

Zinc is an essential micronutrient and natural sources in air are primarily from windblown soil. Other natural sources are volcanos, forest fires, biogenic emissions and sea salt. Most high concentrations of Zn in air are directly related to anthropogenic activities such as Zn and coal mining, the refining and smelting of non-ferrous metals, coal and bottom fly ash, the production of fertilizers, wood preservatives, brass, rubber, paints, bronze and it is also released during galvanization and electroplating activities (Athanassiadis 1969; ATSDR 2012c). Although an essential microelement, inhaled Zn and ZnO from atmospheric dust has been found to cause necrosis and inflammation of mouse lungs (Adamson et al. 2000, Adamcakova-Dodd et al. 2014).

Results from the Cape Point sampling site

The weather data for the Cape Point site based upon the nearest weather station showed that the humidity averaged 80 ± 5% (Fig. 2). Minimum temperatures ranged between 10-17 °C and the maximum temperatures ranged between 15-24 °C with an average of 27 mm rainfall in the 3 months prior to sampling. Fig. 3 shows that the wind direction was mostly from ENE, which faces Gordons Bay and Strand which are 40 km ENE of Cape Point, and the second most common wind direction was from ESE, which is open ocean facing. This corresponds with earlier studies which have determined that the wind direction at this site during summer is predominantly from the SE to SW, and is comprised predominantly of clean ocean air, whereas winters are mostly from the north and northwest and can carry pollutants from continental and polluted air masses (Rautenbach and Smith 2001, Brunke et al. 2016).

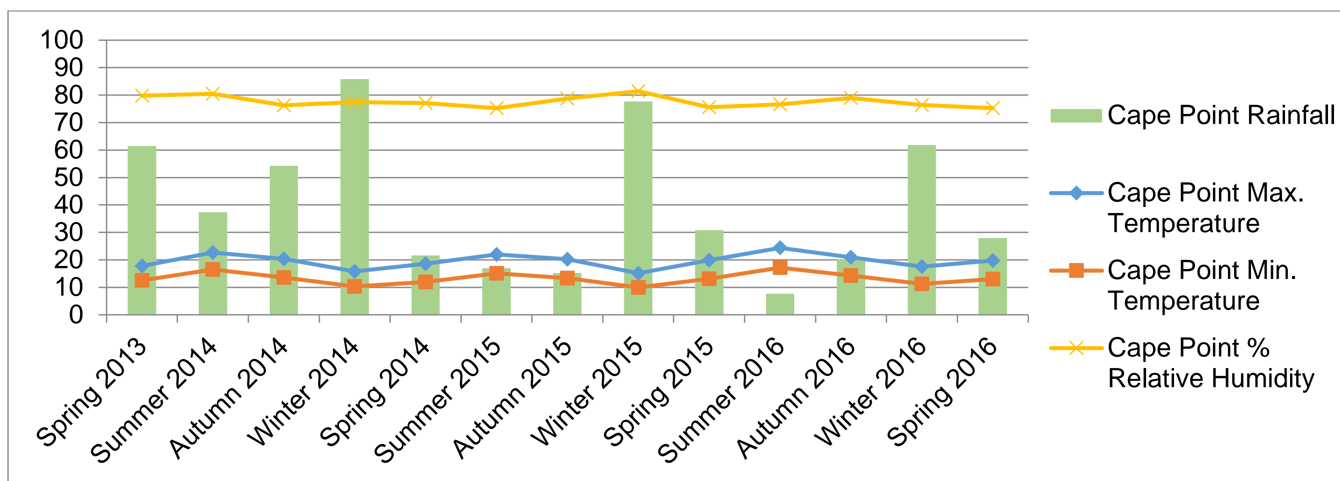


Fig. 2 Weather data for the Cape Point site where the average rainfall is in mm, the minimum and maximum temperatures are in °C and relative humidity is in percentage. Readings are taken from the Cape Point Weather Station (GPS co-oridinates 34.3530 S, 18.4890 E, 228 m elevation)

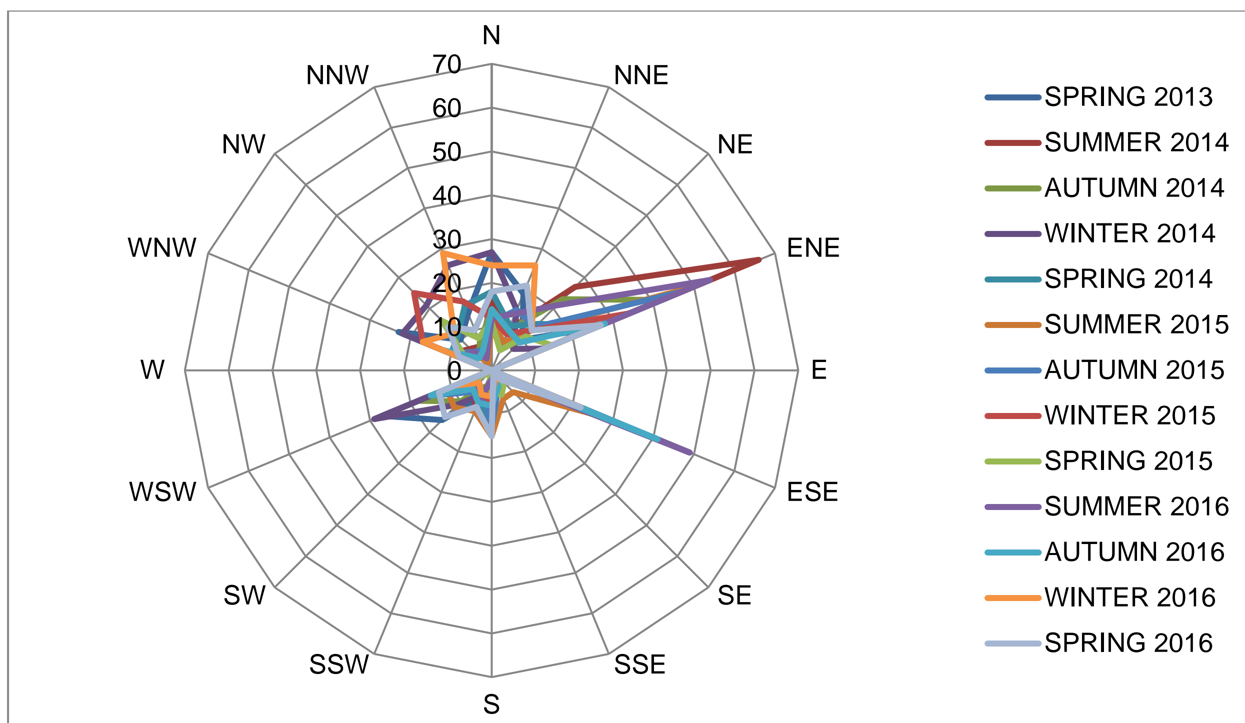
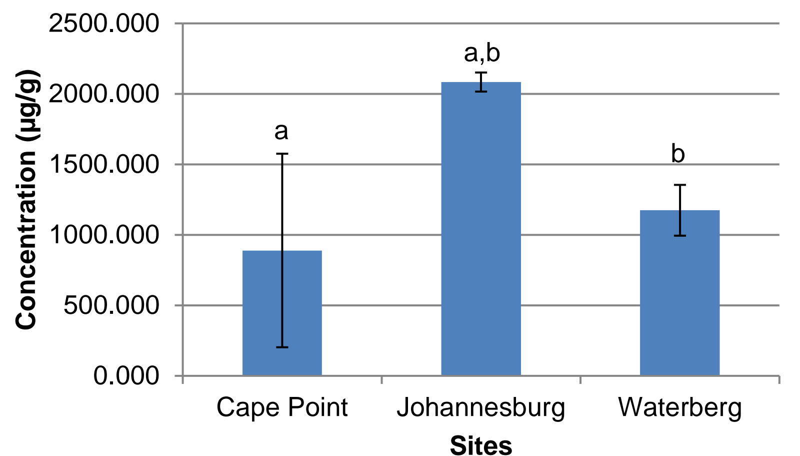


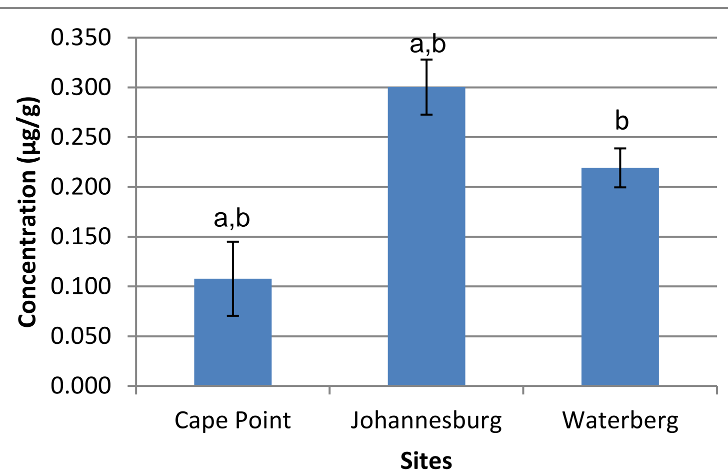
Fig. 3 Prevailing wind direction as observed by the Cape Point weather station for the 3 years prior to sampling, where the number correlates to number of occurrences during the mentioned timeframe

Concentrations of Cd, Cr, Cu, Mn, Pb and V were statistically significantly ($p<0.05$) lower in lichens from the Cape Point site than in lichens from either the Johannesburg or Waterberg site (Fig. 4b-e, g-h), which shows its fitness for purpose as a reference (background) site. Concentrations of Al (Fig. 4a) and Zn (Fig. 4i) were only significantly lower than lichens from the Johannesburg site, and were not statistically lower than lichens from the Waterberg site. The average concentration of Ni (Fig. 4f) in lichens at the Cape Point site was statistically higher than in both the Johannesburg and Waterberg sites. Likely sources of Ni at this site are both natural and anthropogenic in origin. Mountain wild fires which often occur in the dry summer months on the shrubby Fynbos and sea salt (USEPA 1984) distributed from the ocean by strong winds are likely predominant sources of Ni at this site, where fires are known to greatly increase the presence and abundance of a number of metals (Brunke and Labuschagne 2001). Potential anthropogenic sources are industries throughout False Bay including electroplating, battery production, oil refining, coal power plants, steel and paint production in Strand and Somerset West. Another potential source could be the burning of waste and fuels in informal settlements such as Khayelitsha and Mitchells Plain (PGWC 2010) which is in the direction of prevailing winds (ENE).

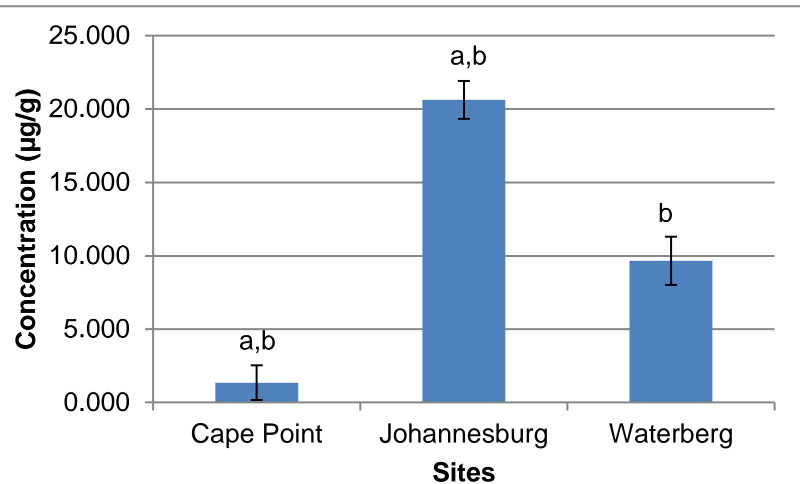
a) Al



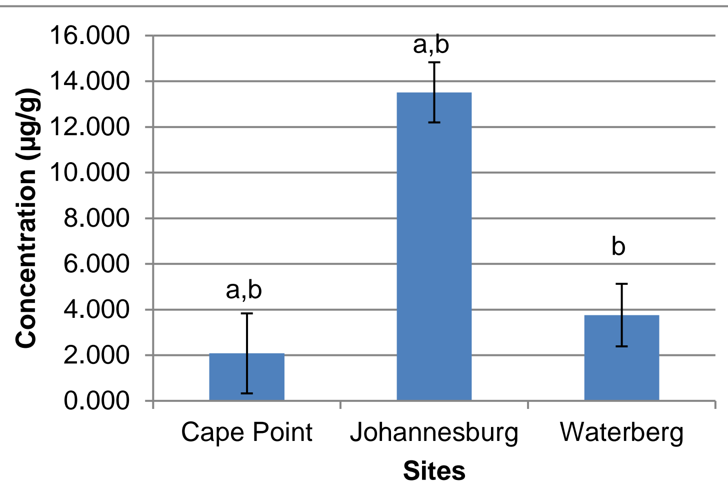
b) Cd



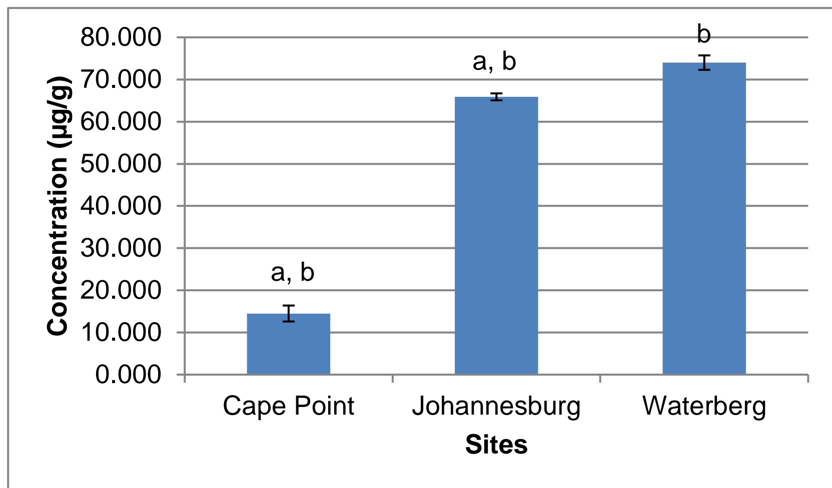
c) Cr



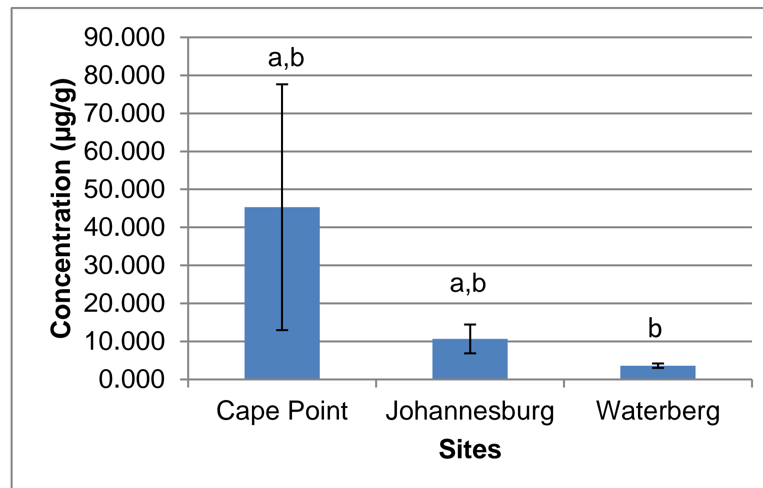
d) Cu



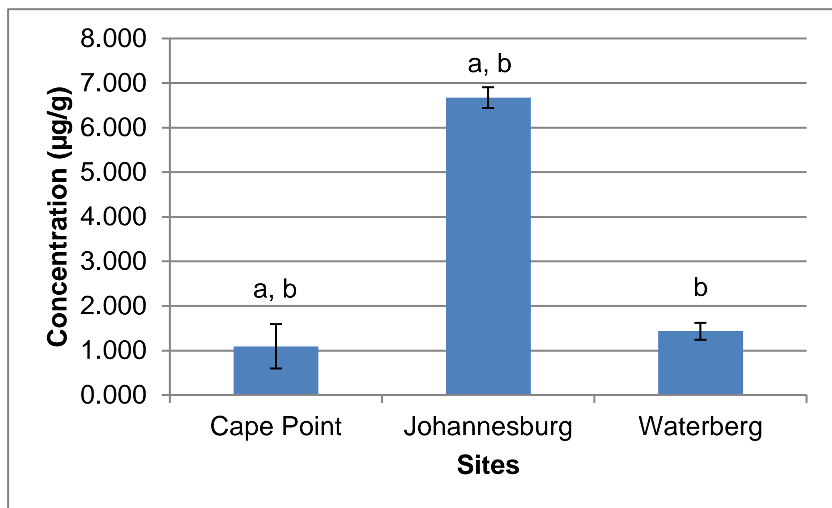
e) Mn



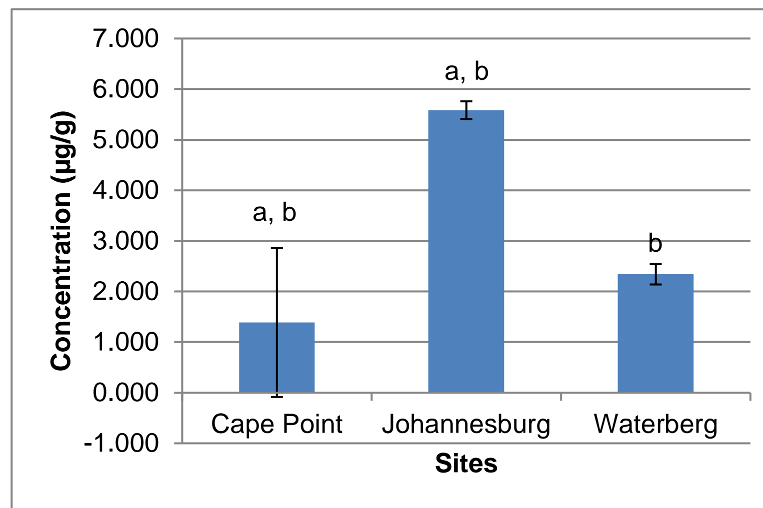
f) Ni



g) Pb



h) V



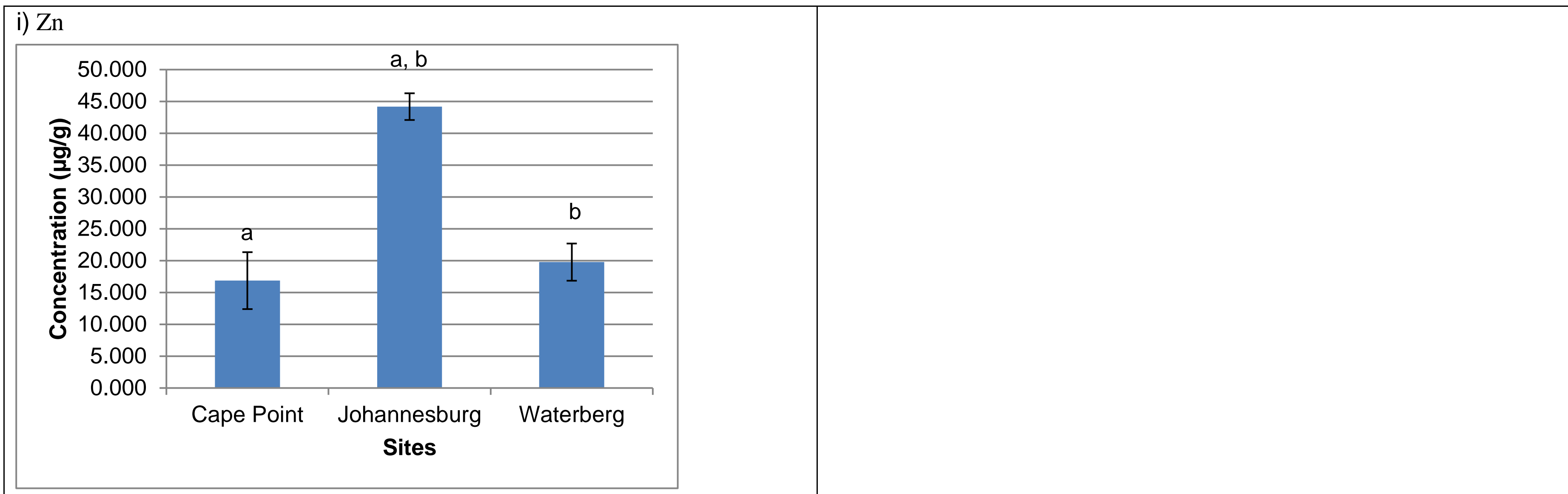


Fig. 4a-i Average concentrations (blank subtracted values) and STDEV of in the bulk lichen sample collected from different sites within South Africa where the following letters correlate to the respective elements: a) Al, b) = Cd, c) =Cr, d) =Cu, e) =Mn, f) =Ni, g) =Pb, h) =V, i) =Zn. Values reflected are in µg/g dry weight. Statistically significant differences are indicated by lowercase letters above the bars where the same letters indicate significance

Although the Cape Point site is far away from most anthropogenic impacts which is the most likely cause for low concentrations of metals in lichens from this site, it should be noted that the high percentage relative humidity at Cape Point could also assist detoxifying metabolic processes, as lichens are more metabolically active when wet (Nash and Gries 1995; Bergamaschi et al. 2007) and may also wash some metals from the lichen surface. When compared with other sites, higher nighttime temperatures and a smaller difference between daytime and nighttime temperatures would also help to ensure that the lichens were not subjected to any additional stress, all of which would facilitate the metabolism and sequestration of toxic metals.

Results from the Johannesburg sampling sites

As can be seen in Fig. 5, the weather data based upon the nearest weather station showed that the humidity averaged 55 ± 8% over the 3 years prior to sampling. Minimum temperatures ranged between 4 - 16 °C and the maximum temperatures averaged between 21- 29 °C. There was an average of 35 mm of rainfall in the months prior to sampling and the wind direction was predominantly from NNE, although winds coming from the NNW and NW were also very common (Fig. 6). Since the percentage relative humidity is lower, and there is a greater difference between day time and nighttime temperatures than the Cape Point site, the higher concentrations of metals in lichens from JHB may be somewhat related to increased morphological and metabolic stress as a consequence of these differences. Rainfall at this site was on average 8 mm higher than the Cape Point site in the three months prior to sampling, which could facilitate the removal of some metals from the surface of JHB lichens, implying that concentrations may be even higher at other times of the year.

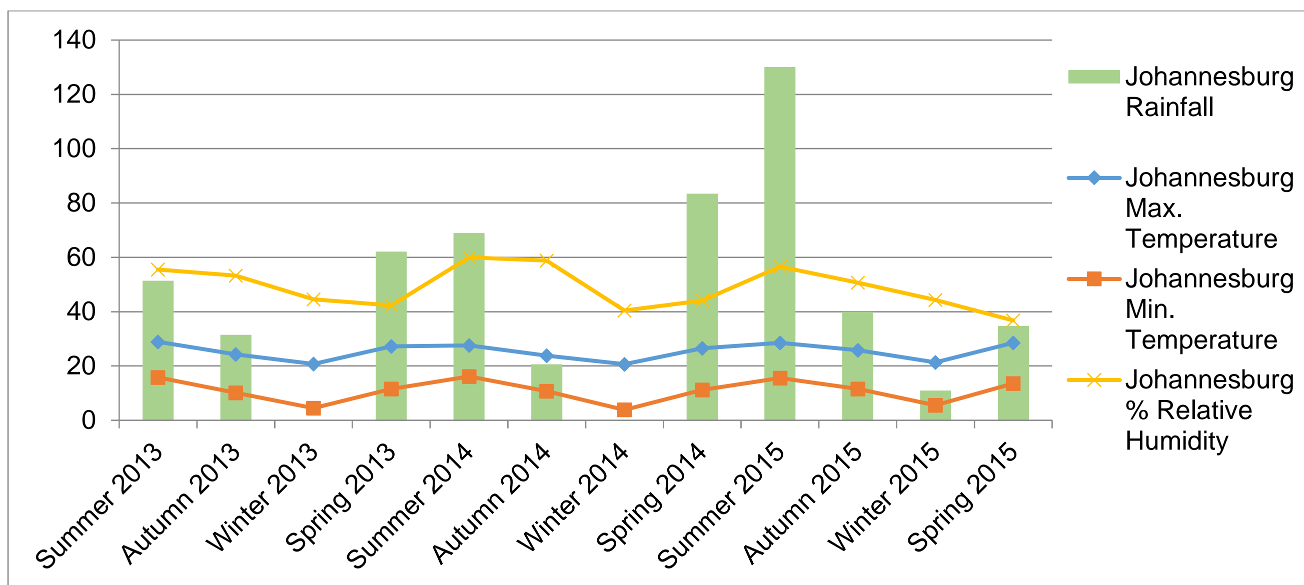


Fig. 5 Weather data for the Johannesburg site where the average rainfall is in mm, the minimum and maximum temperatures are in °C and relative humidity in percentage. Readings are taken from Johannesburg Botanical Garden Weather Station (GPS co-ordinates: 26.1560 S, 27.9990 E, 1624 m elevation)

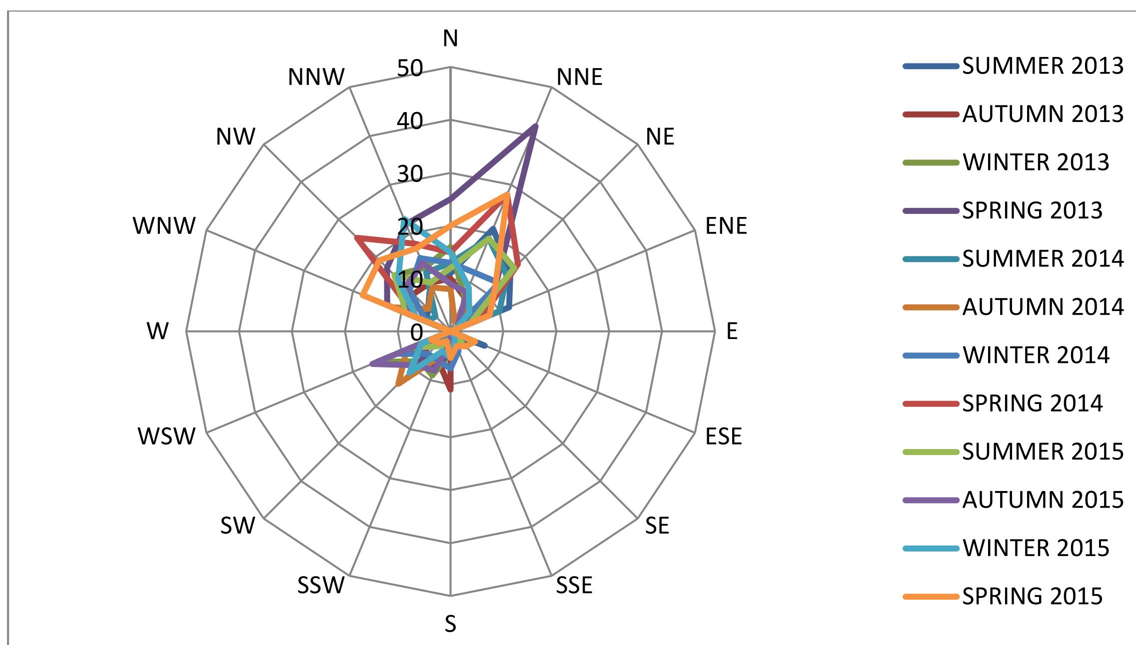


Fig. 6 Prevailing wind direction as observed by the Johannesburg Botanical Gardens weather station 3 years prior to lichen sampling, where number correlates to number of occurrences during the timeframe

As shown in Fig. 4a-d and Fig. 4g-i, concentrations of Al, Cd, Cr, Cu, Pb, V and Zn were statistically higher in lichen samples from the Johannesburg site, than from both the Cape Point and Waterberg sites. Concentrations of Mn in lichens from the Johannesburg site were statistically lower than in Waterberg site, but statistically higher than in the Cape Point site. The concentrations of all metals are likely to be related to automobile associated pollutants such as tyre wear, rust and automotive emissions which are NNW, NW and NNE of the site. Other potential sources of pollutants at this site are several aluminium, steel, copper and brass works within close proximity to the site (≥10 km). Although Pb use in petrol has been largely discontinued in South Africa since 2006 (DME 2006), the Pb present at this site may also be from residual, persistent Pb from its historical use in fuel, as has been found in other studies (Forbes et al. 2009).

Results from the Waterberg sampling sites

As can be seen in Fig. 7, the weather data based upon the nearest weather station to the Waterberg site, showed that the humidity ranged between 33 - 62% over the 3 years prior to sampling. Minimum temperatures ranged from 8 - 23 °C and the maximum temperatures between 26 - 36 °C. There was an average of 70 mm of rainfall in the 3 months prior to sampling and wind direction was predominantly from the NE (Fig. 8).

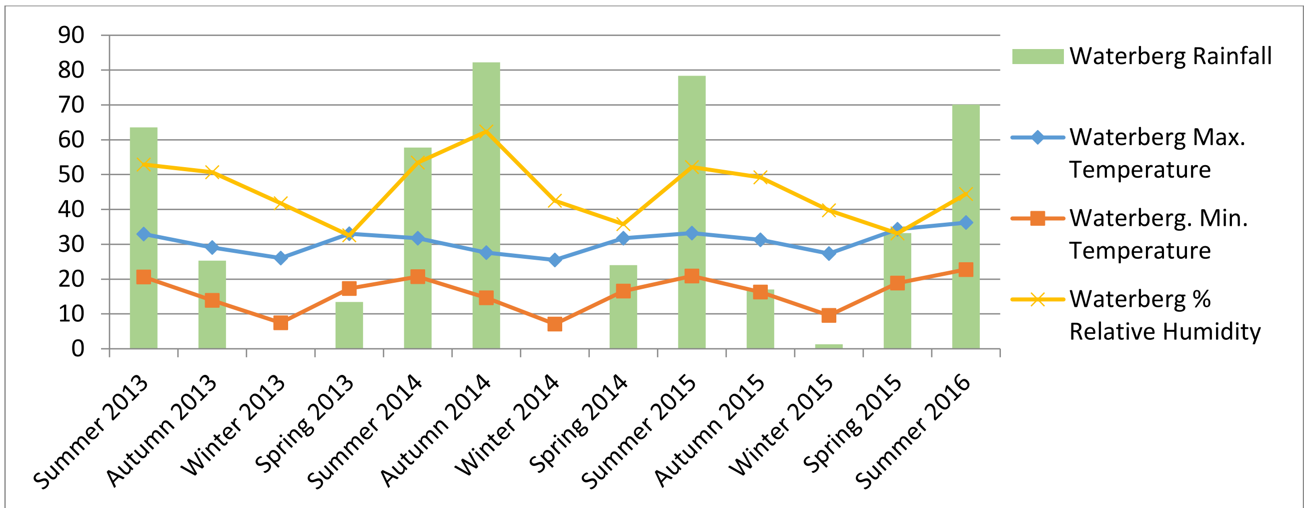


Fig. 7 Weather data for the Waterberg site, where the average rainfall is in mm, the minimum and maximum temperatures are in °C and relative humidity in percentage. Readings taken from Lephalale Weather Station (GPS co-ordinates: 23.6760S, 27.7050 E, 839 m elevation)

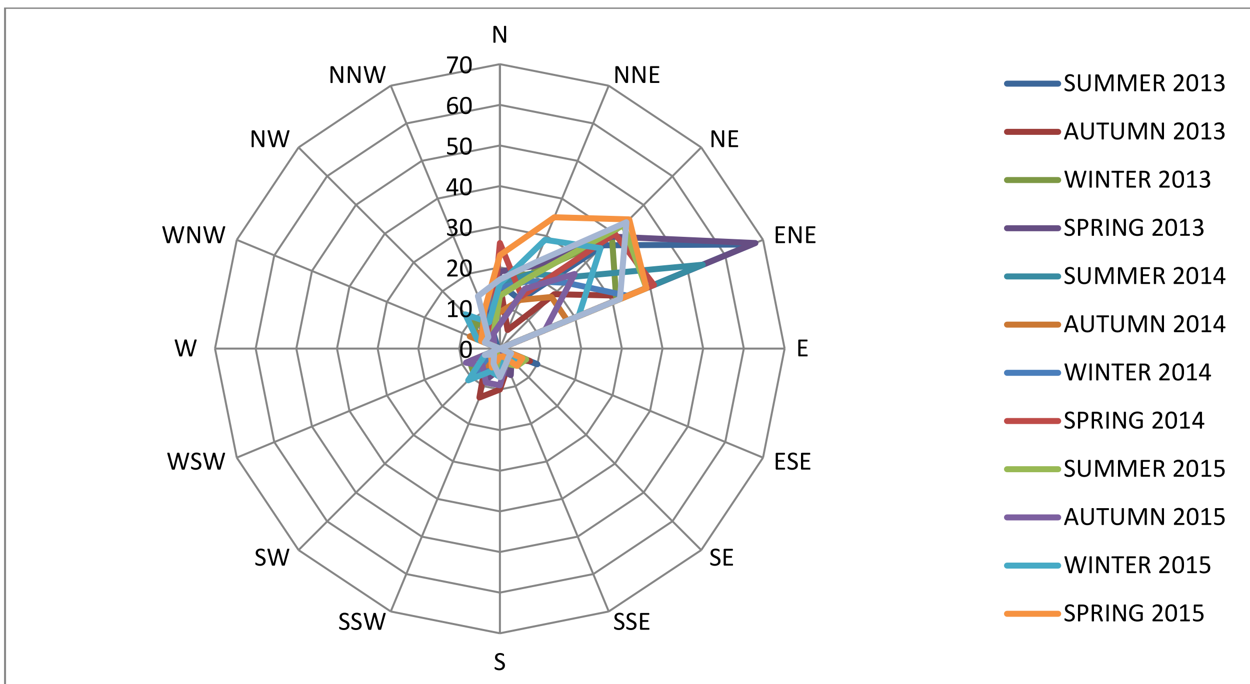


Fig. 8 Prevailing wind direction as observed by the Lephalale Weather Station 3 year prior to sampling, where number correlates to the number of occurrences during the timeframe

The statistically higher concentrations of Mn in lichens from the Waterberg site, in comparison to the Cape Point and Johannesburg sites (Fig. 4e) are likely both geological and anthropogenic in origin, where naturally occurring windblown soils and dusts from the neighbouring Transvaal Supergroup could have been deposited on the surface of the lichens (Beukes et al. 1983). Since the prevailing wind direction was from the NE, the impacts upon this site are most likely to be related to the disturbance of land from farming activities, livestock and crop sprayings, and supplementation (Walter 1988, Spears 2011). Most of the mines in the area are SW and SE of the site. The occurrence of winds from this direction was uncommon, however such impacts may still account for a small percentage of the concentrations observed. These mines include an open-cast haematite ore mine in the nearby (50 km away) town of Thabazimbi, as well as the number of platinum, diamond and clay mines. Concentrations of Cd, Cu, Cr, Pb and V (Fig. 4b-d, g-h) are statistically higher in lichens from the Waterberg site than in lichens from the Cape Point site, and this is likely due to agricultural and mining activities in the surrounding areas.

At this site, the change in humidity between seasons over the 3 years prior to sampling are significant. Daytime and nighttime temperatures at this site are also much higher than the other sites which could increase the risk of lichen desiccation. Such stressors may also reduce the ability of the lichens to tolerate atmospheric pollutants (Bergamaschi et al. 2007). That being said, the rainfall at this site in the three months prior to sampling was more than double that of both the Cape Point and Johannesburg sites, and this may have facilitated the removal of metals from the lichen surface.

Comparison to past studies

When concentrations of Cr, Cu, Mn, Ni and Pb were compared to past studies which used the lichen *Hypogymnia physodes* as a biomonitor of air pollution in the Kruger National Park (KNP) in the year 2000 (Panichev and McCrindle 2004), and *P. austrosinense* which was used as a biomonitor of air pollution in Pretoria in 2007 (Forbes et al. 2009), distinct differences between the concentrations were observed (Table 1). Some natural causes of these differences may be due to site location, where contrasting geologies could be a significant factor. Sampling season could also play a considerable role, where the sampling for both cited studies took place in the dry season, whereas samples from our study were collected during or just after the rainy season at each site. This dynamic is important due to the aforementioned impact of moisture on metabolism and the enhanced removal of metals from the lichen surface (Nash and Gries 1995; Bergamaschi et al. 2007). Species differences between lichens from our study, and those from Panichev and McCrindle (2004) may also account for some of the observations, as different lichens species may have differing tolerances to metals, and different mechanisms for the metabolism, sequestration and detoxification of metals (Sarret et al. 1998; Branquinho et al. 1999; Rola et al. 2016). Furthermore, the current study used quite a large representative sample of 10 g of lichen for the Waterberg and JHB sites to create a bulk sample from which the samples replicates were taken (replicates for Cape Point n=21, Johannesburg n=10, Waterberg n=20). Neither the KNP nor Pretoria studies provided an indication of how much sample was collected and processed to represent the population, where this aspect could create some bias in the results.

Average concentrations of Pb in lichens from the control site of the KNP study (Panichev and McCrindle 2004) are slightly higher than those from Cape Point and Waterberg but are lower than the Johannesburg site, affirming that the source of Pb in JHB is likely anthropogenic in origin. Higher concentrations of Pb in lichens from the control site of the KNP study, in comparison to the Cape Point reference site of the current study, could be a consequence of the sampling taking place during different years, where Pb was phased out as an anti-knock agent in fuel in 2006 (DME 2006), and its use in paints has become less common (Mathee 2014). The KNP lies on the Bushveld Complex which has the largest group of Cr, V and Platinum Group Metal (PGMs) deposits in the world (Von Gruenewaldt and Merkel 1995; Clarke et al. 2009; Fisher et al. 2011), therefore levels of Cr are expected to be naturally higher in the study by Panichev and McCrindle (2004). In the high-risk area of the KNP study, the high Cr concentrations when compared to the reference site of the KNP study could have been due to the disturbance of land as a result of mining activities. In the current study, concentrations of Cr are expected to be lower, as the Waterberg site lies on the Waterberg group which overlies the Bushveld Complex. The Waterberg group can be several kilometres thick and so the PGM, Cr and V minerals in the Bushveld Complex at this site could be considered less accessible than at the KNP site. Outside the town of Thabazimbi however, this Waterberg layer is somewhat thinner and allows platinum mining to take place (MT 2018) which could contribute to higher background concentrations of this site when compared to the Cape Point site.

The average Mn concentrations in lichens from the Pretoria site (Forbes et al. 2009) were higher than all of the three sites from the current study. This finding agrees with geological site information, where lichens from the Pretoria site is expected to have higher concentrations of Mn than the other sites since it lies directly upon the Transvaal Supergroup which is known to have rich Mn deposits (Beukes et al. 1983) and includes the Hekpoort strata, which includes a Mn-rich latosol plinthic catena (CG 2008; Forbes et al. 2009). The Pretoria site also has greater anthropogenic influences than the Waterberg site from our study. These influences are primarily in the form of automobile emissions as this study was done in the year following the banning of leaded fuel and the implementation of methylcyclopentadienyl manganese tricarbonyl (MMT) as a new anti-knock agent in fuels (Forbes et al. 2009). Average concentrations of Ni and Cu are higher at both KNP sites in comparison to all the sites from our study, where these differences may be partly due to seasonal differences between the sampling sites and the solubility of Cu- and Ni- containing compounds.

Table 1: Comparison of current findings with past studies within South Africa, where Pretoria results are from Forbes et al. (2009), and KNP is from Panichev and McCrindle (2004). Results are shown in µg/g (mean±SD) and cells which are crossed out indicate an absence of this data in the cited studies

Sampling site	Species	Cr	Mn	Ni	Cu	Pb
Cape Point	*P. austrosinense*	1.3±1.2	14.5±1.9	45.3±32.3	2.1±1.8	1.1±0.5
Johannesburg	*P. austrosinense*	20.6±1.3	65.9±0.8	10.7±3.8	13.5±1.3	6.7±0.2
Waterberg	*P. austrosinense*	9.7±1.6	74.0±1.7	3.6±0.6	3.8±1.4	1.4±0.2
Pretoria CBD	*P. austrosinense*		97.1±39.1			181.1±98.0
Pretoria Outside CBD	*P. austrosinense*		97.1±39.1			41.5±36.4
Kruger High-Risk Area	*H. physodes*	96.0±11.0		111.0±12.0	231.0±24.0	22.5±8.4
Kruger Control Area	*H. physodes*	64.0±8.0		28.0±5.0	25.0±3.0	3.8±1.8

Conclusions

The lichen, *Parmotrema austrosinense,* has been shown to be a useful biomonitor of air pollution, allowing for comparisons of air quality between different sites for a large range of metals within South Africa for the first time. This study has proven that the urban site had significantly higher concentrations of a number of different metals which are primarily automobile-related, in the form of vehicle emissions, and parts wear and tear. Industrial contributions are also present at this site. We have also demonstrated the appropriateness of Cape Point as a suitable environmental reference site for future air pollution assessments regarding metals. This study has shown that it is extremely useful to include weather data in studies using lichens as biomonitors of air pollution, as this provides a more holistic understanding of factors affecting metal concentrations in lichens, including the identification of potential stressors and conditions affecting lichen metabolism. It is also essential to use the same lichen species for comparative assessments, and to sample during the same season, as these parameters have been shown to bring an unknown bias when comparing the results between different studies. Moreover, it is highly recommended that future studies be expanded to include sampling during different seasons at the same site, as this will provide a baseline for seasonal differences and allow for inter-seasonal comparisons to be made.

Conflict of Interests

There are no known conflicts of interest

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Paper 2- Supplementary Information

Article Title: Spatial study of atmospheric metal concentrations in South Africa using the lichen biomonitor, *Parmotrema austrosinense*

Journal Name: Environmental Monitoring and Assessment

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Online Resource 1: Concentrations shown in µg/g (mean±SD) of Al, V, Cr, Mn, Ni, Cu, Zn, Cd and Pb in the lichen *Parmotrema austrosinense* at three sites with differing anthropogenic impacts in South Africa, sampled between December 2015 and October 2016.

Site	**Al**	**V**	**Cr**	**Mn**	**Ni**	**Cu**	**Zn**	**Cd**	**Pb**
Cape Point Location 1 (n=5)	457.32±37.61	0.69±0.14	0.84±0.54	16.67±0.64	37.18±4.91	2.33±0.67	22.23±1.16	0.16±0.02	1.09±0.07
Cape Point Location 2 (n=3)	993.52±26.34	1.22±0.13	1.77±0.30	13.30±0.20	56.82±12.13	1.99±0.68	20.39±3.75	0.08±0.02	1.39±1.16
Cape Point Location 3 (n=3)	603.36±39.62	0.64±0.11	0.90±0.09	12.03±1.41	11.68±4.01	2.43±2.36	13.13±2.36	0.09±0.02	0.70±0.55
Cape Point Location 4 (n=4)	2161.06±204.49	4.20±0.15	3.28±0.70	15.75±1.29	101.18±6.32	3.05±2.81	14.00±3.04	0.08±0.02	0.94±0.27
Cape Point Location 5 (n=5)	412.73±20.46	0.38±0.08	0.33±0.43	13.53±0.38	21.99±0.96	0.89±1.52	13.90±1.34	0.10±0.02	1.28±0.19

Cape Point average ±SD (n=21)	889.25±686.46	1.39±1.47	1.35±1.18	14.50±1.89	45.30±32.34	2.08±1.75	16.86±4.47	0.11±0.04	1.09±0.50
Johannesburg average±SD (n=10)	2084±67.64	5.58±0.18	20.61±1.29	65.88±0.82	10.66±3.79	13.51±1.32	44.18±2.10	0.30±0.03	6.67±0.23
Waterberg average±SD (n=20)	1174.36±180.12	2.34±0.20	9.66±1.64	74.01±1.71	3.61±0.59	3.76±1.37	19.77±2.92	0.22±0.02	1.43±0.19

4.3. Paper 3

This paper was formatted in accordance with the journal *Environmental Monitoring and Assessment* a Springer journal. At the time when this PhD was submitted for examination, this paper was published.

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Other output from this data:

The work in this chapter was presented as a poster at the 100th Canadian Chemistry Conference 2017, Toronto, Canada (Appendix D).

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Comparison of sample preparation procedures on metal(loid) fractionation patterns in lichens

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Abstract The effects of different sample preparation strategies and storage on metal(loid) fractionation trends in plant material is largely underresearched. In this study, a bulk sample of lichen *Parmotrema austrosinense* (Zahlbr.) Hale was analysed for its total extractable metal(loid) content by ICP-MS, and was determined to be adequately homogenous (<5% RSD) for most elements. Several subsets of this sample were prepared utilising a range of sample preservation techniques and subjected to a modified sequential extraction procedure or to total metal extraction. Both experiments were repeated after 1-month storage at 4 °C. Cryogenic freezing gave the best reproducibility for total extractable elemental concentrations between months, indicating this to be the most suitable method of sample preparation in such studies. The combined extraction efficiencies were >82% for As, Cu, Mn, Pb, Sr and Zn but poor for other elements, where sample preparation strategies 'no sample preparation' and 'dried in a desiccator' had the best extraction recoveries. Cryogenic freezing procedures had a significantly ($p < 0.05$) negative effect on metal extractability, and is therefore inappropriate for sequential extraction procedures in lichens. Biotransformation over a period of a month is suspected for most elements, with the exception of Sr and Zn, where changes in the fractionation patterns were statistically significant ($p < 0.05$), indicating the need for minimal delay in sample cleaning and preservation when species fractionation patterns are of interest. This study also shows that the assumption that species stability can be ensured through cryopreservation and freeze drying techniques needs to be revisited.

Electronic supplementary material The online version of this article (doi:10.1007/s10661-017-6155-4) contains supplementary material, which is available to authorized users.

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Keywords Sequential extraction · Fractionation patterns · Lichen metal(loid)s · Sample preparation · Air biomonitoring

Abbreviations

BCR	Bureau Community of Reference
CRM	Certified reference material
ICP-MS	Inductively coupled plasma mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification

MDL	Method detection limit
PFA	Perfluoroalkoxy alkane
SET	Sequential elution technique
XAS	X-ray absorption spectrometry

Introduction

Sequential extractions have been widely applied in the determination of the chemical fractionation of metals and metalloids in sediments (Hjorth 2004), soils (Li et al. 2010), sewage sludge (Jamali et al. 2009) and in determining the bioavailability of metals for uptake by plants (Tokahoğlu and Kartal 2004). In this article, we refer to the fractionation of metals in relation to their bioavailability and chemical solubility since fraction availability and water solubility vary under reducing and oxidative conditions. The speciation of a metal(loid) is strongly related to its mobility and bioavailability (Michalke 2003); therefore, these kinds of studies are extremely important. Fairly recently, sequential extractions have also been used in air pollution monitoring studies, where fractionation trends in street dusts (Banerjee 2003) and air filters (Fernández Espinosa et al. 2002; Feng et al. 2009) have been assessed. However, problems have emerged from the use of these monitoring techniques in air pollution assessments. Although the use of air filters is very effective for day-to-day evaluations, they do not reflect long-term exposures, and require the use of expensive sampling equipment.

Biomonitors, such as epiphytic lichens, are commonly used in air pollution monitoring studies as they rely almost solely upon gases, airborne dust particles and dissolved metals in rainwater to supply them with micro- and macro-nutrients (Loppi and Pirintsos 2003). The lack of a cuticle makes them able to assimilate contaminants over prolonged periods (de Bruin 1990; Forbes et al. 2015). Furthermore, they are abundant in the environment, cheap and easy to collect, giving them an advantage over other monitoring methods (Jeran et al. 1996).

A number of studies have explored the use of sequential extractions to evaluate the distribution of elements in the intra-, inter- and extra-cellular portions of lichens (Branquinho et al. 1999; Hauck et al. 2002; Monnet et al. 2005; Pérez-Llamazares et al. 2011; Paoli et al. 2014). However, as far as we are aware, the use of Bureau Community of Reference (BCR) sequential extractions in lichens has not been assessed to date. Since the lichen surface serves as a trap for atmospheric dust, the application of sequential extractions could be useful in evaluating the fractionation of metals in both the surface dusts and that which has been assimilated by the lichens. A vital component of such studies is to appropriately preserve the samples, thus preventing changes in the species distribution patterns.

Preservation, also known as fixation, techniques employed in lichen-based air-biomonitoring studies vary greatly and are often not validated. The aims of these techniques are to preserve the original elemental 'fingerprint' by disrupting microbial activity, whilst simultaneously increasing the ease with which the sample can be homogenised. Whilst some researchers recommend not using any drying or freezing procedures in determining the total metal content in lichens (Giordano et al. 2005), others recommend air drying (Cansaran-Duman et al. 2009; Demiray et al. 2012), oven drying (Ng et al. 2005), freeze drying (Godinho et al. 2008; Hanedar 2015) and cryogenic freezing in liquid nitrogen (Gandois et al. 2014; Pollard et al. 2015). As can be seen in Fig. 1 (and Table S1 in the Supplementary Material), there is little agreement about the choice of fixation techniques in lichen analysis, although oven drying appears to have received the most attention since the year 2000. However, the choice of methods, such as oven drying, may in fact liberate volatile elements from the sample matrix. Moreover, in studies such as those conducted by Hjorth (2004) in lake sediments, the choice of some preservation techniques, such as freeze drying, have been found to alter the speciation patterns of major and trace elements. For these reasons, the choice of fixation techniques in plants may in fact have a much larger effect on the total metal(loid) concentrations than is currently assumed, and the role of these techniques in sequential extractions also needs to be assessed.

Several modifications of the sequential extraction scheme developed by Tessier et al. (1979) have been suggested over the years to increase the selectivity of the different extraction media in fractionation studies. One such example is the modification suggested by Fernández Espinosa et al. (2002), which claims to be able to reduce the chances of chemical species being co-extracted and to improve species selectivity.

In this work, a modification of the sequential extraction technique by Fernández Espinosa et al. (2002) was

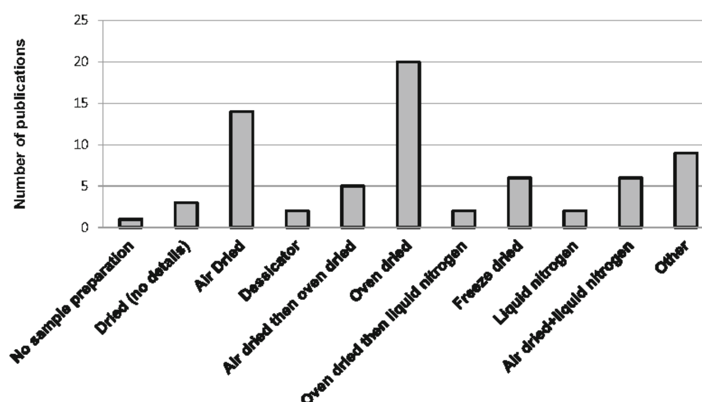

Fig. 1 Number of publications for different sample fixation strategies published between 2000 and 2016 according to Science Direct, Springer Link, RCS, Taylor & Francis online and ACS using the search term 'metals in lichen', limited to results from the first four pages (for further information, refer to Table S1 in the Supplementary Material)

assessed for its novel use in the extraction of metal(-loid)s from lichens. Furthermore, the stability over time of both total and sequentially extracted metals in lichens without fixation was assessed, shedding some light onto this poorly researched area. Since sample preservation techniques may alter the distribution of species fractions through inter-conversions or losses, this has been assessed for the first time in plants and recommendations made in this regard. These types of assessments are not only extremely important but also are an essential component of method development, and will help evaluate some of the critical issues surrounding fractionation patterns of metal(loid) studies in plants, especially lichens. Moreover, this study aims to serve as an important reference for future metal biomonitoring and speciation studies for air quality assessments.

Materials and methods

Sample collection and cleaning

Lichen samples, *Parmotrema austrosinense* (Zahlbr.) Hale, were collected from the Johannesburg Botanical Gardens, South Africa (GPS co-ordinates 26.159211°S, 28.001003°E), which is 6 km from the city centre. Samples were taken >100 m away from the main road to prevent bias in the results. Samples were collected from all around the tree to negate the effects of the prevailing wind conditions and were collected between 2 and 4 m above ground level, and therefore avoided the stemflow (Monaci et al. 2012) and soil backsplash zone. Samples were collected at least 5 days after a major rain event as this may wash pollutants from the thallus surface (Gailey et al. 1985; Brown and Brown 1991; Kularatne and de Freitas 2013). In this way, surface loading of metals can be re-established. *P. austrosinense* was chosen as it is abundant within the sampling site and is common throughout South Africa. Furthermore, it has been used in a previous study within South Africa (Forbes et al. 2009). It is a foliose lichen, where lichens of this thallus type have been found to have greater bioaccumulation potentials than lichens with a fruticose thallus (Glenn et al. 1991). A degree of heterogeneity in chemical species may be expected between samples within a population (Madrid and Cámara 1997; Gräfe et al. 2014). Furthermore, previous studies have shown that there is often an uneven distribution of accumulated metals on the surface of lichens in accordance with their age (Bargagli et al. 1987). Therefore, to account for this, a large representative sample of whole lichen thalli was collected using plastic forceps and stored in acid-washed polypropylene bottles. Care was taken to ensure that minimal adhering materials were stored with the samples, thus limiting contamination of the lichen surface. Upon returning to the laboratory, the samples were cleaned of adhering debris under a magnification lamp, and gently tapped to remove any remaining extraneous material. Samples were not washed, as evidence has suggested that the washing procedure can alter the metal composition of lichens and the adsorbed atmospheric dust was also of interest and washing would remove these particles (Frati et al. 2005; Cansaran-Duman et al. 2009; González-Miqueo et al. 2010; Kroukamp et al. 2016). Samples were handled with nitrile gloves, torn into smaller pieces and passed through an Endecott mesh sieve, where fractions greater than 1 mm and less than 4.699 mm were collected for further processing. The complete mass of the cleaned sample was 10 g.

Sample preparation and loss in mass

Five samples (0.5 g each) were accurately weighed from the bulk material on an analytical balance (XP205, Mettler Toledo). One of the samples did not undergo any sample preparation, whereas the other four underwent drying in a desiccator for 24 h, oven drying (Memmert Drying Oven) at 50 °C for 24 h, freeze drying (Edwards Tissue Dryer, ETD4) or cryogenic freezing in liquid nitrogen, respectively. Each sample was re-weighed after the drying or freezing procedure to determine the loss in mass. The findings from this component of the study are described in the "Loss in mass" section. The samples were finely homogenised in a clean and dry porcelain pestle and mortar, where this step took place over liquid nitrogen for the cryogenically frozen sample. The bulk, unpreserved sample was kept in a fridge (4 °C) for a period of a month, and the sample preparation techniques repeated on this sample. This was done to assess the validity of air drying lichen samples and storing them for an undisclosed period of time before chemical analysis as has been done previously (Demiray et al. 2012).

Closed vessel microwave digestion procedure

Homogeneity was determined by collecting five subsets of 0.5 g from the bulk material (whole thalli), and each portion was finely ground in a porcelain pestle and mortar. From each ground subset, triplicates of 0.1 g were digested in a Mars 6 microwave digestion system (CEM) using 10 mL HNO_3 (65%, Merck, suprapur) and 1 mL H_2O_2 (30%, Merck, suprapur) (Milestone Application Note HPR-FO-55 2014). The digests were filtered through pre-wet (using 0.5 mL MeOH) Teflon syringe filters (0.45 μm, Membrane Solutions) and diluted to 50 mL using deionised water (18 MΩ cm^{-1}; Milli-Q Millipore). An aliquot (1 mL) of this was taken and further diluted to 10 mL with deionised water for analysis. The results were averaged and the standard deviations between the original five subsets were determined.

Sequential extraction procedure

Triplicates (0.1 g per replicate) of each different sample preparation method were placed into acid-washed centrifuge tubes (Griener Bio-one). The samples were subjected to the following extraction steps based upon a method adapted from Fernández Espinosa et al. (2002):

1. Water extraction (15 mL): samples were placed in acid washed, capped centrifuge tubes and stirred with micro-magnetic stirrer beads for 3 h.

   1.1. Extractions were then centrifuged at 6000 rpm for 10 min (Hettich Zentrifugen, EBA 20) and the supernatant was collected by pipette. The sample was filtered through pre-wet Teflon syringe filters (0.45 μm) and stored at 4 °C for analysis.

2. Hydroxylamine hydrochloride (10 mL): pellet from water extraction step was re-suspended and stirred with micro-magnetic stirrer beads (0.25 M, pH 2, time 5 h).

   2.1. Repeat of step 1.1

3. Peroxide and ammonium acetate step: the pellets from the previous extraction step were quantitatively transferred into perfluoroalkoxy alkane (PFA) vials (Savillex) using H_2O_2 (7.5 mL; Merck, suprapur) and evaporated to near dryness on a hotplate (Stuart) at 95 °C. Another addition of 7.5 mL H_2O_2 was made and evaporated to near dryness. Ammonium acetate (15 mL, 2.5 M, pH 3) was added to each sample. The samples were stirred at room temperature for 1.5 h.

   3.1. Repeat of step 1.1

4. Modified step: the pellets from the previous extraction step were returned to the hotplate at 95 °C in closed vessels and digested with 3 mL of HNO_3 (65%; Merck, suprapur) and 1 mL of HCl (30%; Merck, suprapur). The samples were evaporated to dryness, re-dissolved in 0.23 mL of HNO_3 (65%; Merck, suprapur) and diluted to 15 mL. This step should be adequate to ensure the complete extraction of metals from the sample considering that HNO_3 has been used in this regard (Bargagli et al. 2002). A clear and colourless solution was observed for this final extraction step.

All extraction media were freshly prepared on the day of extraction and used within 24 h.

Analysis, quality assurance and quality control

All analyses took place within 2 days of the sample preparation. Samples were diluted on the day of analysis. Dilution factors for the matrices were pre-determined using 10 ppb standard solutions with Ga, Re and Ru as internal standards in the respective extraction matrices. It was found that a 1:10 dilution for the hydroxylamine hydrochloride extraction matrix, and a 1:15 dilution factor for the ammonium acetate matrix was required to ensure that the internal standards were within the range of 70–130% recovery (Keith 1996). The extracts from the water and final extraction steps, as well as the total extractable metal, were found to require a 1:10 dilution. Sets of standards were freshly prepared on the day of analysis and were appropriately matrix matched to each type of matrix. Procedural blanks were done in triplicate. Samples were analysed on a NexION ICP-MS (PerkinElmer, X-series). Isotopes free of isobaric interferences were chosen for each analyte of interest, and transition elements and metalloids were analysed using a collision gas (He, ≥99.999%, Afrox) to reduce polyatomic interferences. Isotopes which were least abundant, thereby preventing detector saturation, and free of isobaric interferences were chosen for the analysis of alkali earth metals. Samples were analysed for Al, As, Ca, Cd, Cu, Co, Cr, Fe, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Sb, Sc, Se, Sn, Sr, Th, Ti, U, V and Zn. Gallium, Re and Ru were used as internal standards to appropriately match the range of first ionisation potentials of the various analytes and were within the range of 70–130% recovery, thereby indicating that there were no major matrix effects. A certified reference material (CRM) of tea leaves (INCT-TL-1) was used for quality assurance and was digested in the same manner as discussed in the "Closed vessel microwave digestion procedure" section. Analytes Ca, Co, Cr, Cu, K, Mg, Mn, Na, Ni, Pb, Sc, Sr and V were found to be within the 95% confidence interval as indicated on the CRM certificate. Al, As, Cd and Zn were found to be outside these limits. The Al recovery of 126% was likely due to this analyte being more than 10% higher than the last calibration standard. Zn recoveries of 198% recovery in the tea leaves CRM could be indicative of Zn contamination in this sample. As and Cd concentrations in this CRM were below the method detection limit (MDL). Limits of detection (LODs) were determined by 3 $S_{y/x}/b$ and limits of quantification (LOQs) were determined by 10 $S_{y/x}/b$, where '$S_{y/x}$' is the standard deviation of the calibration curve and 'b' is the slope of the curve.

Statistical analysis

Statistical analysis was performed using the statistical program, SPSS 22.0. Appropriate post hoc tests (including one-way ANOVA with post hoc tests of Dunnet's *T*-3 test, Scheffes analysis, homogeneity of variance and Brown-Forsythe or the Student's *t* test) for parametric and non-parametric data were used, and statistically significant differences were determined with significance set at $p < 0.05$. Only results which are statistically significant and above the detection limits of the instrument are discussed here.

Results and discussion

Loss in mass

The change in mass resulting from drying and freezing procedures is often not mentioned in other studies, where concentrations are commonly reported in terms of dried or frozen weight. Since losses due to vaporisation and volatilisation of species may occur in addition to loss of water, by neglecting to mention this information, direct comparisons between different sample preparation strategies may be biased. In this study, it was found that the average loss of mass due to drying the samples in a desiccator, oven at 50 °C and freeze dryer were 5.77, 7.72 and 5.64%, respectively. Findings from this study regarding the percentage loss of mass due to freeze drying procedures were found to be similar to those reported in the study by Carreras and Pignata (2002). Interestingly, there was no observed change in mass using cryogenic freezing procedures. These results show the importance of considering the method of drying employed when comparing the results of different studies.

Homogeneity of elements for total extractable metal analysis, extraction efficiency and storage for sequential extractions

As can be seen in Table 1, samples were found to be adequately homogenous (<5% RSD) for most elements of interest, with the exclusion of alkali earth elements Ca and Na and trace elements As, Ni, Se, Sr and Ti, of which Se was below the LOQ. The heterogeneity of Ca could be due to contamination of the samples by the porcelain pestle and mortar. Observed heterogeneity in Ni correlates with the findings from a study by Carreras

Table 1 Test for homogeneity and stability of elements in the bulk lichen sample, where significant differences ($p < 0.05$) are indicated by Y

Element	Homogeneity (% RSD)	Recovery of combined sequential extraction steps in comparison to total extractable metal values (%) and % RSD					Statistically significant difference before and after storage for 1 month						
							Extraction step 1					Extraction step 2	
		NO	DES.	OVEN	FRE.	CRYO	NO	DES.	OVEN	FRE.	CRYO	NO	DES.
Li	2.61						X	X	–	X	X	–	–
Na	6.72						X	X	X	X	X	–	–
Mg	3.64						X	X	X	X	X	Y	Y
Al	1.97	32.0 ± 9.4	33.8 ± 14.4	31.1 ± 7.6	23.4 ± 5.2	16.3 ± 7.5	X	X	X	X	Y	X	X
K	4.18						Y	X	X	Y	X	Y	X
Ca	10.01						X	X	X	X	X	Y	Y
Sc	1.01	–	–	–	–	–	–	–	–	–	–	–	–
Ti	5.71	28.6 ± 3.4	35.3	33.4 ± 4.8	27.8 ± 4.5	24.1 ± 4.5	–	–	–	–	–	–	–
V	2.37	25.3 ± 1.0	21.3 ± 51.4	19.8 ± 3.6	19.9 ± 1.8	20 ± 2.7	Y	X	Y	X	Y	X	X
Cr	4.28	–	–	–	–	–	–	–	–	–	–	–	–
Mn	3.08	101 ± 6	92.5 ± 18.8	93.7 ± 17.6	84.1 ± 12.8	74.8 ± 8.9	X	X	X	X	Y	X	X
Fe	1.7	28.6 ± 13.0	27.1 ± 17.1	26.4 ± 5.5	22.8 ± 9.7	17.9 ± 6.7	X	X	X	X	Y	X	X
Co	2.14	–	–	–	–	–	–	–	–	X	–	–	–
Ni	10.76	46.5 ± 9.3	64.8 ± 27.0	56.7 ± 10.4	50.8 ± 15.7	34.2 ± 6.8	X	Y	X	X	Y	X	X
Cu	4.21	100 ± 46	66.7 ± 23.8	66.1 ± 19.3	67.1 ± 12.6	34.8 ± 6.55	X	X	Y	X	X	Y	X
Zn	4.8	166 ± 47	138 ± 29	125 ± 43	107 ± 13	133 ± 21	X	X	X	X	X	X	X
As	7.17	134 ± 36	112 ± 51	107 ± 26	113 ± 32	106 ± 27	X	X	X	X	X	–	–
Se	63.77						–	–	–	–	–	*	*
Sr	7.01	124 ± 50	223 ± 129	105 ± 28	160 ± 85	62.7 ± 29.8	X	X	X	X	Y	X	–
Sn	3.71	–	–	–	–	–	X	X	X	X	X	–	–
Pb	3.32	82.0 ± 3.34	70.9 ± 10.0	69.1 ± 4.6	66.3 ± 3	58.8 ± 4.1	X	X	X	Y	X	X	X
U	6.02						–	–	–	–	–	–	–

Element	Statistically significant difference before and after storage for 1 month												
	Extraction step 2			Extraction step 3					Extraction step 4				
	OVEN	FRE.	CRYO	NO	DES.	OVEN	FRE.	CRYO	NO	DES.	OVEN	FRE.	CRYO
Li	–	–	–	–	–	–	–	–	–	–	–	–	–
Na	–	–	–	–	–	–	–	–	–	–	–	–	–
Mg	Y	X	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	–
Al	X	X	X	Y	Y	Y	Y	X	X	X	Y	Y	X

Table 1 (continued)

Element	Statistically significant difference before and after storage for 1 month												
	Extraction step 2			Extraction step 3					Extraction step 4				
	OVEN	FRE.	CRYO	NO	DES.	OVEN	FRE.	CRYO	NO	DES.	OVEN	FRE.	CRYO
K	Y	X	Y	Y	Y	–	–	Y	X	Y	X	X	Y
Ca	Y	Y	Y	X	X	X	Y	X	X	X	X	X	X
Sc	X	–	–	–	–	–	–	–	–	–	–	–	–
Ti	–	–	–	X	X	Y	X	X	X	X	X	X	–
V	X	X	X	–	Y	–	Y	–	–	–	–	–	–
Cr	–	–	–	–	–	–	–	–	–	Y	–	Y	–
Mn	X	X	X	X	X	X	X	Y	Y	Y	Y	Y	–
Fe	Y	Y	X	X	X	Y	X	X	Y	Y	Y	Y	–
Co	–	–	–	–	–	–	–	–	–	–	–	–	–
Ni	X	X	X	–	–	–	–	–	Y	X	X	X	–
Cu	Y	Y	X	X	X	X	X	X	Y	X	X	Y	–
Zn	X	X	X	X	X	X	X	Y	X	X	X	X	X
As	–	–	–	X	X	Y	X	Y	Y	Y	X	Y	–
Se	*	–	–	*	–	–	–	–	–	–	–	–	–
Sr	X	X	X	X	X	X	X	X	X	X	X	X	X
Sn	–	–	–	–	X	X	X	–	X	X	Y	Y	–
Pb	X	X	X	Y	X	X	X	Y	X	X	X	X	–
U	–	–	–	–	–	–	–	–	–	–	–	–	–

Values which are not statistically significantly different ($p > 0.05$) are indicated by *X*. Only values above the limit of detection (LOD) are reported. NO: no sample preparation, DES.: dried in a desiccator, OVEN: dried in an oven at 50 °C for 24 h, FRE.: freeze dried, CRYO: cryogenically frozen in liquid nitrogen, * below LOQ, - below detection.

and Pignata (2002), which found a great degree of heterogeneity in Ni concentrations between sampling sites. The primary hypothesis for this was that metals, such as Ni, occur predominantly in the particulate form, thus implying an uneven exposure to this metal between different lichens within the same population. Other possible reasons for the observed heterogeneity for the specified metals are uneven distributions of calcium oxalate crystals on the lichen surface. Formation of these crystals occur in response to toxic metals entering the lichen, and facilitate their sequestration and detoxification (Bačkor and Loppi 2009). There is also evidence of uneven accumulation of metals on the surface of foliose lichens according to their age (Bargagli et al. 1987), which may further account for some of the observed heterogeneity in the samples. In contrast to the study by Carreras and Pignata (2002), this study found Co, Fe and Mn to be adequately homogenous within the sample matrix. This may be due to a smaller bulk sample (6 g), or a larger geographical study area comprising a number of different pollution sources in their study.

In terms of total extractable metal concentrations, cryogenically frozen samples exhibited the best overall reproducibility between 'no storage' and '1-month storage' for As, Cr, Cu, Fe, K, Li, Mg, Mn, Sr, Ti and Zn (0.05–13.5% RSD; results not shown). This stability is attributed to the low temperature of liquid nitrogen; which prevents the loss of volatile analytes, the increased ease and efficiency of homogenisation as opposed to other methods and the limited contact time with the pestle and mortar. Using this technique, it was found that samples could be stored for a period of 1 month at 4 °C for total extractable metal analysis without using sample preservation techniques if the mentioned elements are of interest. Samples involving no sample preparation were fairly reproducible, where the best reproducibility was found for Ca, Cr, Li, Mn, Ni, Sn and Sr (2–8% RSD; results not shown). Freeze drying procedures had poor reproducibility with the exception of Cu, Mn, Na, Ni, Ti and V (2.5–14.4% RSD; results not shown) and are only recommended if these analytes are of interest. Losses observed from using this technique, and other techniques which involve the heating (oven drying) or drying (desiccator drying) of the sample, may be due to the remobilisation of elements within the lichen into more volatile forms over time. These findings contrast the results of Hjorth (2004) in lake sediments, which state that freeze drying is a convenient way to preserve and stabilise samples if the total extractable metal and metalloid concentration is of interest. Presumably, variations in vacuum pressure and length of vacuum time in freeze drying techniques between months may play a role in the volatilisation of certain pre-disposed metals. For all techniques other than cryogenic freezing, the amount of processing time required to achieve the desired homogeneity differed, and since the homogenisation process generates heat, this could be a further contributing factor to varying degrees of liberation of analytes in comparison to cryogenically frozen samples.

The combined extraction efficiencies (sum of extraction concentrations/total extractable metal concentrations × 100%) for most metals in comparison to the total extractable metal concentrations was found to be relatively poor with the exception of As, Cu, Mn, Pb, Sr and Zn (Table 1, column 3). Despite seemingly low recoveries, they are higher than those reported in other speciation studies, such as those reported for As which gave recoveries of 7–25% in the fruticose lichens *Alectoria ochroleuca* and *Usnea articulate* (Kuehnelt et al. 2000), and 2–9.3% in the foliose lichen, *Parmelia sulcata* (Farinha et al. 2004). The results from the latter study used a sequential extraction with water, $CaCl_2$ and H_3PO_4 as the first, second and third extraction media, respectively, and assumed that most As was primarily bound in a refractory form. This contrasted to findings from our study which show that most of the As is bound to organic and sulphides which are released under oxidising conditions (Fig. 2c, d). Samples which underwent no sample preparation for Cu, Fe, Mn, Pb, Sr and V and those which were dried in a desiccator for Al, Cu, Fe, Mn, Ni, Pb, Ti and V had higher average accumulative extraction recoveries than most other sample preparation techniques, although the % RSDs for the latter were very high. Observed poor recoveries could be due to a number of factors, including losses due to the adsorption of analytes to the walls of the vessels employed in each extraction step. Another explanation could be concentrations in the extracts after dilution being below the LOD, thus giving a 'false low', and varying levels of interaction between the sample and the extraction matrix. Although plant material is known to exhibit hydophobic behaviour due to its low density (Sánchez-Moreno et al. 2010), a pronounced and comparatively poor interaction of cryogenically frozen samples with the extraction media was observed. This may account for poor extraction efficiencies using this technique. It is evident that the gross assumption that cryofixation and freeze drying preservation techniques are able to ensure that the original species integrity is maintained is one which requires re-visiting.

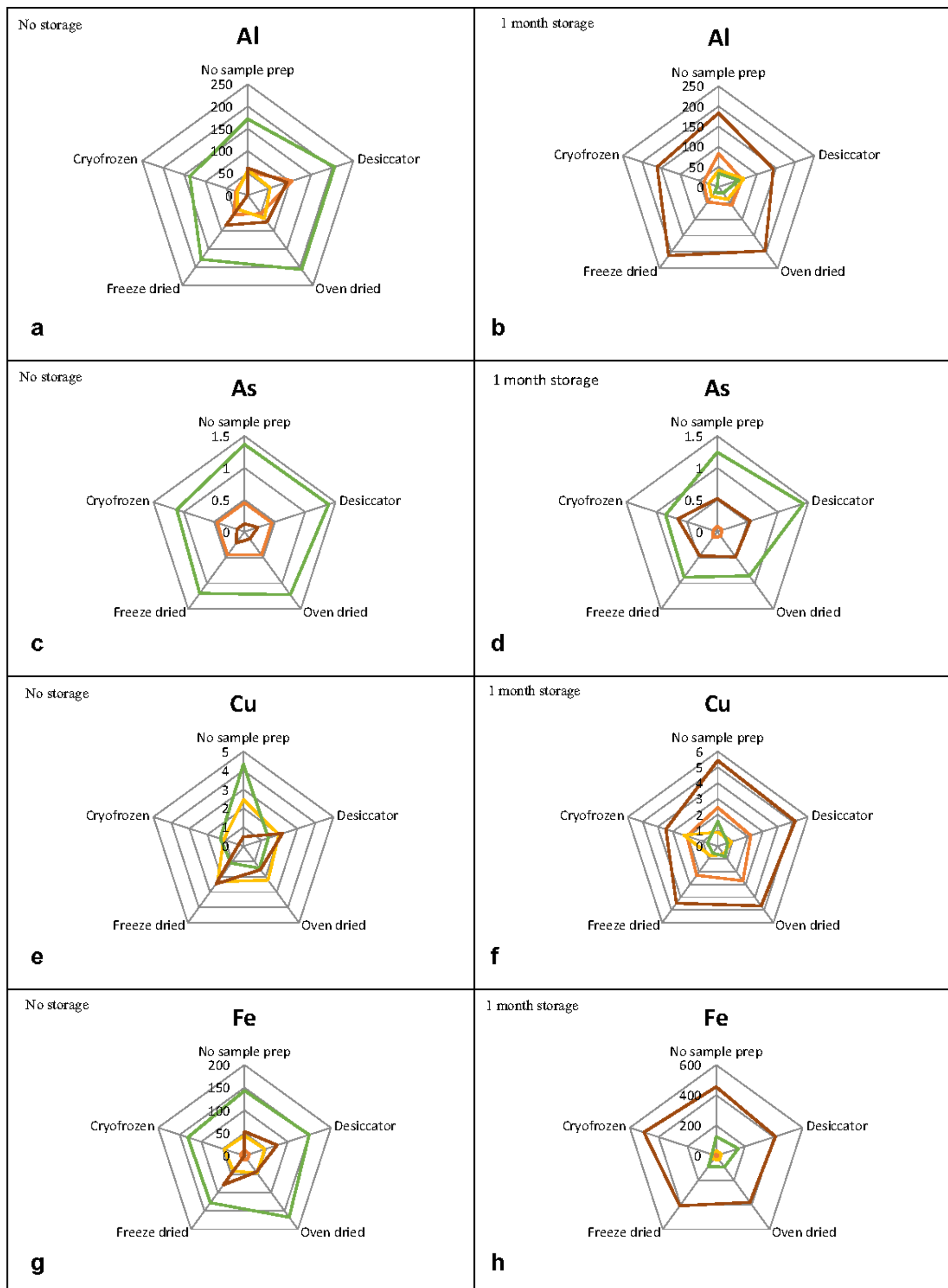

Fig. 2 Average concentrations (blank subtraction values) of selected metal(loid)s in the bulk lichen sample between different extraction media and sample preparation strategies. Bulk material was stored for a period of 1 month in a fridge at 4 °C without fixation. Values reflected are in μg/g dry weight, where *orange* = soluble/exchangeable metals, *yellow* = metals bound to carbonates and oxides and which are reducible, *green* = metals bound to organic matter and sulphides and which are oxidizable, *red* = non-bioavailable/residual

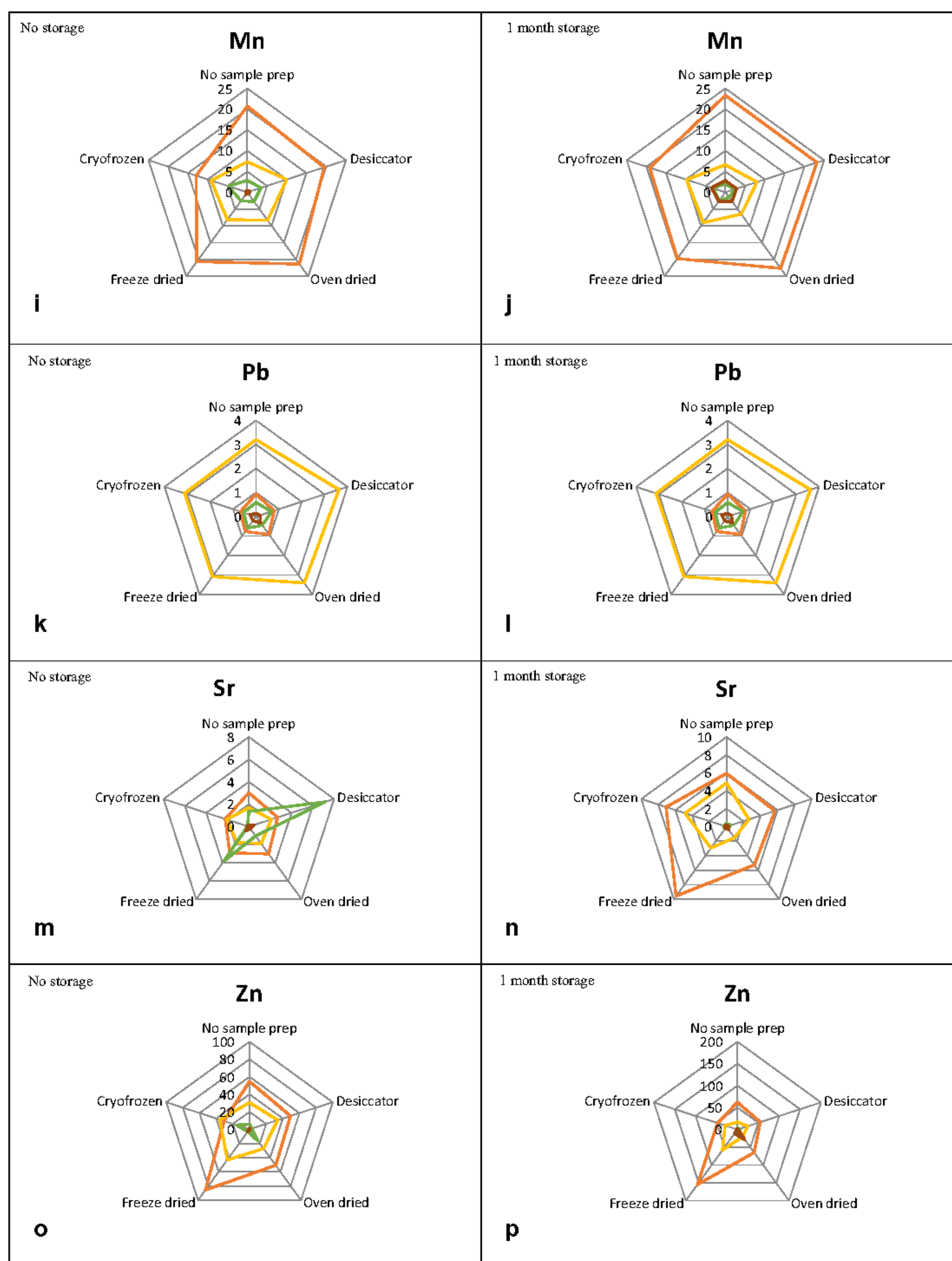

Fig. 2 (continued)

Regarding storage for sequential extractions (Table 1), most of the analytes deviated over storage of 1 month, where a few of these specific analytes can be more effectively visualised in Fig. 2a–p. According to Fig. 2a, b, Al was almost completely transformed from being more available in the oxidizable fraction to being non-bioavailable, and these results were statistically significant ($p < 0.05$; Table 1), thus indicating that storage over a month period for the sequential extraction of Al is not appropriate. As can be seen in Fig. 2i, j, there was a slight

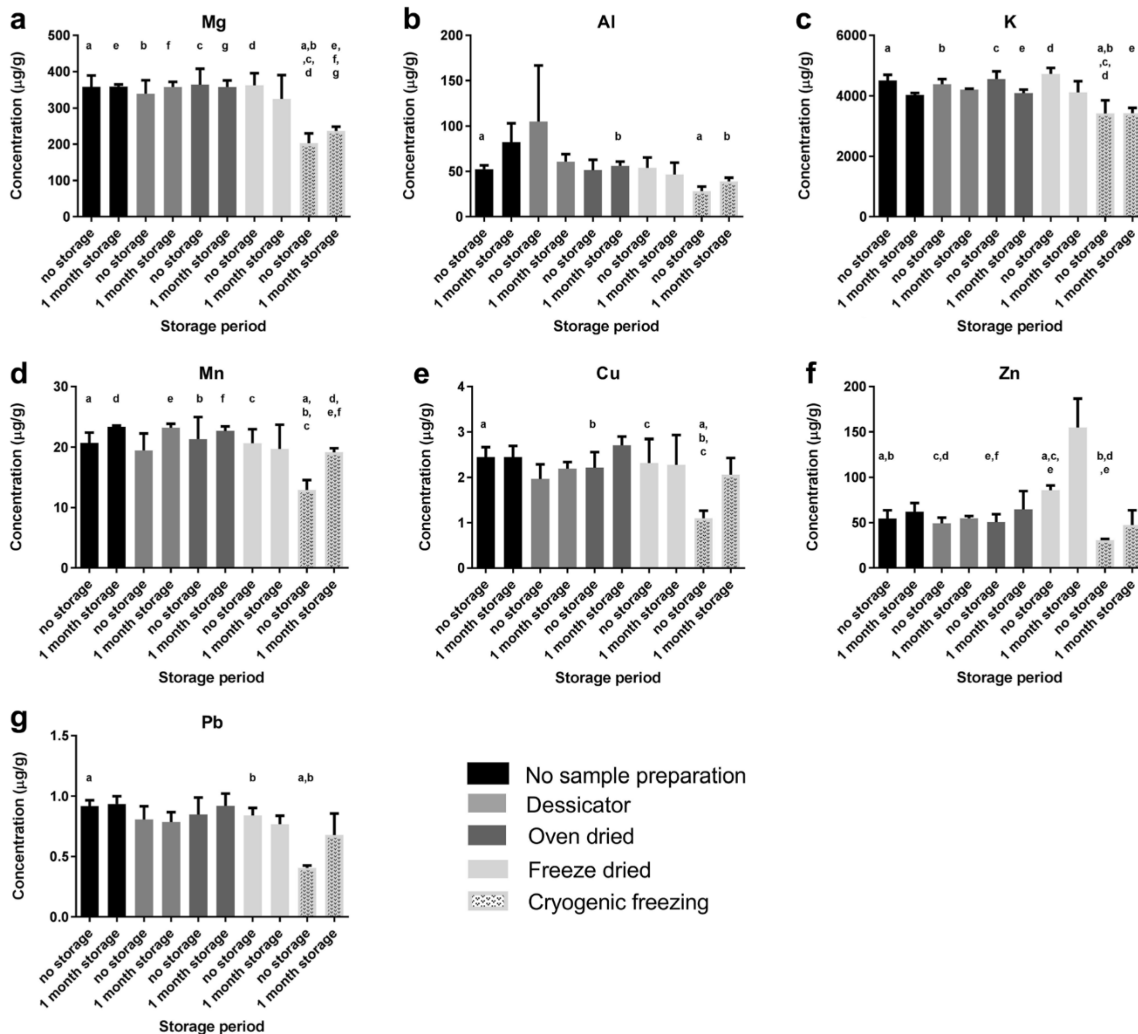

Fig. 3 Average concentrations (blank subtracted values) of selected metal(loid)s in the bulk lichen sample from water extraction step. Only elements for which results were significantly different ($p < 0.05$) are reported, where significance is indicated by corresponding letters above bars. Values are based upon the dry weight of the samples (error bars are ±standard deviation)

remobilisation of Mn into the non-bioavailable fraction between no storage and 1-month storage samples, where this difference in the non-bioavailable fraction was statistically significantly different ($p < 0.05$; Table 1) for all sample preparation types. Iron concentrations between no storage and 1-month storage shifted from being predominantly in the oxidizable form to the non-bioavailable form (Fig. 2g, h) with additional transformation being observed from reducible into the non-bioavailable fraction, where these differences were statistically significant ($p < 0.05$; Table 1). Sample preparation strategies appeared to have a great effect on the availability of Cu in the various extraction media (Fig. 2e, f). This was especially prominent before biotransformation could take place during storage (no storage results), where Cu was more available in the reducible and oxidizable fractions using no sample preparation techniques. Freeze drying and drying in a desiccator found more Cu to become available for extraction in the final extraction media. Between no storage and 1-month storage samples, there was a large transformation of Cu into the non-bioavailable fraction and some mobilisation into the soluble fraction which may indicate detoxification mechanisms of the lichen to sequester Cu from the thallus onto the lichen surface.

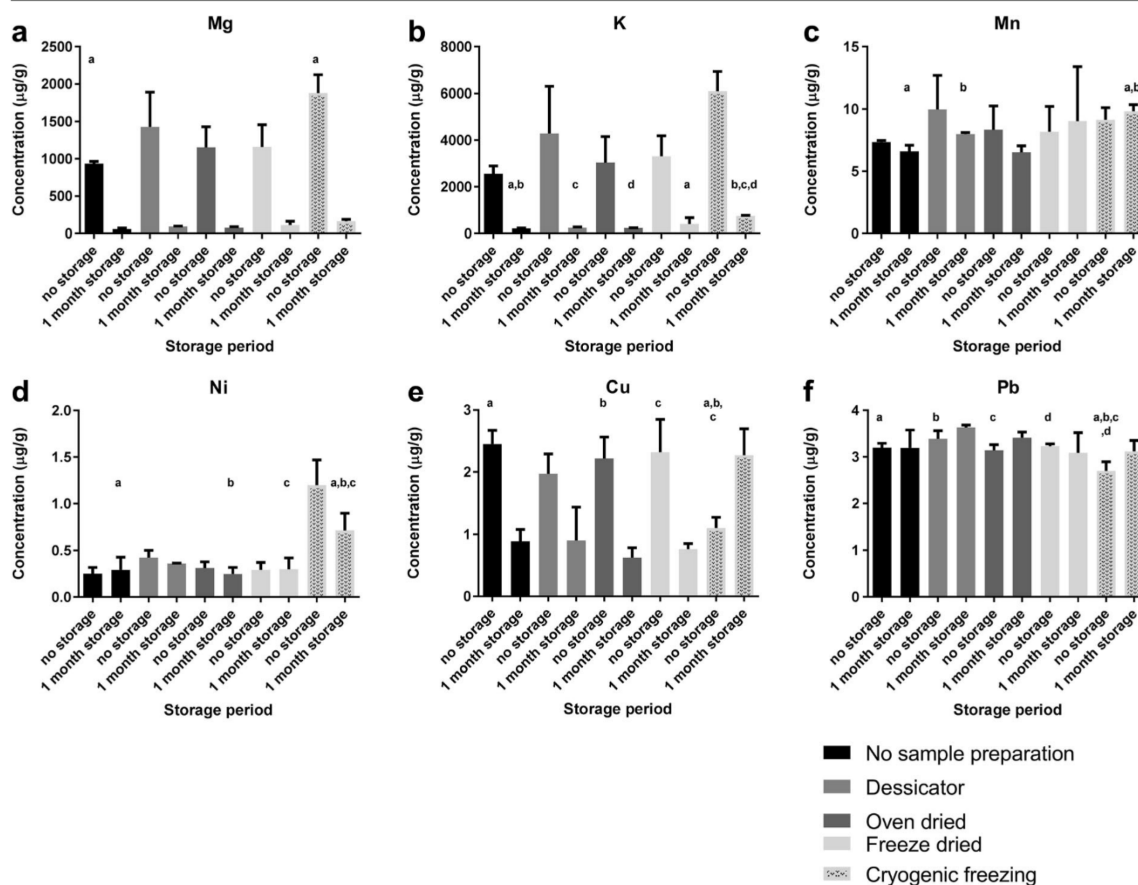

Fig. 4 Average concentrations (blank subtracted values) of selected metal(loid)s in the bulk lichen sample from hydroxylamine hydrochloride extraction step. Only elements for which results were significantly ($p < 0.05$) are reported, where significance is indicated by corresponding letters above the bars. Values are based upon the dry weight of the samples (error bars are ±standard deviation)

Storage for longer periods of time may be suitable for a few elements such as Zn and Sr. Although Zn showed a change in the average concentrations of the oxidizable and non-bioavailable fractions between no storage and 1-month storage samples (Fig. 2o, p), these differences were not statistically significant (Table 1). Results from cryogenic freezing procedures, however, were shown to exhibit statistically significant differences ($p < 0.05$; Table 1), and this was apparent for a number of other elements, therefore indicating that this sample preparation strategy should not be recommended for sequential extractions of metal(loid)s in lichens. Strontium averages between no storage and 1-month storage (Fig. 2m, n) varied greatly depending upon the sample preparation strategy; however, differences between months were statistically significant only for cryogenic freezing procedures (Table 1), confirming earlier observations that this technique is not suitable for sequential extractions. As can be seen in Fig. 2k, l, Pb trends between no storage and 1-month storage samples did not deviate greatly. Sample preparation strategies such as freeze drying, no sample preparation and cryogenic freezing were statistically significantly different ($p < 0.05$) between no storage and 1-month storage, indicating an effect of these sample preparation strategies on the availability of Pb.

Evaluation of the merits of different sample preparation strategies for the sequential extraction of metals from lichens

It should be noted that a sequential elution technique (SET) has previously been applied in the determination of

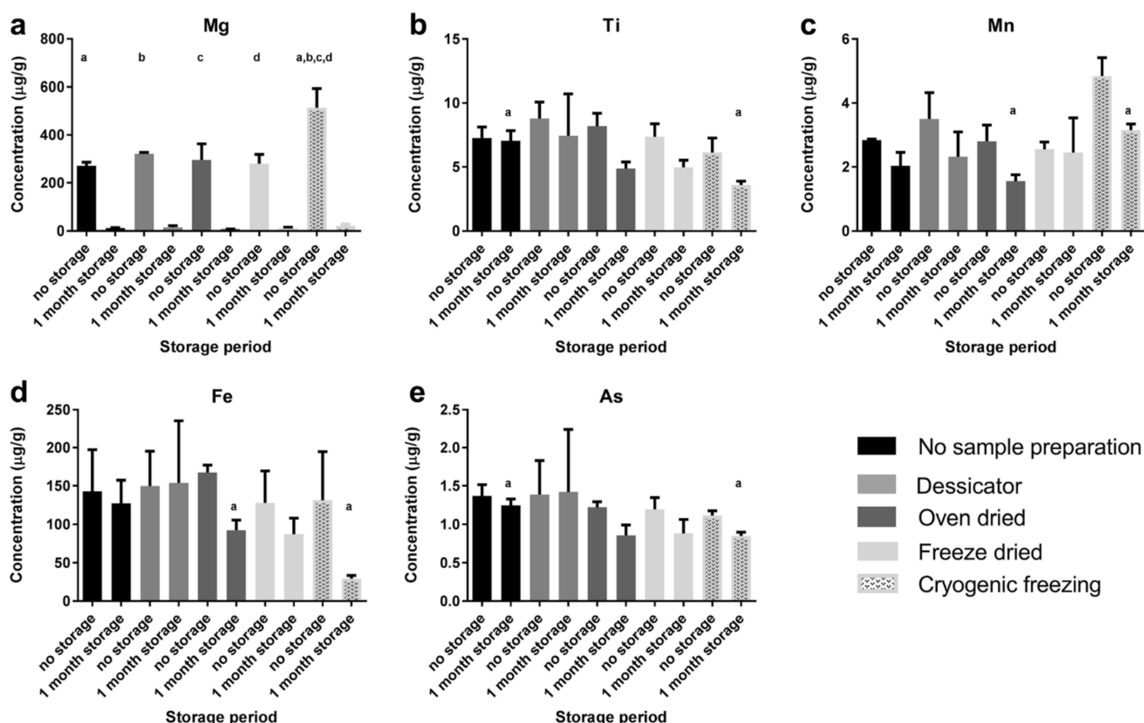

Fig. 5 Average concentrations (blank subtracted values) of selected metal(loid)s in the bulk lichen sample from peroxide and ammonium acetate extraction step. Only elements for which results were significantly different ($p < 0.05$) are reported, where significance is indicated by corresponding letters above the bars. Values are based upon the dry weight of the samples (error bars are ±standard deviation)

elemental distributions within cryptogams (primarily bryophytes) in order to determine metabolic pathways (Pérez-Llamazares et al. 2011). This technique thus has a different focus to that of this study, as the SET determines extra-, inter- and intra-cellular concentrations of elements in addition to surface particulate fractions, which therefore requires that the permeability of the cell membrane is not altered for analysis by SET. Inherent potential errors which have resulted in SET not having been widely employed in field studies or for routine use include potential loss of particulate matter during washing to determine intercellular contents, and difficulty in evaluating efficiency of the extractions, particularly for elements which have been less studied using the SET (Pérez-Llamazares et al. 2011). In this regard, only a limited number of elements have been studied to date in lichens (Ca, Cd, Cu, Fe, K, Mg, Mn, Na, Pb and Zn), and most of these have been laboratory-based experiments involving exposure of lichens to the contaminants in solution (Pérez-Llamazares et al. 2011). In this study, we have therefore focused on the comparison of sample preparation strategies for the determination of metal(loids) in lichens based on field studies reported in the literature (Fig. 1 and Table S1 in the Supplementary Material).

We found that the loss of moisture in the first month (no storage) due to sample preparation techniques was 5.94, 7.82 and 5.16% for desiccator, oven dried and freeze dried samples, respectively. The second month resulted in water losses of 5.60, 7.62 and 6.13% for desiccator, oven dried and freeze dried samples, respectively. Consequently, change in the water percentage of the samples between the 2 months was considered to be negligible and thought not to contribute towards alterations in the observed chemical fractionation patterns between months.

As shown in Fig. 3 in the water extraction step (step 1), the cryogenically frozen samples were found to have significantly lower recoveries for Al, Cu, K, Mg, Mn, Pb and Zn in comparison to many of the other sample preparation techniques. It was thought that this was due to a poorer level of interaction between the extraction matrix and the cryogenically frozen samples than those observed in other approaches, as was visually evident during sample

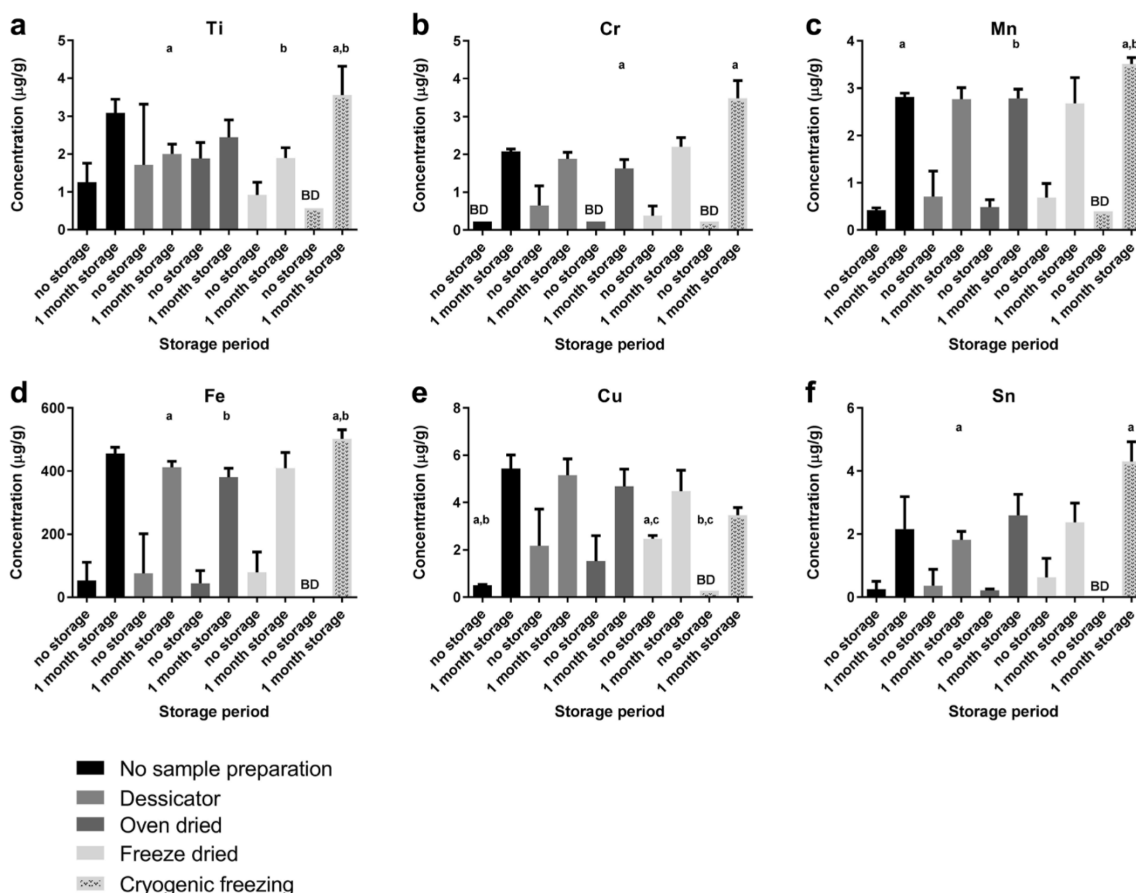

Fig. 6 Average concentrations (blank subtracted values) of selected metal(loid)s in the bulk lichen sample from the final extraction step using nitric acid. Only elements for which results were significantly different ($p < 0.05$) are reported, where significance is indicated by corresponding letters above the bars. Values are based upon the dry weight of the samples (error bars are ±standard deviation)

preparation. For almost all of the mentioned elements, these concentrations were higher in the subsequent extraction steps. From this information, we can deduce that the cryogenic freezing of samples is not an appropriate sample preparation technique for the sequential extraction of analytes from lichens, as it appears to adversely affect the availability and interaction capabilities of the lichen with the extraction media (specifically water). This opinion contrasts to those of Krämer et al. (2000) and Laurette et al. (2012), who assume that cryofixation is the ideal method of preserving the original species integrity in herbs, and oilseed rape and sunflowers, respectively. It should be noted, however, that their studies speciated metals using X-ray absorption spectroscopy (XAS) which does not rely upon the extractability of the various metal(loid) species; thus, for XAS, cryofixation may indeed be an appropriate method of sample preparation. It is necessary to further evaluate the results from this study using chromatography-based species separation methods. Such studies would be able to indicate whether or not the observed differences in partitioning are solely due to the interaction of the matrix with the sample or if these differences are due to changes in elemental speciation patterns.

Although freeze drying has been said to reduce water-based reactions (Ure 2002), in some cases, it has been found to modify the major chemical speciation patterns (Ure 2002) and eliminate particular volatile species from the sample in sediments (Hjorth 2004) and wheat and Indian mustard plants (Eiche et al. 2015). Zinc and Cu, however, are not volatile and concentrations observed in no storage samples using freeze drying were statistically significantly higher ($p < 0.05$) than the other sample

preparation techniques, in the water (Fig. 3e, f) for these elements, and in the final extraction step for Cu (Fig. 6e). Although this observation cannot be fully explained, it can be hypothesised that this trend may be attributed to the slightly higher degree of heterogeneity (4.80 and 4.21% for Zn and Cu, respectively) observed for these elements, respectively. In terms of Zn in the first extraction step, it is also possible that the vacuum experienced during freeze drying procedure may have resulted in Zn cations being drawn from the cells into the intracellular spaces, allowing them to be extracted via shaking (Hauck et al. 2002).

As previously mentioned, many of the elements which had poor recoveries in the cryogenic freezing sample preparation water extraction step were recovered in the subsequent extraction steps. In the case of Cu (Figs. 3e, 4e and 6e), K (Figs. 3c and 4b), Mg (Figs. 3a, 4a and 5a), Mn (Figs. 3d, 4c, 5c and 6c) and Ni (Fig. 4d), differences in recoveries were mostly statistically significant ($p < 0.05$). Samples which did not undergo any preparation and those which were dried in an oven and a desiccator did not exhibit any statistically significant difference and hence are not indicated in the results but can be viewed in Table 1 and Fig. 2. Samples which did not undergo any sample preparation and those which were dried in an oven and a desiccator did not exhibit any statistically significant differences ($p < 0.05$) to one another, indicating that all of these techniques are appropriate for sequential extraction methods, provided that the sample is promptly processed.

Conclusions

Total extractable metal(loid) and speciation determinations in biomonitors may provide a useful contribution to environmental assessments. This study has proven that the cryogenic freezing of samples for total extractable metal analysis is the preferred sample preparation technique for lichens in comparison to other methods. A sample mass of 10 g, and of a particle size >1 and <4.699 mm, was found to exhibit adequate homogeneity for most elements. Although the use of sequential extractions is not considered to be completely able to isolate different chemical species of an analyte due to the co-extraction of species, this study has shown that sequential extractions do show some promise in providing a general indication of metal fractionation patterns in lichens for certain elements. If speciation studies are of interest, samples should be analysed as soon as possible and should not be stored over extended time periods without fixation since biotransformation of chemical species is likely to occur. It is further suggested that column chromatography be used to check if the statistical differences observed during sequential extractions for cryogenically frozen samples are due to the level of interaction of the sample with the extraction matrix or due to a change in the chemical species of the analyte as a consequence of this technique. This study demonstrates the influence of sample preparation, preservation and storage on the concentration and speciation of atmospheric metal(-loid)s in lichens. The poor choice of these may adversely affect the integrity of the analytical results obtained; therefore, the impact which these variations may pose should be carefully considered when comparing results from different studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Paper 3- Supplementary Information

Article Title: Comparison of sample preparation procedures on metal(loid) fractionation patterns in lichens

Journal Name: Environmental Monitoring and Assessment

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Table S1 Comparison of popularity of different sample preparation techniques for total metal analysis

Sample preparation method	Thallus type	Species of lichen	Location	Method of Analysis	Analytes	Reference
No sample preparation	Foliose-fruticose	*Pseudevernia furfuracea (foliose-fruticose)*	Italy	ICP-MS*	Al, As, Cd, Cr, Co, Cu, Fe, Mo, Ni, Pb, Ti V, Zn	(Giordano , et al., 2005)
Dried	Foliose	*Remototrachyna awasthii (foliose)*	India	ICP-MS	Al, As, Cd, Cr, Fe, Pb, Mn, Zn	(Bajpai, et al., 2013)
Dried	Foliose	*Hypogymnia physodes (foliose)*	Finland	ICP-OES*	Cd, Cr, Cu, Fe, Ni, Pb, V, Zn	(Lippo, et al., 1995)
Dried	Squamulose, crustose, crustose, foliose	*Cladonia furcata (squamulose), Hypocenomyce scalaris (squamulose), Lepraria incana (crustose), Lepraria elobata (foliose)*	Poland	FAAS*	Pb, Zn	(Pawlik-Skowrońska & Bačkor, 2011)
Air dried	Foliose	*Pseudevernia furfuracea*	Turkey	FAAS	Cr, Fe, Mn, Pb, Zn,	(Cansaran-Duman, et al., 2009)
Air dried	Foliose	*Xanthoria parietina (foliose)*	Turkey	ICP-MS	Al, As, Cd, Co, Cu, Fe, Hg, K, Mn, Ni, Pb, S, Ti, Tl, V, Zn	(Demiray, et al., 2012)
Air dried	Foliose	*Parmelia caperata(foliose)*	Italy	FAAS, GFAAS*, CVAAS*	Al, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn	(Loppi & Pirintsos, 2003)
Air dried	Foliose	*Parmelia sulcata (foliose)*	Ghana	TINAA*	Al, As, Cd, Co, Cu, Hg, Mn, Sb, Th, V	(Boamponsem, et al., 2010)
Air dried	Umbilicate	*Umbilicaria decussata (umbilicate)*	Antarctica	FIAS hydride generator*	Hg	(Bargagli, et al., 2005)
Air dried	Fruticose, foliose, foliose, foliose, foliose, umbilicate,	*Alectoria (fruticose), Heterodermia (foliose), Hypogymnia sp. (foliose), Hypotrachyna (foliose),*	Himalayas, Kenya, Italy	ET-AAS*, INAA*	As, Br, Cd, Ce, Co, Cr, Cs, Cu, Fe, Hf, K, La, Mg, Mn, Pb, Rb, Sb, Sc, Se, Sm, Th, Zn, V	(Bergamaschi, et al., 2004)

	foliose, foliose, foliose, foliose, squamulose, umbilicate, umbilicate, fruticose, foliose, foliose	*Parmotrema(foliose), Umbilicaria cylindrica (umbilicate), Usnea sp. (foliose), Brodoa intestiniformis (foliose), Parmotrema lobulascens (foliose), Parmotrema reticulatum (foliose), Rhizoplaca melanophthalma (squamulose), Umbilicaria decussata (umbilicate), Umbilicaria subglabra (umbilicate), Letharia vulpina (fruticose), Pseudevernia furfuracea*				
Air dried on blotting paper	Foliose	*Hypogymnia physodes (foliose)*	Russia	MC-ICP-MS*	^{204}Pb, ^{206}Pb, ^{207}Pb, ^{208}Pb	(Spiro, et al., 2004)
Dried at room temperature	Foliose	*Parmelia caperata (foliose)*	Italy	ICP-OES, ZE-TAAS, FIMS	Al, As, B, Ba, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, S, V, Zn	(Bargagli, et al., 2002)
Dried at room temperature	Foliose	*Parmotrema austrosinense (foliose)*	South Africa	FAAS	Pb, Mn	(Forbes, et al., 2009)
Dried at room temperature (25 °C)	Fruticose	*Usnea longissima (fruticose), Usnea diffracta (fruticose)*	Russia	γ-ray spectrometry	$^{134}Cs/^{137}Cs$	(Ramzaev, et al., 2014)
Dried in paper bags	Foliose-Fruticose	*Pseudevernia furfuracea*	Italy	ICP-OES, ICP-MS	Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn	(Bari, et al., 2001)
Dried in paper bags	Foliose	*Xanthoria parietina (foliose)*	Slovakia	ICP-MS, ICP-OES	Al, As, Ca, Cd, Cu, Cr, Fe, Hg, Mn, Ni, Pb, S, Ti, V, Zn	(Paoli, et al., 2014)
Stored in paper bags and dried at room temperature	Foliose, fruticose	*Nephroma antarcticum (foliose), Usnea sp. (fruticose)*	Chile	ICP-MS, HR-CSAAS*, FIMS	Al, Ba, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, S, Sr, V, Zn	(Monaci, et al., 2012)
Dried in a desiccator	Foliose	*Pseudevernia furfuracea*	Italy	ICP-OES, FAAS, ZE-TAAS, FIMS*	Al, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, Pb, V, Zn	(Adamo, et al., 2007)
Dried in a desiccator	Squamulose, foliose, crustose, squamulose, foliose	*Cladonia rangiformis (squamulose), Parmelia sulcata (foliose), Pertusia sp. (crustose), Cladonia convoluta (squamulose), Xanthoria parietina (foliose)*	Greece	GFAAS	Cd, Cr, Cu, Fe, Mn, Ni, Pb	(Tsikritzis, et al., 2002)

Air dried in paper bags and then oven dried at 70 °C for 24 hr	All foliose	*Peltigera aphthosa (foliose), Peltigera neopolydactyla (foliose), Peltigera scabrosa (foliose), Nephroma arcticum(foliose)*	Canada	ICP-MS	Al, Ca, Cd, Co, Cr, Cu, K, Mg, Mn, Mo, Na, Ni, P, Pb, Ti, V, Zn	(Darnajoux, et al., 2015)
Dried in paper envelopes for 2 weeks and then dried at 90 ˚C for 24 hr	Squamulose, fruticose	*Cladonia humulis (squamulose), Stereocaulon japonicum (fruticose)*	Japan	ICP-MS	Al, Ca, Cu, Fe, Mg, Mn, Na, Zn	(Nakajima, et al., 2013)
Air dried or Oven dried at 40 °C	Foliose	*Parmelia sp. (foliose)*	Vulcano Island, Archipelago	INAA, ICP (Type of ICP not mentioned)	Al, As, Au, Ba, Br, Ca, Co, Cr, Cs, Cu, Fe, Hf, K, Mg, Mo, Mn, Na, Ni, P, Pb, Rb, Sb, Sc, Sr, Ti, Th, U, V, Y, Zn	(Dongarrá & Varrica, 1998)
Air dried then oven dried at 40 °C	All foliose	*Parmelia conspersa (foliose), Xanthoria calcicola (foliose)*	Sicily	ICP-MS	Ca, Cu, Mg, Mn, Ni, P, Pb, Sr, Ti, V, Zn	(Varrica, et al., 2000)
Air dried for 15 hr and oven dried at 90 ˚C	Crustose, foliose, fruticose	*Acarospora gwynii, Buellia grimmae, B. Pallida, Lecanora expectans, L. fuscobrunnea, Lecidea cancriformis, L. siplei, Rhizocarpon flavum, Rinodina olevaceobrunnea, Physcia caesia, Usnea longissimi,*	Antarctica	FAAS	Cr,Cu, Fe, Pb, Zn	(Upreti & Pandey, 2000)
Oven dried at 40 °C	Foliose	*Pseudevernia furfuracea*	Italy	ICP-MS	Al, As, Ca, Cd, Co, Cu, Fe, K, Mg, Mn, Mo, Ni, Pb, Ti, V, Zn	(Adamo, et al., 2003)
Oven dried at 40 ± 2 °C	Foliose	*Dirinaria picta (foliose)*	Singapore	ICP-MS	As, Cd, Cu, Ni, Pb, Zn	(Ng, et al., 2005)
Oven dried at 60 ˚C for 24 hr	Foliose	*Pyxine cocoes (foliose)*	India	ICP-MS	Al, As, Cd, Cr, Cu, Fe, Pb, Zn	(Bajpai & Upreti, 2012)
Oven dried at 60 °C for 24 hr	Fruticose	*Flavocetraria nivalis (fruticose)*	West Greenland	FAAS, GFAAS,	Pb, Zn	(Søndergaard, et al., 2011)
Oven dried at 65 ˚C	All foliose	*Hypogymnia physodes (foliose), Xanthoria parietina (foliose), Parmelia sulcata(foliose)*	Poland	FAAS	Cu, Fe, K, Mg, Mn, Ni, Pb, Zn	(Parzych, et al., 2016)
Oven dried at 80 °C	All foliose	*Dirinaria applanata, Parmelia isidiza, Parmelia tincorum, Coccocarpia pellita, Heterodermia diademata, Dirinaria*	Nepal	FAAS	Cr, Ni, Pb, Zn	(Pandey, et al., 2002)

		consimilis, Parmelia praesorediosa, Leptogium denticulatum, Phaeophyscia hispidula, Heterodermia pellucida, Pyxine messnerina				
Oven dried at 85 °C for 48 hr	Squamulose	*Cladonia convoluta, Cladonia foliacea*	Turkey	Alpha particle transmission	^{210}Po, ^{210}Pb	(Sert, et al., 2011)
Oven dried at 85 ˚C for 48 hr	All squamulose	*Rhizoplaca melanophthalma, Cladonia convoluta, Cladonia pyxidata*	Turkey	Direct gamma assay	^{210}Po, ^{210}Pb	(Uğur, et al., 2003)
Oven dried at 85 ˚C for 24 hr	Foliose	*Xanthoria parietina*	Turkey	ICP-MS	As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Se, Th, U, V, Zn	(Gür & Yaprak, 2011)
Oven dried at 90 °C for 24 hr	Squamulose	*Cladonia pyxidata, Cladonia ret, Cladonia cartosa*	Poland	FAAS	As, Cd, Cu, Pb, Zn	(Osyczka, et al., 2016)
Oven dried at 95 °C	All foliose	*Parmelia physodes, Parmelia sulcata, Usnea strigosa*	Canada	INAA	V	(Juichang, et al., 1995)
Oven dried at 100 °C	Foliose	*Parmelia caperata*	Nigeria	FAAS, GFAAS	Cd, Cr, Co, Cu, Mn, Ni, Pb, Zn	(Ite, et al., 2014)
Oven dried at 105 °C	Foliose	*Pseudevernia furfuracea*	Italy	ICP-MS	As, Cd, Cr, Pb, V, Cu, Zn, Fe, Al, Mn	(Basile, et al., 2008)
Oven dried at 105 °C	Both fruticose	*Evernia sp. And/or Usnea sp.*	France	ICP-MS	Al, As, Cd, Cr, Cu, Ni, Pb, Zn, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$	(Doucet & Carignan, 2001)
Oven dried at 105 °C	Fruticose, foliose, foliose, foliose, foliose	*Ramalina polymorpha, Xanthoria parietina, Physcia astellaris, Flavoparmelia caperata, Physcia adscendes*	Turkey	FAAS, GFAAS	Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn	(Mendil, et al., 2009)
Oven dried at 105 ˚C	Foliose, fruticose, foliose, foliose	*Pseudevernia furfuracea, Usnea Longissimi, Lobaria pulmonaria, Peltigera praetextata*	Turkey	FAAS	Cr, Cu, Fe, Ni, Pb, Zn	(Cicek, et al., 2008)
Oven dried at 110 ˚C	Foliose	*Hypogymnia physodes*	Siberia	FAAS	Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn	(Valeeva & Moskovchenko, 2002)
Oven drying at 120 °C	Foliose	*Pseudevernia furfuracea*	Italy	ICP-MS	Al, B, Ba, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Sr, Ti, Zn	(Giordano, et al., 2010)
Oven dried at 105 ˚C to constant weight	Foliose	*Usnea barbata*	Argentina	SF-ICP-MS*	Ir, Pt, Rh	(Pino, et al., 2010)

Oven dried at 105 ˚C for 24 hr or freeze dried	Fruticose, fruticose, foliose	*Evernia prunastri, Ramalina farinacea, Hypogymnia physodes*	France	ICP-OES, ICP-MS, CVAAS	Al, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Ti, Zn	(Cloquet, et al., 2015)
Oven dried at 60 ˚C for 48 hr and then frozen and pulverized over liquid nitrogen	Fruticose	*Usnea aurantiaco, Usnea antarctica,*	Antarctica	LA-ICP-MS*, ICP-MS	Pb	(Guerra, et al., 2011)
Oven dried at 105 ˚C for 1 hr then frozen and pulverized over liquid nitrogen	Foliose	*Hypogymnia physodes*	France	ICP-MS	Pb isotopes	(Cloquet, et al., 2009)
Air dried and then freeze dried	Foliose	*Usnea umblyoclada*	Argentina	FAAS	Cu, Co, Fe, Mn, Ni, Pb Zn	(Carreras & Pignata, 2002)
Frozen in a deep freeze, freeze dried then re-frozen	Foliose	*Xanthoria parietina*	Turkey	ICP-OES, ICP-MS	As, Cd, Co, Cr, Cu, Fe, Mn, Pb, Zn	(Hanedar, 2015)
Freeze dried	Fruticose	*Ramalina sp., Evernia sp., Usnea sp. ,Bryoria sp.*	USA, Canada, France	INAA, Potentiometry, ICP-MS	Al, As, Cl, Cr, Cu, In, Na, Pb, Se, Zn	(Wen & Carignan, 2009)
Freeze dried	Foliose	*Flavoparmelia caperata*	Portugal	ICP-MS, INAA	As, Ca, Co, Cr, Cs, Fe, Hf, K, La, Mg, Mn, Na, Rb, Sb, Sc, Sm, Zn	(Godinho, et al., 2008)
Freeze dried	Fruticose	*Usnea sp., Bryoria sp., Evernia sp.*	USA, Canada	TIMS*, ICP-MS	Al, Pb	(Carignan, et al., 2002)
Air dried and then frozen in liquid nitrogen	Foliose	*Flavoparmelia caperata*	Italy	ICP-MS	As, Cd, Cr, Fe, Ni, Pb, V, Zn	(Paoli, et al., 2012)
Air dried and then frozen in liquid nitrogen	Foliose, fruticose, fruticose, foliose/fruticose	*Pseudevernia furfuracea, Evernia prunastri, Ramalina fastigiata, Cetraria islandica*	Serbia, Montenegro, Greece, Bulgaria, Italy	ICP-MS, INAA	U	(Loppi, et al., 2003)
Dried and then frozen in liquid nitrogen	Foliose, foliose, fruticose	*Xanthoria parietina, Parmelia sulcata, Evernia prunastri*	France	ICP-MS	Al, As, Cd, Ce, Cr, Cs, Cu, Fe, La, Mn, Nd, Ni, Pb, Sb, Sm,Sn, V, Yb, Zn	(Gandois, et al., 2014)
Air dried, cryogenically frozen in liquid nitrogen and stored in a desiccator	Foliose	*Hypogymnia physodes*	Russia	ICP-MS	Pb	(Pollard, et al., 2015)
Air dried and then cryogenically frozen in liquid nitrogen	Fruticose	*Evernia prunastri*	Italy	ICP-MS	Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Mg, Mn, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn	(Frati, et al., 2005)
Air dried and cryogenically frozen in liquid nitrogen	Foliose	*Flavoparmelia caperata*	Italy	FAAS, GFAAS	Cd, Cr, Cu, Ni, Pb, Zn	(Loppi, et al., 2004)

Liquid nitrogen	Foliose	*Hypogymnia physodes*	Slovenia	INAA	Ag, As, Ba, Br, Ce, Cd, Co, Cr, Cs, Fe, Ga, Hf, Hg, K, La, Mo, Na, Rb, Sb, Sc, Se, Sr, Sm, Tb, Th, U, W, Zn	(Jeran, et al., 1996)
Liquid nitrogen	Foliose	*Hypogymnia physodes*	Slovenia	INAA	As, Ba, Br, Ca, Cd, Ce, Co, Cr, Cs, Fe, Ga, Hf, Hg, K, La, Mo, Na, Rb, Sb, Sc, Se, Sm, Sr, Th, U, W, Zn	(Jeran, et al., 2002)
Air dried and frozen at -20 °C	Fruticose	*Evernia prunastri*	Italy	ICP-MS	As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sb, Tl, V	(Paoli, et al., 2015)
Frozen in liquid nitrogen and freeze dried	Foliose	*Parmelia sulcata*	Netherlands	INAA	Co, Sc, Zn	(Sloof, 1995)
Dried in paper bags, stored in coolers at 10 °C for 18 days and then frozen at -17°C. Ground and frozen in liquid nitrogen.	Foliose	*Hypogymnia physodes*	Canada	CVAAS	Hg	(Sensen & Richardson, 2002)
Oven dried at 105 °C and dry-ashed at 450 °C	Foliose	*Hypogymnia physodes*	Finland	FAAS	Cr, Ni	(Kansanen & Venetvaara, 1991)
Air dried and then ashed at 450 ˚C for 7 hr	Foliose	*P. reticulatum*	New Zealand	GFAAS	Cr, Cu, Pb, Zn	(Kularatne & de Freitas, 2013)
Dried in a Heraeus furnace and ground with a spex mill	All foliose	*Flavoparmelia caperata, Xanthoparmelia conspersa, Physcia adscendes, Parmotrema perlatum, Xanthoria parietina*	Turkey	EDXRF*, FAAS	Ba, Co, Cr, Cu, Fe, Mn, Ni, Sn, Ti, Pb, Zn	(Koz, et al., 2010)
Dried at < 40 °C	Foliose	*Hypogymina physodes*	Finland	ICP-OES & ICP-MS	Al, Ba, Ca, Cr, Cu, Fe,Mn, Na, Pb, Ti, V, Zn	(Salo, et al., 2012)
Warm dried	Fruticose, fruticose, foliose	*Evernia prunastri, Ramalina farinacea, Hypogymnia physodes*	France	ICP-MS, MC-ICP-MS	Zn, Pb, Zn isotopes, Pb isotopes	(Cloquet, et al., 2006)
Not mentioned	Foliose	*Hypogymnia physodes*	Romania	ICP-OES, ICP-MS	56 elements including REEs	(Rusu, et al., 2006)

*ICP-MS: Inductively Coupled Plasma Mass Spectrometry, ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry, FAAS: Flame Atomic Absorption Spectroscopy, GFAAS: Graphite Furnace Atomic Adsorption Spectroscopy, CVAAS: Cold Vapour Atomic Absorption Spectroscopy, TINAA/INAA:Instrumental Neutron Activation Analysis, FIAS: Flow Injection Analysis System, ETAAS: Electrothermal Atomic Absorption Spectrometry, MC-ICP-MS: Multicollector ICP-MS, ZE-TAAS: Electrothermal Atomic Absorption Spectrometer with a Graphite furnace and background Zeeman Corrector, FIMS: Flow Injection Mercury System, SF-ICP-MS: Sector Field ICP-MS, LA-ICP-MS: Laser Ablation ICP-MS, TIMS: Thermal Ionization Mass Spectrometry, EDXRF: Energy Dispersive X-ray Fluorescence

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4.4.Paper 4

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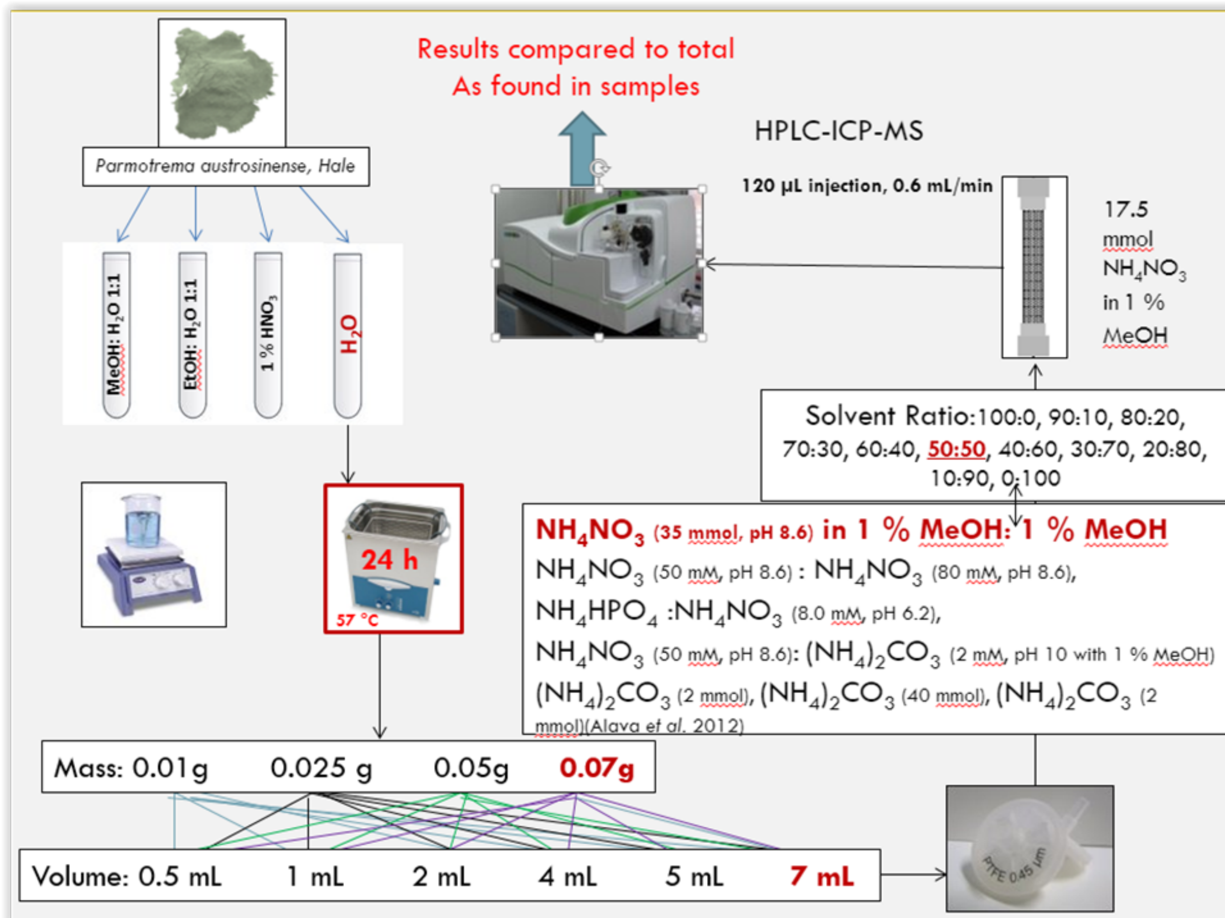
Graphical abstract



Optimized extraction of inorganic arsenic species from a foliose lichen biomonitor

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Abstract

To assess the two most toxicologically relevant species of As, namely arsenite (As III) and arsenate (As V), chromatographic separations often require two separate chromatographic columns to address the co-elution of arsenobetaine (AsB) with As III. This issue is typically observed using conventional isocratic methods on anion exchange columns, increasing cost and analysis time. Here we optimize the extraction of inorganic As from a lichen air biomonitor, and develop a method for the chromatographic separation of five common As species on a PRP X-100 anion exchange column, resulting in the complete baseline separation of all species under study. This method was then applied to lichen biomonitors from an urban and rural site to demonstrate its use. In order of abundance, the various arsenic species in lichens from the urban site in South Africa were As V>As III>AsB>dimethylarsinic acid (DMA)>monomethylarsonic acid (MMA), and As V>AsB>As III>DMA>MMA for the rural site, where MMA was present in extremely low, non-quantifiable concentrations in lichens from both sites. Total concentrations of As were higher in samples from the urban site (6.43 ± 0.25 µg/g) than those from the rural site (1.87 ± 0.05 µg/g), with an overall extraction efficiency of 19% and 40% respectively. The optimized method utilised relatively inexpensive solvents and is therefore low-cost and eco-friendly in comparison to conventional chromatographic techniques. This is the first study which addresses the optimized extraction and characterization of As species in a South African lichen biomonitor of air pollution.

Key words:
Arsenic speciation; HPLC-ICP-MS; Extraction; Biomonitor; Air pollution; Lichen

Introduction

The speciation of arsenic in biomonitors of air pollution poses an interesting analytical challenge, where samples are often complex, containing several different arsenic species. Such speciation studies are of significance due to the high relative abundance of toxic inorganic arsenic species attached to particulate matter in air, in comparison to their methylated counterparts (Chung et al. 2014). In some cases this particulate matter has been reported to be As-enriched by 10-1000 times higher than continental crust concentrations (Johnson and Braman 1975, Cullen and Reimer 1989). Organic arsenic species, although generally of lower abundance in the atmosphere, may arise from a number of different sources. These include the production of volatile organo-As species through microbe and yeast metabolism (Cullen and Reimer 1989, Koch et al. 1999, Bentley and Chasteen 2002, Chung et al. 2014), the spraying of arsenic-containing pesticides such as monosodium methyl arsonate (MSMA), or the use of arsenic-based preservatives such as biocides composed of aryl- and alkyl-arsenicals in wood production (Cullen and Reimer 1989). Organic arsenic species, however, are not of as great toxicological relevance as inorganic forms due to their lower toxicity, where some species are regarded as non-toxic (Machado et al. 2006). Since all arsenic species are not equal in terms of their toxicity, studies evaluating the total arsenic concentration would not be able to accurately reflect upon the bioavailability and toxicity of airborne arsenic (Chakraborti et al. 2013). As such, it is important to have an analytical method which can accurately differentiate between the various chemical forms of arsenic in air.

Lichens are effective biomonitors of air pollution and have been integrated into a number of regional and national air pollution surveys where several arsenic species have been found to be present in the thallus (Machado et al. 2006). There is some uncertainty about the appropriateness of using lichens as biomonitors of As species in air due to their ability to metabolize and methylate the various chemical forms (Farinha et al. 2004, Mrak et al. 2008). On the other hand, the ability of lichens to methylate As compounds may in fact prove useful in biomonitoring studies in terms of understanding temporal variations in exposure to As, therefore speciation of As in lichens is worthy of further investigation.

Given the great number of arsenic species present in nature, sufficient resolution of peaks can be difficult to attain in chromatographic applications (Dembitsky and Rezanka 2003, Quaghebeur and Rengel 2005). Moreover, there are a considerable number of factors which can affect the equilibrium between species, including improper sample handling and storage, the introduction of acidic or basic solvents during extraction, and other factors (Kroukamp et al. 2016). It is therefore essential that the methods used in sampling, extraction and separation ensure that the integrity of the original chemical species remains intact so that meaningful information can be gathered.

There is currently no consensus regarding the best extraction solvent for the various species of arsenic from plant and plant-like materials, although many studies use methanol:deionized water (MeOH: DIW; 1:1) which has proven to be effective in extracting organo-arsenic compounds (Koch et al. 1999, Zhang et al. 2002, Machado et al. 2006, Bergqvist et al. 2014). Other studies, although few in number, use dilute HNO_3, such as that used on signalgrass (*Brachiaria brizantha*) (Amaral et al. 2014), and some recent studies such as those conducted on a hyperaccumulating fern (*Pterris vittatta*), rice seedlings and tobacco leaf have employed the use of ethanol:DIW (EtOH: DIW; 1:1) (Zhao et al. 2015). Kuehnelt et al. (2000) found that DIW extracted As more efficiently than the more commonly employed MeOH:DIW, where these findings were attributed to the greater extraction of inorganic forms of As and arsenoribose. Despite these findings, summed extraction efficiencies of the various identified As species using these methods are often relatively poor. In the study by Kuehnelt et al. (2000) for example, only 7—25% extraction yield was achieved for fruticose lichens *Alectoria ochroleuca* and *Usnea articulata* respectively, and were based upon the separation and quantification of twelve arseno-compounds.

There is also no standardization when it comes to how these species are extracted, where ultrasonic baths (Farinha et al. 2004), mechanical shakers (Machado et al. 2006) and microwave techniques (Quaghebeur et al. 2003) have all been employed. The sample mass-to-volume of extractant matrix has also not been extensively investigated, where masses are often large (0.2—1 g) and the volume of the solvent can vary greatly (15—50 mL/g) (Koch et al. 1999, Kuenhelt et al. 2000, Farinha et al. 2004, Machado et al. 2006, Mrak et al. 2006, Mrak et al. 2008, Farinha et al. 2009). The effective separation of arsenic species in plant and plant-like materials is also a challenge, where co-elution of species is commonplace in chromatographic analyses (Kuehnelt et al. 2000, Mrak et al. 2006), the most common of which is the co-elution of arsenobetaine (AsB) and arsenite (As III) when using isocratic anion exchange methods. This issue has up until now been addressed by using different analytical columns and mobile phases, which could introduce additional analytical challenges, or by including cation exchange chromatographic methods, thereby increasing analysis time and cost. Gradient methods have also been used (Watts et al. 2008, Alava et al. 2012), but are often not the method of choice due to difficulties in controlling species interconversions, changes in the chromatographic baseline, hidden peaks due to the solvent gradient, and long re-equilibration times. Since As III is often of key analytical interest due to its high toxicity, the baseline separation of As III from AsB using a single analytical run and an isocratic method would be highly advantageous.

The critical assessment of these methods in the evaluation of ultra-trace concentrations of arsenic species, such as is often found in lichen biomonitors, is crucial to understanding atmospheric exposure levels and the relative toxicity of arseno-compounds in air. Lichens have been extensively used in monitoring arsenic air pollution, however studies are

often limited to total As analysis (Mrak et al. 2007, Pisani et al. 2011). Few speciation studies characterizing As in lichens are available (Koch et al. 1999, Farinha et al. 2004, Machado et al. 2006, Mrak et al. 2008, Farinha et al. 2009) and so methods appraising this are still an area requiring further exploration.

In this work, the optimized extraction of inorganic forms of As (As III and arsenate; As V) and the subsequent separation and semi-quantification of five common arsenic species in the lichen biomonitor, *Parmotrema austrosinense,* (Zahlbr.) Hale using High Pressure Liquid Chromatography – Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS) was investigated. The extraction parameters were qualitatively optimized for As III and As V due to their environmental relevance, high toxicity and species prevalence in atmospheric dusts (Machado et al. 2006). Consequently, less abundant forms of As such as arsenosugars and cationic species were not of interest and were therefore outside the scope of this study. The development of an isocratic anion exchange chromatographic method which could baseline separate and semi-quantify AsB, As III, monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and As V was also investigated. Organic As species, MMA and DMA were evaluated as they are the most common toxic organic forms of arsenic found in lichen, fungi and algae (Koch et al. 1999, Machado et al. 2006) where MMA is more toxic than DMA (Bissen and Frimmel 2000). Arsenobetaine was evaluated to ensure that co-elution with As III was avoided, which would otherwise skew the analytical results for this target inorganic analyte.

The optimized method was thereafter applied to two sampling sites within South Africa to evaluate the species of arsenic present in lichens at an urban and rural site. The intention of this was to demonstrate the appropriateness of the proposed method in the application to lichens as biomonitors of air pollution, where observed differences could be tentatively linked to site impacts. Such a study involving the characterization of arsenic species in the South African lichen biomonitor, *P. austrosinense* has not been published to date. The method developed in this study is one of the few methods which can demonstrate relatively high extraction yields for the semi-quantitative data gained using simple solvents and laboratory equipment. It is also the only published method, which the authors are aware of, which has been able to baseline resolve As III from AsB using an isocratic method on a PRP X-100 anion exchange column. Furthermore, a relatively simple and inexpensive mobile phase, without a large waste footprint, makes the proposed method a cost-effective and eco-friendly option.

Materials and methods

Sample collection

Bulk lichen, *P. austrosinense,* was collected for initial method development from the Johannesburg Botanical Gardens (JHB), South Africa (GPS co-ordinates -26.159875 S, 27.99346 E) which is within 10 km of 6 major hospitals, 3 crematoria and the city centre. It is also between 15 and 40 km of two major gold mines. Moreover many residential households burn fossil fuels for cooking and heating purposes. This site was chosen as the impacted urban site for this study since As is often associated with the burning of fossil fuels, mining of gold bearing minerals and crematoriums, the latter of which can lead to highly localized As contamination (USEPA 1998, Chakraborti et al. 2013). Samples were collected in the same manner as has been done in previous work (Kroukamp et al. 2017), being 100 m away from the main road, between 2—4 m above ground level and from all around the tree to prevent bias due to car emissions, soil contamination and the prevalent wind direction respectively (Monaci et al. 2012). As done previously (Kroukamp et al. 2017), a large representative sample of lichen material was collected using plastic forceps and care taken to prevent extraneous (i.e. non-lichen material such as bark) material from being stored with the sample. Samples were then stored in acid-washed polypropylene bottles.

Upon returning to the laboratory, the samples were further cleaned of any remaining substratum under a magnifying lamp, and gently tapped to remove small particles of extraneous material resulting from the cleaning procedure. Since As in the atmosphere is considered to be completely contained within the troposphere and solely present in the particulate form (Farinha et al. 2004) the samples were not washed as this would remove particulate As which had been deposited on the surface of the lichens from the air. This approach supports that of Frati et al. (2005) who found that washing procedures had an effect on the metal content in lichens. Nitrile-gloved hands were used to shred the sample into small pieces to improve homogeneity of the bulk material, and fractions from 1—4.699 mm were collected from an Endecott sieve for further processing. The whole mass of the cleaned sample which was used for further analysis was 10 g. The sample was not freeze dried, oven dried or frozen in liquid nitrogen since a past study found that these procedures may liberate volatile elements, such as As, from the sample matrix (Kroukamp et al. 2017). Instead samples used for method development were air dried and stored in acid-washed polypropylene vials in a cool, dark place until processing. On the day of analysis, the lichen samples were ground using a porcelain pestle and mortar and sieved through an Endecott 420 µm sieve to ensure a homogenous particle distribution for extraction. The initial stock samples used for method development were stored for a total of 6 months from the beginning to the end of the optimization process.

Once evaluated, the optimized extraction was applied to freshly-collected *P. austrosinense* lichen samples from the urban site, JHB, (as described above) and from a rural site in the Waterberg Mountain area, South Africa (GPS co-ordinates -24.4880278 S, 27. 8137778 E), where potential sources of pollution are primarily in the form of agriculture and livestock farming. Samples were collected in the same manner as described earlier.

Instrumentation

All analyses took place using a NexION® 300X ICP-MS (PerkinElmer Inc. Shelton, Connecticut). For chromatographic applications, the ICP-MS was coupled to a Flexar™ HPLC with Chromera™ software. In order to ensure that the system was functioning properly prior to use, daily performance checks, including nebulizer gas flow and torch alignment were done prior to analysis where the instrument was optimized for maximum sensitivity of In with robust plasma conditions such that oxides (CeO/Ce) <2.5% and doubly charged ions (Ce^{++}/Ce) < 3%. After having met the daily performance requirements, the following parameters were optimized on the ICP-MS to ensure that the As signal was maximized: RF power, torch alignment, nebulizer gas flow, plasma gas flow, auxiliary gas flow and torch sampling depth. The torch alignment and nebulizer gas flows were subject to daily changes, as is normal for ICP-MS, and will therefore not be mentioned, however the other optimized parameters for the study are shown in Table S1 (Supplementary Material).

All calibration standards and samples were prepared on the day of analysis. As part of the method development for the analysis of As species in lichens, m/z 75 and 77 were monitored to check for the presence of $^{75}ArCl^+$ interference on $^{75}As^+$. Since no $ArCl^+$ could be detected, the lichen samples were analyzed in standard mode. The column was regenerated after every 60 samples using mildly acidified MeOH and a blank was run after every sample to ensure that there were no memory effects or further species eluting from the column.

Optimization of extraction of arsenic from lichens

Preliminary investigations into the elution times of As III and As V

Standards (100 ppb) of As III and As V were prepared from stock solutions (1000 mg/L, Inorganic Ventures). Standards were analysed in triplicate, both independently and as a mixture, using the system described in Tables S1 and S2 (Supplementary Material) to determine the elution times of As III and As V.

Choice of extraction technique and variation of injection volume

To evaluate the most appropriate extraction technique (hot plate/ ultrasonic bath) and injection volume for the lichen samples, 0.01 g of homogenous pulverized sample was accurately weighed on an analytical balance (XP205, Mettler Toledo) into centrifuge tubes (Griener Bio-one) and extracted in triplicate using either 5 mL of MeOH:DIW (1:1, AR methanol, MilliQ 18 MΩ/cm) or 5 mL DIW for 1 h using either an ultrasonic bath (Integral systems) or a magnetic stirrer (FMH instruments, speed 5, room temperature, micro magnetic Teflon-coated stirrer bars), and analysed with the instrument and parameters described in Tables S1 and S2 (Supplementary Material). In all cases, samples were quantitatively transferred using 4 rinsings of 0.5 mL of water and filtered through pre-wet (using 0.5 mL MeOH) PTFE syringe filters (0.45 µm, Membrane solutions) prior to analysis. Injection volumes of 10, 50 and 100 and 120 µL were evaluated to determine which injection volume resulted in the best normalized (according to the individual sample mass) signal-to-noise ratios (S/N) for the As species without resulting in significant peak tailing.

Choice of extraction solvent

The sample to solvent ratio was decreased to improve detection limits, using the optimal injection volume from the previous step. For evaluation purposes, powdered lichen samples (0.05 g) were extracted in triplicate using 1 ml of either deionized water, EtOH:DIW (1:1, AR ethanol), MeOH:DIW (1:1) or 1% HNO_3 (65% suprapur, Merck in deionized water) respectively. Extractions took place using either an ultrasonic bath or a magnetic stirrer for a period of 1 h (triplicate for each solvent using each of the extraction apparatus). Peak positions for the arsenic species in each solvent were confirmed through spiking of the pure solvent with As III and As V, both individually and as a mixture.

The chromatograms were normalized according to the individual sample mass to ensure that the results were not mass-biased, and the S/N were compared. The method with the best S/N ratio for the inorganic arsenic species (As III and As V) and which did not result in the interconversion of the arsenic species was selected as the preferred extraction technique.

Mass/volume extraction experiments

The optimized parameters from the previous step were used. Each mass of the pulverized lichen material (0.01 g, 0.025 g, 0.05 g, 0.07 g) was extracted in different volumes of DIW (0.5 mL, 1 mL, 2 mL, 4 mL, 5 mL, 7 mL), using an ultrasonic bath for 1 h.

Chromatograms were normalized according to mass and volume and compared. The results with the best S/N ratio for As III and As V were chosen for further method development.

Extraction time dependent study

The optimized parameters from the previous step were used. The sample extraction time using an ultrasonic bath was optimized by evaluating samples in triplicate at extraction times of 5,10, 20,30 min, as well as 1, 2, 3, 5,10, 15, 20, 24 and 29 h and S/N compared among the various chromatograms. Temperature changes in the ultrasonic bath were monitored to check the dependency of the extraction yield upon the extraction temperature and to determine whether or not there were any observed interconversions of arsenic species, which would indicate temperature dependence.

Chromatograms were compared and the results with the best S/N ratio for As III and As V chosen for further method development.

Variation of the mobile phase composition to resolve arsenic species

Although As III and As V are of primary interest due to their toxicity and the fact that they are the most likely forms to be found in airborne dust, this study also included the evaluation of two moderately toxic methylated forms of As, namely MMA and DMA which have been found in some lichen studies (Machado et al. 2006). Although considered to be non-toxic, AsB was included in the analysis to ensure that the method developed did not allow the co-elution of As III with AsB as is commonly found in anion exchange speciation methods.

With the extraction of the most toxicologically relevant arsenic species (As III and As V) optimized, dilute mixed standards of AsB (Sigma Aldrich, Fluka ≥95%), MMA (prepared from monosodium acid methane arsonate, (Sigma Aldrich, Supelco 99.5%), DMA (prepared from Cacodylic acid, Sigma Aldrich ≥ 99.0%), As III and As V were freshly prepared and analysed with the original mobile phase of 70:30 ammonium nitrate (50 mM, pH 8.6) and DIW (Millipore, MilliQ, 18 $M\Omega.cm^{-1}$). Despite being suitable for the initial method development, where the focus was on improving the extraction of As III and As V from the lichen matrix, it was found that the 70:30 NH_4NO_3 (50 mM, pH 8.6): DIW solution resulted in the co-elution of AsB and As III on the PRP X-100 column and caused salting of the nebulizer and injector when analysing a large number of samples. To address this issue, other mobile phases such as NH_4NO_3 (50 mM, pH 8.6) : NH_4NO_3 (80 mM, pH 8.6), NH_4HPO_4 (8.0 mM, pH 6.2, Fluka TraceSELECT®

≥99.999%): NH_4NO_3 (8.0 mM, pH 6.2), NH_4NO_3 (50 mM, pH 8.6): $(NH_4)_2CO_3$ (2 mM, pH 10 with 1% MeOH, Promark Chemicals, AR) and NH_4NO_3 (50 mM, pH 8.6) in 1% MeOH : 1% MeOH (Millipore, MilliQ) were all tested for their suitability for the baseline separation of AsB and As III at ratios of A:B 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. A gradient method of 2 mM $(NH_4)_2CO_3$, followed by 40 mM $(NH_4)_2CO_3$, followed by 2 mM $(NH_4)_2CO_3$ as recommended by Alava et al. (2012) was also evaluated for its suitability.

Since lichens contain a number of arsenic species leading to complex chromatograms, the resolution study utilised pure individual and mixed standards of As III, AsB, DMA, MMA and As V. Standards were prepared in deionized water to mimic the extraction solvent used in the extraction of the samples. An argon humidifier was used in all studies to reduce salt-build up at the nebulizer tip, thereby eliminating aspiration issues over a large number of samples.

Analysis of lichen samples

The elution times of the individual As species were identified through the use of freshly prepared standard solutions. The optimized method of 0.07 g of unwashed, air dried, powdered sample was extracted using 7 mL of H_2O (MilliQ) in an ultrasonic bath for 24 h. The ICP-MS conditions shown in Table S1 (Supplementary Material) along with the optimized mobile phase from the previous step were used for further evaluations. To check elution times of the different arsenic species in the lichen matrix, a mixed species spike of As III, As V, MMA, DMA and AsB was added to the lichen matrix. Ten replicate samples each from the urban and rural sites were prepared as described earlier, and the concentrations of As III, As V, MMA, DMA and AsB determined. A blank was run after every sample to ensure that there were no further species eluting from the column and to check for memory effects. A method blank was also prepared in the same manner as the samples.

Total As concentration analysis

For total As determinations, powdered lichen samples were prepared as reported previously (Kroukamp et al. 2017) where 0.1 g was weighed in triplicate and digested in a CEM Mars 6 microwave digestion unit using 10 mL of HNO_3 (65%, Merck, suprapur) and 1 mL of H_2O_2 (30%, Merck, suprapur) with a ramp time of 20 min to 180 °C and a hold time of 20 min. This digestion method was a modification of the Milestone Application Note for lichen digestions (HPR-FO-55, Milestone 2014) where the volume of HNO_3 was increased to 10 mL due to a lower acid volume limit on the Mars 6 microwave digestion system as a result of 100 mL digestion vessels being used. Water was not used to achieve the necessary volume as this would dilute the acid and would likely result in an incomplete sample digestion.

Samples were filtered through a quantitative filter paper (Merck, 0.22 µm hardened ashless) and the filtrate was diluted to 50 mL using deionized water.

Quality control (QC) standards for total arsenic analysis included the BCR Reference Material no 482, Trace elements in Lichen (*Pseudevernia furfuracea*) and the Tea Leaf CRM, INCT-TL-1 (Institute of Nuclear Chemistry and Technology) and were prepared in the same manner as the samples.

Samples and the QC standards were analysed with dilutions of 1:10 and 1:5 and were also analysed without any dilution where the internal standard, ruthenium, was monitored to determine whether or not there were any significant matrix effects and was also used to compensate for long term drift. Samples were analysed on the same day as the dilution and no more than 72 h after the digestion had taken place. Total arsenic concentrations were determined using a PerkinElmer NexION® 300X ICP-MS with a collision gas (He) to manage any polyatomic interferences which may have arisen from acid impurities. Adding a collision gas also had the added benefit of providing collisional focussing of the ion beam thereby improving analyte sensitivity (Tanner et al. 2002).

Data processing

All chromatogram signals from method development were qualitatively evaluated by normalizing according to mass and replotted using Microsoft excel. Data processing of chromatograms for the lichen samples was performed on Chromera Chromatography software. In some cases, the software did not permit the identification of a peak due to software post-processing limitations. In such cases the results were considered to be outliers and due to this and other factors discussed later, the data is regarded as semi-quantitative.

Results and discussion

Optimization of extraction of arsenic

Preliminary investigations into the elution times of As III and As V for method development

The purpose of this study was to determine what the elution times were for the toxic, inorganic forms of arsenic (As III and As V) in a 70:30 50 mM NH_4NO_3: DIW mobile phase (Table S2, Supplementary Material) using a PRP-X-100 column at 0.6 mL/min in the absence of artefacts which could be introduced by the lichen matrix. During the initial method development for this study, As III and As V were found to elute at approximately 3 min 45 s and 12 min 15 s respectively.

Choice of extraction technique and variation of injection volume

The extraction method using an ultrasonic bath and an injection volume of 120 µL was found to have the best S/N for As III and As V when compared to magnetic stirring over the same time period. The increased extraction using ultrasonic bath may be related to the elevated temperature in the ultrasonic bath (42 °C at 1 h) in comparison to the magnetic stirring at ambient temperature. An injection volume of 120 µL was needed in order to achieve good S/N ratios for the As species under study due to the low natural concentrations of these As species in the lichen matrix. Despite the large injection volume, no significant tailing of the As III and As V peaks were observed. Deionized water showed promise as the extractant of choice as it resulted in the best S/N for the inorganic species (As III and As V), where MeOH:DIW 1:1 was found to give the best S/N for the other species of arsenic where this is likely due to MeOH facilitating the dissolution of organoarsenicals as further revealed in the next section.

Choice of extraction solvent for further evaluations

Deionized water was found to be the most effective extraction solvent for the inorganic As species since metal arsenites and arsenates are both highly soluble in water (Magalhães 2002), confirming our earlier findings in this study and those of Kuehnelt et al. (2000) who had found that water exhibited the best extraction efficiency in comparison to MeOH:DIW (1:1) in the fruticose lichens, *Alectoria ochroleuca* and *Usnea articulata.* The extraction solvent of MeOH:DIW (1:1) provided good but lower S/N (Table S3, Supplementary Material) of As III and As V and higher S/N for the unknown As species in the samples, thought to be organic arseno-compounds due to the prevalence of organo-arsenicals in lichens (Koch et al. 1999, Machado et al. 2014), showing that this solvent is more suited to the extraction of the unidentified organo-arsenic compounds (Fig. 1).

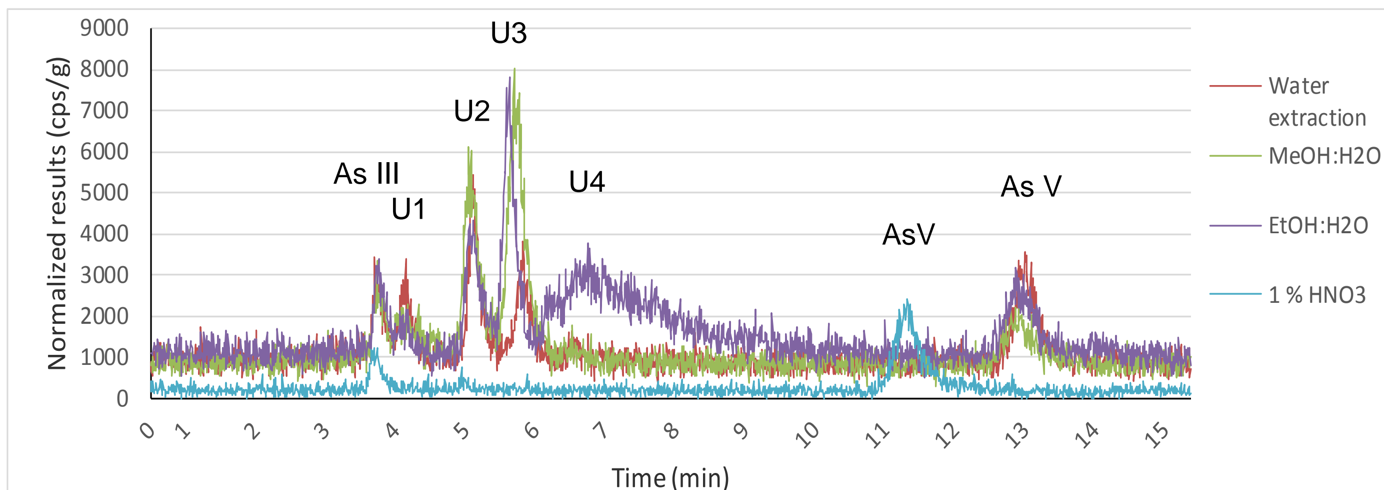

Fig. 1 Averaged, mass normalized HPLC-ICP-MS peak intensities (cps/g) showing the suitability of the various extraction solvents (DIW, MeOH:DIW, EtOH: DIW, 1% HNO_3) used in the ultrasonic bath extraction of powdered lichen material over a period of 1 h. Inorganic arsenic species (As III and As V), and unknown arsenic species (U1—4) are shown. Compounds were separated using a Hamilton® PRP X-100 column (Mobile phase: 70:30 50 mM NH_4NO_3:DIW, pH 8.6, flow: 0.6 mL/min)

Studies involving the use of EtOH:DIW as an extraction solvent as recommended by Zhao et al. (2015) who analysed As in the hyperaccumulating fern (*Pterris vittata*), rice seedlings and tobacco leaf, showed some promise as an appropriate extraction solvent. However, the poor S/N (Table S3, Supplementary Material) and the appearance of an additional peak (U4, Fig. 1) which was not found to be present in any of the other extractions raised caution to the use of this solvent for further method development. It is plausible that these additional peaks resulted from impurities in the ethanol solvent or were due to the improved extraction of an additional arsenic species, although this hypothesis would need to be verified through further experimentation. If an additional As species, or many, are present this would imply that EtOH is selective to certain arsenicals but poor in its overall recovery of arsenic species as observed by the poor S/N ratios in comparison to the other extraction solvents, however studies of this type will not be covered in the scope of this study.

Our findings contrasted to those by Amaral et al. (2014) who had recommended the use of 1% nitric acid as an appropriate extraction solvent, as we found that As V S/N (Table S3, Supplementary Material) increased dramatically in the presence of 1% HNO_3, the As III S/N increased slightly, and the organic species which were observed in the other solvents were undetectable (Fig. 1). These results agreed with finding by Cullen and Reimer (1989), where the use of 1% HNO_3 as an extraction solvent compromised the integrity of the various As species in the lichen matrix as it is a strong oxidiser, making it unsuitable as an extraction solvent in such applications. The observed vertical baseline shift between the other extraction solvents and HNO_3 (Fig. 1) is likely due to the evaluation taking place after the ICP-

MS components (nebulizer, torch, spray chamber) had to be cleaned due to a salt crystallization on the various sample introduction components of the ICP-MS. Conditions between analyses were the same, taking place using the same mobile phase, daily performance parameters and analyte intensities for the daily tuning solution, therefore the results are valid. Moreover, decisions regarding the suitability of the solvent were based upon the S/N ratios and the integrity of the various arsenic species and consequently vertical shifts are not of consequence. The observed horizontal shift of As V in this matrix (Fig. 1) is likely due to the change in the pH and ionic strength of the solution as peak positions were confirmed through spiking.

Mass/volume extraction experiments

It was found that 0.07 g of lichen sample extracted in 7 mL of DIW in an ultrasonic bath yielded the best S/N ratios for the mass normalized intensities, therefore these values were used for further method development.

Effect of extraction time

The triplicate samples were normalized according to mass, averaged for each extraction time and replotted in Excel. Based upon S/N, an extraction time of 24 h yielded the highest S/N for the inorganic arsenic species of interest. Since the relative abundance of the different peaks remained very much the same over the 24 h extraction period (maximum temperature of ultrasonic bath: 57 °C) the extraction procedure did not appear to affect the integrity of the arsenic species present in the lichen matrix. This confirms findings of Mrak et al. (2006) who found that at up to 90 °C, the various forms of arsenic remained intact. At temperatures exceeding 90 °C, however, they noted a decrease in the AsB concentration and an increase in trimethylarsine oxide (TMAO).

It can be seen from Figure S1 (Supplementary Material) that an increase in the time of extraction resulted in an increase in temperature in the ultrasonic bath over the first 2 h, thereafter the ultrasonic bath temperature stabilized at 57 °C. As such, the observed improvement in extraction efficiency is in fact most likely dominated by the length of time of extraction and not so much due to the increase in temperature, as suggested in the initial method development in earlier sections of this article. To elaborate, extending the time of extraction would ensure adequate dispersion of the sample slurry through continuous agitation (Blasco et al. 2006), improving sample-to-extraction matrix contact. Moreover, it would expose the sample to a higher number of ultrasonic-generated imploding bubbles which are known to result in high local pressures and temperatures (Kazi et al. 2009). These findings are somewhat different to those of Machado et al. (2006) in *Parmelia caperata* and Mrak et al. (2006) in *Hypogymnia physodes* and *Cladonia Rei,* where an increase

in temperature was solely responsible for improved extractability of As from lichens. Although temperature may play a role in improved extraction of the lichen matrix in our study, it is unlikely to be the only causative factor.

Variation of mobile phase composition to resolve arsenic species

None of the tested ratios of mobile phases, NH_4NO_3 (50 mM, pH 8.6):NH_4NO_3 (80 mM, pH 8.6), NH_4HPO_4 (8.0 mM, pH 6.2):NH_4NO_3 (8.0 mM, pH 6.2) were able to separate AsB from As III. The gradient method developed by Alava et al. (2012) showed some degree of separation of these species, however this mobile phase caused blockages of the nebulizer due to salt build up after just a few samples. Although a mobile phase of 30:70 NH_4NO_3 (50 mM, pH 8.6):NH_4CO_3 (2 mM, pH 10) + 1% MeOH gave a good separation of As species, an elution time of 30 min was required for a complete elution of all of the arsenic species and high throughput was limited by blockages and salt formation in the nebulizer and injector after just a few samples.

It was found that the addition of MeOH (1%) to the DIW in 70:30 NH_4NO_3 (50 mM, pH 8.6):DIW resulted in a better separation of AsB and As III. Further optimizations involved the adjustment of this ratio, where an end mobile phase composition of 17.5 mmol NH_4NO_3 in 1% MeOH at 0.6 mL/min resulted in the complete baseline resolution between all five target arsenic species (Fig. 2). This is the only published method that the authors are aware of which is able to baseline resolve these five arsenic species using an isocratic method on a PRP X-100 anion exchange column. Attempts were made to improve the method elution times by increasing the mobile phase flow rate to 1 mL/min. Although this improved the sharpness of the MMA peak, AsB and As III were no longer baseline resolved and so the flow rates were returned to 0.6 mL/min.

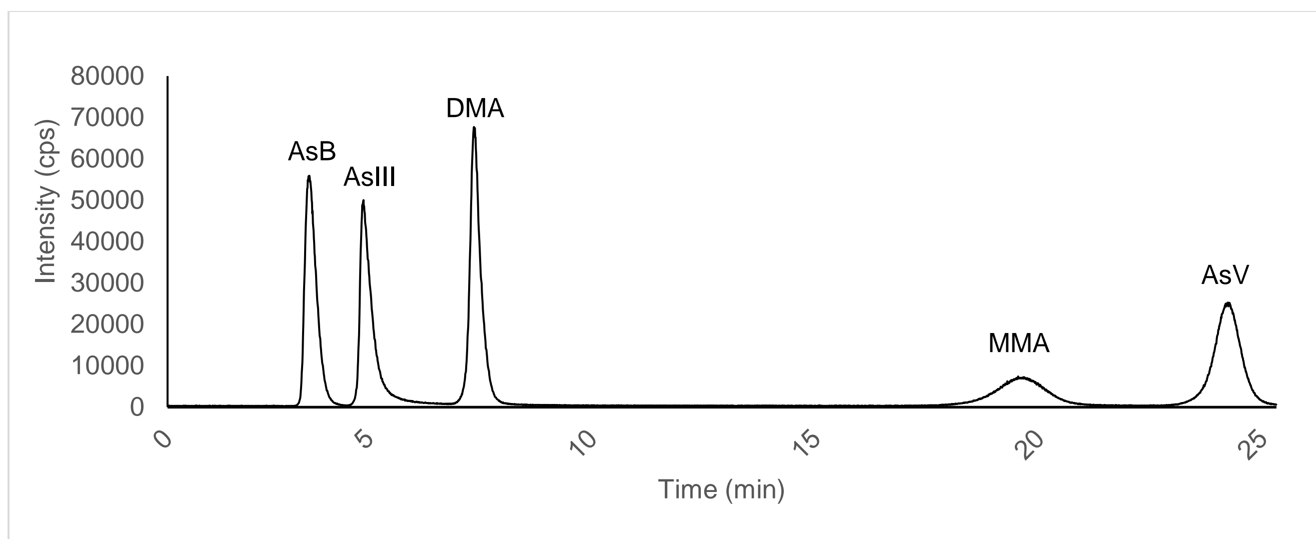

Fig. 2 The complete separation of five arsenic species (AsB, As III, DMA, MMA and AsV) in deionized water in under 25 min, with a mobile phase of 17.5 mmol NH_4NO_3 in 1% MeOH using a PRP X-100 anion exchange column (4.6 x 150 mm, 5 µm) by HPLC-ICP-MS

Analysis of lichen samples

During initial method validation using the lichen extracts, elution times of the AsB, As III and DMA were the same as those observed in pure standards, as confirmed through spiking. Spikes of MMA and As V eluted slightly earlier in comparison to spikes in water and are likely due to the change of matrix, although the change could be effectively dealt with by expanding the peak search window. In samples spiked with a mixture of the five target arsenic species, peak positions were similar to those observed in the single spikes of each species into the lichen matrix. The method blank (Fig. S2, Supplementary Material) was not found to contribute to the As baseline when compared to a blank which had not been through the sample preparation procedure.

Initial studies involving the bulk lichen material from the urban site showed a change in species over an extended storage time. At the start of method development, the S/N were highest for the inorganic arsenic species and by the end of method development, S/N were highest for the organic forms of arsenic. The storage of shredded (but not pulverized) lichens for a period of 6 months in a cool dark place could have provided the lichens with the time and conditions needed to metabolize and methylate the inorganic forms of As initially found to be present in the sample. This finding confirms the hypothesises of Farinha et al. (2004) and Mrak et al. (2008) that lichens are actively involved in the metabolism and biotransformation of arsenic species. Although some may consider the biotransformation of arsenic by a biomonitor as non-beneficial, such a process can be useful in providing insight into how recent the impacts are, allowing changes in arsenic contamination within an environment as a result of air pollution to be determined.

Due to these effects, a new bulk lichen material sample was collected from each site for the final method assessments. Here the elution times for MMA and As V were found to have increased to 21 and 43 min respectively, as a consequence of column degradation over time. Despite this change in elution times, the separation was proven to remain unaffected and the results allowed for an understanding of arsenic speciation in lichens. As such, elution times for all species were re-evaluated in water and the lichen matrix, and the optimized method run accordingly. Correlation co-efficients for AsB, As III, DMA, MMA and As V (n=5+blank) were 0.99999, 0.99990, 0.99998, 0.99999 and 0.99995 respectively. As can be seen in Fig. 3 the repeatability of the developed method was excellent.

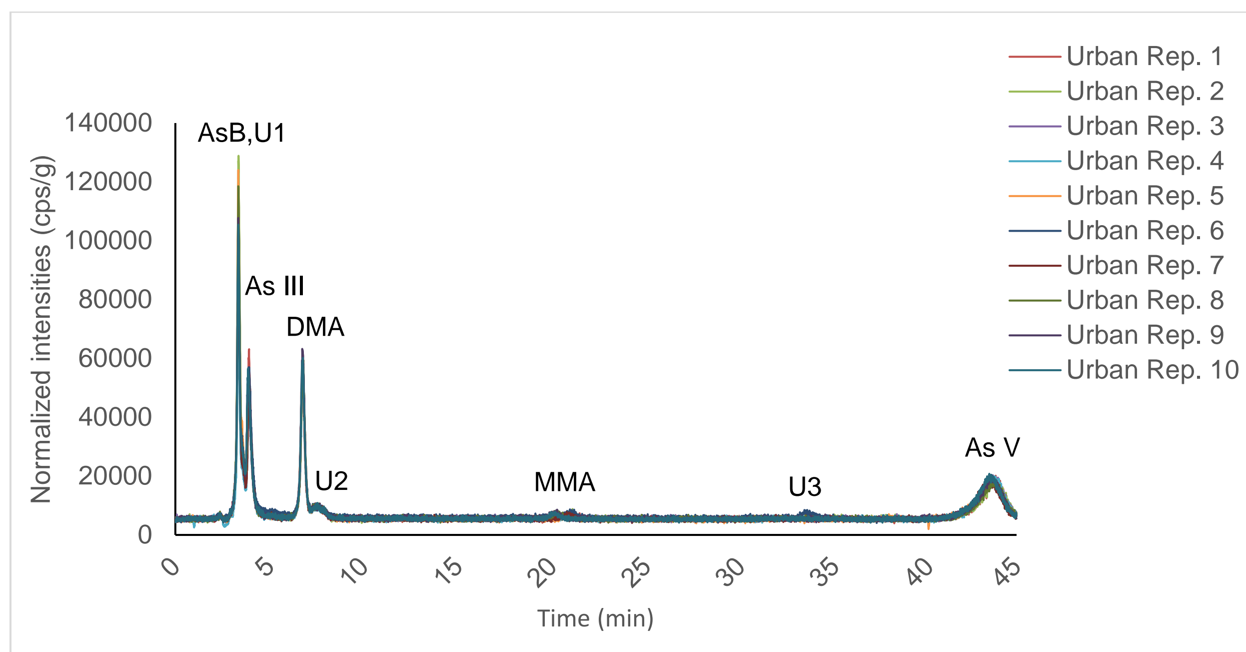

Fig. 3 Mass normalized intensities (cps/g) of As species found in freshly-collected lichen, *P. austrosinense*, from the urban site, JHB as determined by HPLC-ICP-MS. Results show 10 replicate analyses of lichen samples from this site which took place using a DIW extraction carried out in an ultrasonic bath over 24 h, separated on a Hamilton PRP X-100 column (mobile phase: 17.5 mmol NH_4NO_3 in 1% MeOH, pH 8.6, flow: 0.6 mL/min, injection volume: 120 µL). Five arsenic species (AsB, As III, DMA, MMA and As V), as well as unknown arsenic species (U1—3) are shown

It can be seen from Fig. 3 and Fig. 4 that AsB and As III were no longer baseline resolved, likely due to the elution of an additional As species from the lichen, *P austrosinense* (Fig. 4, U1) just after AsB. Despite this, the most toxicologically relevant form of As, As III, was still sufficiently resolved for semi-quantitation. Fig. 3 shows that there were two additional unidentified As species in the urban site (U2 & U3) in comparison to the rural site (Fig. 5). These are likely to be other organo-arsenic species or arsenosugars as has been found in studies by Koch et al. (1999), and may have resulted from different sources of pollution such as the application of pesticides in this park or nearby industrial activities, the volatile metabolic products of different microbes in surrounding soils (Huang et al. 2011),

meteorological factors or lichen metabolic processes. Since the same lichen species was used throughout the study, the latter scenario is less likely. Concentrations of As III and As V were lower in the rural site in comparison to the urban site, indicating differences in ambient air quality (Fig. 6). Arsenobetaine +U1 were found to be in high abundance in lichens from both sites, although concentrations for the urban site were higher than the rural site. Since lichens are the product of a symbiotic relationship between algae and fungi (Forbes et al. 2009), it is highly likely that AsB in these samples had arisen from the fungal component, where many fungi are known to possess high concentrations of this arseno-compound (Nearing et al. 2014a, Nearing et al. 2014b). Based upon the semi-quantitative results, the summative As concentration of freshly-collected lichens from the urban and rural sites were 1.22 ± 0.50 μg/g and 0.74 ± 0.06 μg/g respectively. In order of abundance, the various arsenic species in lichens from the urban site were As V>As III>AsB+U1>DMA>MMA, and As V>AsB+U1>As III>DMA>MMA for the rural site, where MMA was present in extremely low, non-quantifiable concentrations in lichens from both sites.

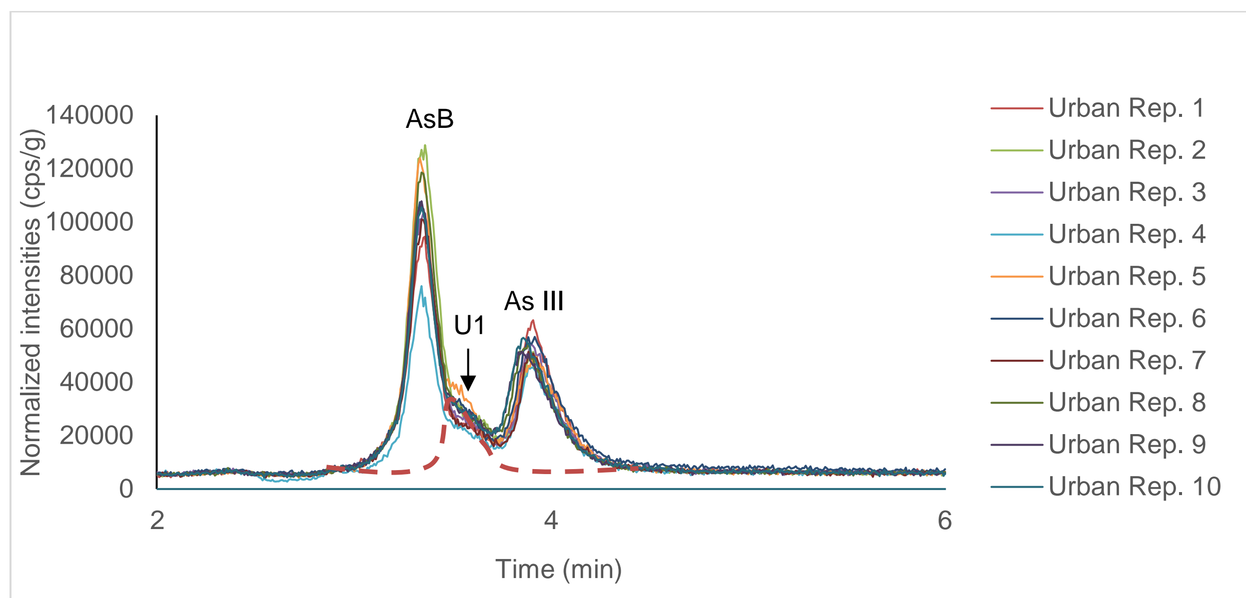

Fig. 4 Magnification of chromatogram (Fig. 3, 2—5 min) showing extracted AsB and As III in lichens from an urban impacted site as determined by HPLC-ICP-MS. The dotted line shows the hypothetical elution time and peak shape for the unknown arsenic species eluting just after AsB which prevents the complete baseline separation of AsB from As III

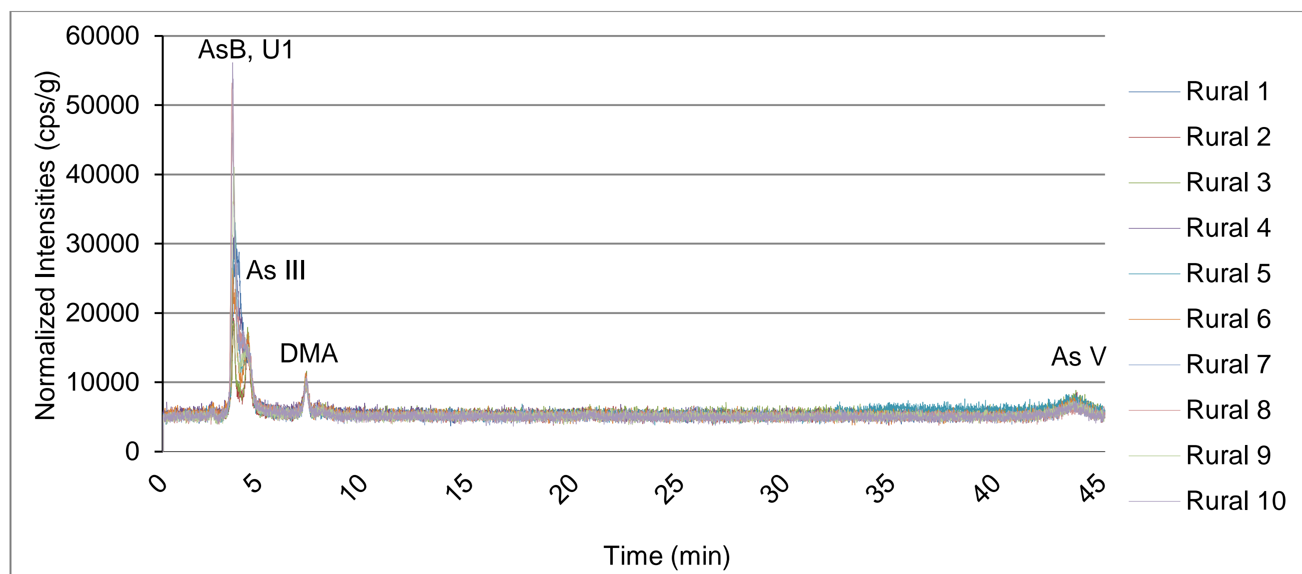

Fig. 5 Mass normalized intensities (cps/g) of As species found in freshly-collected lichen, *P. austrosinense*, from the rural site, Waterberg as determined by HPLC-ICP-MS. Results show 10 replicate analyses of lichen samples from this site which took place using a DIW extraction carried out in an ultrasonic bath over 24 h, separated on a Hamilton PRP X-100 column (mobile phase: 17.5 mmol NH_4NO_3 in 1% MeOH, pH 8.6, flow: 0.6 mL/min, injection volume: 120 µL). Five arsenic species (AsB, As III, DMA, MMA and As V), as well as an unknown arsenic species (U1) are shown

Total arsenic analysis and recoveries from speciation analysis

A dilution factor of five was found to be most appropriate for the lichen samples. Total recoveries for the lichen and tea leaf samples were 104 and 121% for the lichen and tea leaf CRMs respectively and were within the 95% confidence interval stipulated on the CRM certificate. The internal standard recoveries for all samples were well within the limits of 70—130% set by the USEPA (Keith 1996).

Since arsenic may enter the environment from gold mining (Villaescusa and Bollinger 2008, Chakraborti et al. 2013), crematoriums, medical waste incineration (USEPA 1998), burning of fossils fuels (Garelik et al. 2005) and smelting activities (Crecelius et al. 1974), it can be expected that urban sites, such as JHB, would exhibit higher concentrations of arsenic than a rural site which does not have these activities. This hypothesis agrees with the findings from our study where the total concentrations of As were higher in samples from the urban site (Total: 6.43 ± 0.25 µg/g) than those from the rural site (1.87 ± 0.05 µg/g). The burning of fossil fuels, such as coal, and the burning of pressure-treated wood for the cooking of food and heat generation are believed to be the major contributors to arsenic concentrations at the urban site. Cremation services and gold mining are also likely sources of As air pollution at this site, although probably minor contributors. Sources of As at the rural site are likely to be predominantly natural in origin, although neighbouring farming activities may also be a contributing factor.

The sum of the water-extractable concentrations of the monitored As species were 19% and 40% of the total arsenic concentration in lichens from the urban and rural sites respectively (Table 1). The difference in the extraction yields between sites can partly be contributed to the higher percentage of AsV (88% of the summed species concentrations) in lichens from the rural site in comparison to the urban site (81% of the summed species concentrations) which will cause higher yields since AsV is water-soluble. The rest of the difference could be due to the high RSDs for AsB+U1 from the rural site resulting from the apparent higher concentration of the unknown interfering As species eluting on the tail end of AsB in lichen samples from this site. Since only five As species were under investigation in the present study, the extraction yields are an improvement on those reported by Kuenhelt et al. (2000) who evaluated twelve As species in the fruticose lichens, *Alectoria ochroleuca* and *Usnea articulata* with yields of 7% and 25% respectively. Koch et al. (1999) reported high extraction yields of As from the lichens, *Bryoria sp.* and *Alectoria sp.* however most reported values were below the LOD and LOQ, and the actual measurable recoveries accounted for only 0.625—10% of the total arsenic species.

Table 2 Concentration of four of the five As species evaluated in this study (AsB, As III, DMA, As V) in lichens from an urban (n=10) and rural (n=10) site as determined by HPLC-ICP-MS in comparison to the total As concentrations in lichens from these sites. Results are shown in µg/g (mean±SD). The fifth As species under study, MMA, was not included since it was below the method detection limit**s** (MDL for MMA:1.00E-04 µg/g)

Site	**AsB+U1**	**As III**	**DMA**	**As V**	**Total As**	**Summative extraction efficiency (%)**
Urban Site	8.11E-02±1.13E-02	9.57E-02±4.55E-03	5.59E-02±4.37E-03	9.92E-01±5.06E-01	6.43E+00±2.52E-01	19
Rural Site	2.91E-02±1.26E-02	4.94E-02±7.73E-03	1.58E-02±6.87E-04	6.54E-01±4.65E-02	1.87E+00±5.46E-02	40
Calculated LOD	6.00E-05	6.00E-05	4.29E-05	2.14E-04	3.89E-04	

In agreement with studies by Koch et al. 1999, Machado et al., 2006 and Farinha et al. 2009, we found that the As III and As V were the dominant As species in the freshly collected lichen, *P. austrosinense,* from the urban impacted site of our study. For the rural site, As V was the dominant arsenic species however As III was found in low concentrations in lichens from this site pointing to a different source of As contamination. In agreement with findings by Farinha et al. (2009) and Koch et al. (1999) we too found arsenate in all of our samples and this was considered to be the main arsenic species. Our results contrasted to those of Machado et al. (2006) who found that arsenite was the most abundant As species at their sites, where the order of abundance of As species in lichens from a background site of their study was As III > As V > DMA > MMA, and As III = As V > DMA > MMA in discontinuously exposed transplanted lichens which had been exposed to pollution for a period of 2 months. This difference could be due to a different lichen metabolic processes or rates, or different pollutant sources, as the pollution sources for the study by Machado et al. (2006) were a coal-fired power plant and industrial area respectively. The higher abundance of As III observed in their study is counter to what one would expect, given that the main emission byproduct from coal combustion has been found to be As V (Goodarzi and Huggins 2005, Shah et al. 2007). As such, differences between two lichen species metabolisms could be a major factor affecting the As species observed, where the study by Machado et al. (2014) used the foliose lichen *Parmelia caperata*. Moreover, the length of storage time before processing could also be a factor since As V usually converts to As III before it is methylated (Challenger 1945, Cullen 2014), although it is hard to draw conclusions regarding this, since the storage period was not mentioned in their study. It is also plausible that this lichen species may be able to convert As V to As III more readily than *P. austrosinense* following the mechanism proposed by the Challenger pathway (Challenger 1945, Cullen 2014).

Conclusions

This study has shown that the total concentrations of As were higher in lichen samples from the urban site (Total: 6.43 ± 0.25 µg/g) than those from the rural site (1.87 ± 0.05 µg/g). In the method development related to the extraction of inorganic As species, the ultrasonic extraction of pulverized lichen material over a period of 24 h using deionized water resulted in the highest S/N for the As III and As V species present. Optimised chromatographic parameters included an injection volume of 120 µL and a mobile phase of 17.5 mmol NH_4NO_3 in 1% MeOH, pH 8.6 at 0.6 mL/min which resulted in the complete baseline resolution of five target As species, namely AsB, As III, DMA, MMA and As V, using a PRP X-100 anion exchange column. This study confirmed that lichens methylate inorganic arsenic species over an extended storage time. As such, if a direct comparison of the lichen with the environment is needed, fresh samples should be collected, where metabolites can help to elucidate information regarding how recent the impact is. Since lichens have a complex array of arsenic species present in their thallus, it is possible that additional As species may elute shortly after AsB which could affect the baseline separation of AsB and As III. Nevertheless, meaningful semi-quantitative toxicological information can still be gathered in a relatively short time. Based upon the semi-quantitative results, the summative As concentration of freshly-collected lichens from the urban and rural sites in this preliminary study were 1.22 ± 0.50 µg/g and 0.74 ± 0.06 µg/g respectively. Consequently, the extraction efficiency of As species from the lichen using the proposed method was 19% and 40% of the total arsenic concentration in lichens from the urban and rural sites respectively, demonstrating an

improved extraction efficiency in comparison to other published methods. It should be noted that more samples would need to be collected in future studies and other factors affecting speciation, such as meteorological data should be included to further substantiate these results. In order of abundance, the various arsenic species in lichens from the urban site were As V>As III>AsB>DMA>MMA, and As V>AsB>As III>DMA>MMA for the rural site, where MMA was present in extremely low, non-quantifiable concentrations in lichens from both sites. These differences in the speciation patterns between an urban and rural site were likely reflective of differences sources. Future studies could involve the adjustment of the pH to determine whether or not a lower pH could help resolve AsB and As III, however care should be taken to ensure that this does not promote the interconversion of As species.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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Paper 4- Supplementary Information

Optimized extraction of inorganic arsenic species from a foliose lichen biomonitor

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Supplementary Material

Table S1: Experimental conditions for the ICP-MS and HPLC

Instrument	Condition	Name or Value
ICP-MS	Model	NexION® (PerkinElmer, 300X)
	RF Power	1500 W
	Plasma/Coolant gas flow	17 L/min
	Auxiliary Gas flow	1.4 L/min
	Torch Sampling Depth	- 1.0 mm from standard set point
	Injector	Quartz, 2.0 mm
	Spray Chamber	Quartz baffle-type cyclonic spray chamber
	Nebulizer	Quartz Meinhard® Nebulizer
HPLC	Model	Flexar™ HPLC with Chromera™ Software
	Autosampler Loop size	250 µL
	Autosampler Injection Mode	Partial loop injection
	Anion Exchange Column	Hamilton® PRP X-100 (5 µm, 4.6 x 150 mm)

Table S2: Extraction parameters used for method development

Parameter	Specification
Mobile Phase	Ammonium nitrate (50 mM, pH 8.6, Sigma Aldrich ACS ≥98%) pH adjusted with ammonium hydroxide (25%)
Flow Rate	0.6 $mL.min^{-1}$

Table S3: Signal to noise ratio for choice of solvent extraction studies

Sample ID	**Signal to noise ratio**					
	As III	**U1**	**U2**	**U3**	**U4**	**As V**
Water extraction	3	3	6	4	0	4
1:1 MeOH:H_2O	3	2	7	9	0	2
1:1 EtOH:H_2O	2	1	3	7	3	2
1% HNO_3	3	0	0	0	0	7

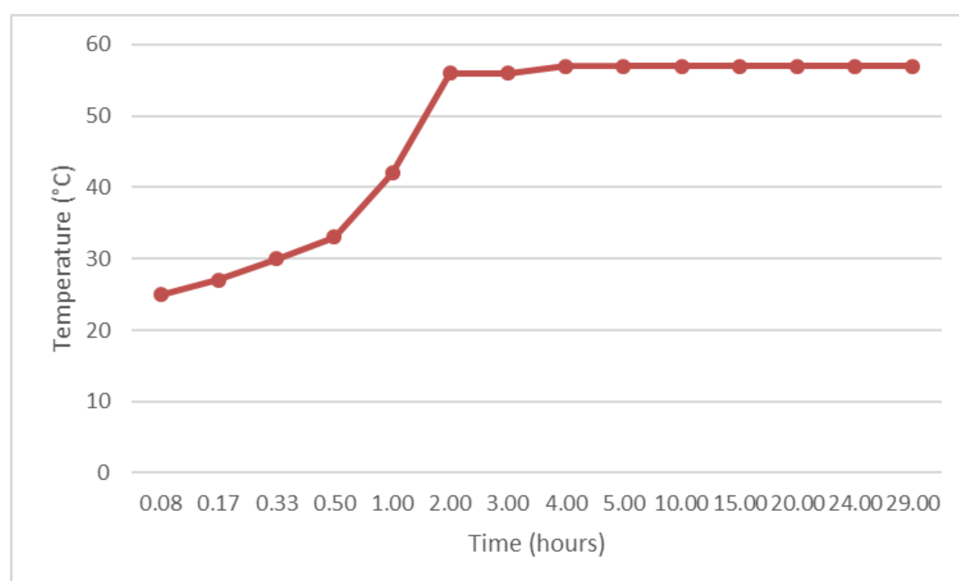

Fig. S1 Effect of extraction time on the temperature of the ultrasonic bath water

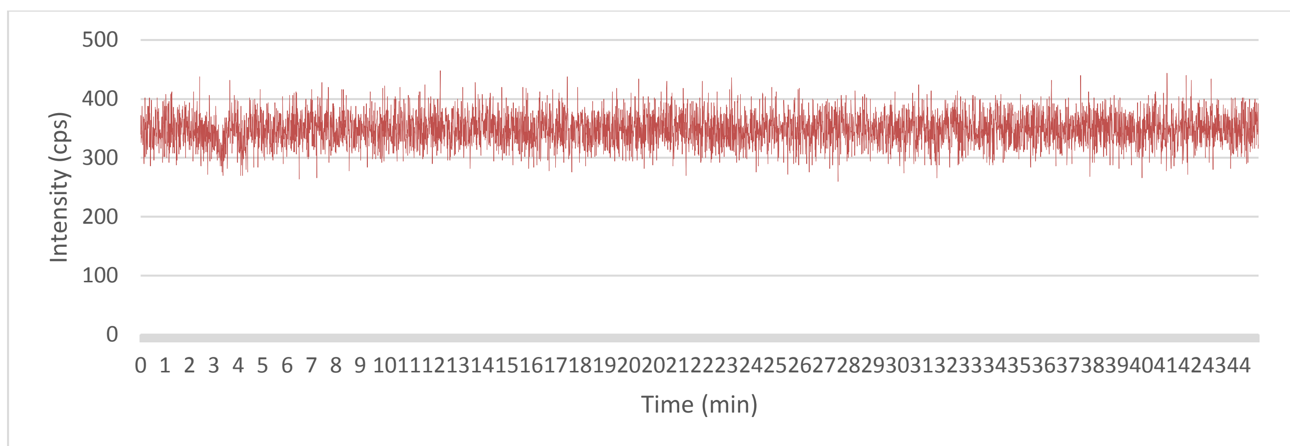

Fig. S2 Method blank showing no As contribution from the analytical method employed as determined by HPLC-ICP-MS. Method blank was prepared in the same manner as the samples, where DIW was extracted in an ultrasonic bath over 24 h, filtered through a pre-wet 0.45 µm PTFE filter and separated on a Hamilton PRP X-100 column (Mobile phase: 17.5 mmol NH_4NO_3 in 1% MeOH, pH 8.6, flow: 0.6 mL/min, injection volume: 120 µL)

4.5. Paper 5

This paper was formatted in accordance with the journal *Chemosphere,* an Elsevier journal. At the time when this PhD was submitted for examination, this paper was ready for submission pending acceptance of Paper 4 since this paper requires citation of the work presented in Paper 4.

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Species distribution patterns of arsenic in a lichen biomonitor

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Abstract
As stand-alone approaches, chromatographic separations of arsenic in lichens using HPLC-ICP-MS or the use of sequential extractions have historically been shown to have low analyte recoveries and poor analyte selectivity respectively. This study substitutes the typical water extraction step of a sequential extraction with a chromatographic separation of five arsenic species using HPLC-ICP-MS, followed by a three-step sequential extraction and analysis with ICP-MS. The method was applied to lichens from a rural and urban site to demonstrate the applicability thereof, and the sum of arsenic concentrations from the extraction steps were compared to the total arsenic concentration. Short term species stability of the As species in the lichen matrix was also evaluated over 1 month in the water-extractable fraction, where As species concentrations changed week by week, providing insight into biotransformation mechanisms. In the water extraction step, dimethylarsinic acid (DMA) and arsenobetaine and an unknown As species (AsB+U1) were statistically ($p<0.05$) higher in the urban site than the rural site. Analyte recoveries using the combined method were higher than other studies reported in literature, with percentage recoveries of 104% and 111% of As in the urban and rural sites respectively. Arsenic concentrations were found in the following order of abundance at both sites: oxidizable>reducible>water-extractable>residual. Concentrations of As in the oxidizable and non-bioavailable fraction were statistically lower ($p<0.05$) in the rural site than in the urban site. Based upon the information gained from this study, we could draw concise conclusions regarding source apportionment, timeline for exposure, and the magnitude of the pollution event.

Highlights

- Chromatographic separation of five arsenic species in a lichen matrix
- Water extraction step of sequential extraction substituted by HPLC-ICP-MS method
- HPLC-ICP-MS followed by sequential extraction improves As species/fraction recoveries in lichens
- Method provides information on source apportionment and timing of pollution events
- *P. austrosinense* likely reduces As bound to carbonates and oxides due to oxalic acid

Keywords
Arsenic species; Air pollution; Biomonitoring; Lichens; Sequential Extraction; HPLC-ICP-MS

Abbreviations

%RSD	Percentage relative standard deviation
As III	Arsenite
As V	Arsenate
AsB	Arsenobetaine
cps	Counts per second
DIW	Deionized water
DMA	Dimethylarsinic acid
HPLC-ICP-MS	High pressure liquid chromatography inductively coupled plasma mass spectrometry
MDL	Method detection limits
MMA	Monomethylarsonic acid
PGM	Platinum group metals
PTFE	Polytetrafluoroethylene

S/N	Signal-to-noise ratio
T0	Initial water extraction and chromatographic separation week 1
T1	Water extraction and chromatographic separation week 2
T2	Water extraction and chromatographic separation week 3
T3	Water extraction and chromatographic separation week 4
TETRA	Tetra-arsenic oxide
TMAO	Trimethylarsine oxide

1. Introduction

Carcinogenic metalloids, such as some species of arsenic, are known to elicit a toxicological response in humans (Sun *et al.* 2014, Sattar *et al.* 2016, Zhang *et al.* 2018). In air, most As is attached to, or forms part of, suspended particulate matter, whether it be from naturally occurring dusts, or particles resulting from industrial or mining processes (ATSDR 2007, Chung *et al.* 2014). Concentrations of As in the particles are often significantly higher (10-1000 x) than those in the continental crust, where this is due to the adsorption of gaseous As from air or complexation processes which entrap volatile arsenicals (Cullen and Reimer 1989). If inhaled, these particles can greatly increase the incidence of lung cancer and other respiratory problems (Person *et al.* 2015). Since there are a number of different chemical forms of As with varying degrees of toxicity, separation of chemical species according to their solubility i.e. chemical fractionation, or affinity for the packing material in chromatographic columns i.e. chromatographic separation, is essential in determining As bioavailability and potential detrimental effects (Michalke 2003, Farinha *et al.* 2004, Mrak *et al.* 2006, Farinha *et al.* 2009, Kroukamp *et al.* 2017).

Many monitoring methods have been used to evaluate As in air, however it is the use of biomonitors, such as epiphytic lichens, which have proven to be especially useful due to the fact that they can be collected over large tracts of land, allowing for comprehensive spatial and temporal information regarding atmospheric pollution to be gathered (USDA 2004, Bargagli *et al.* 2005, SOER 2009). Through their lack of cuticle, ability to passively trap and hyperaccumulate metals (Nieboer *et al.* 1976, Nash 1996, Loppi and Pirintsos 2003), and sole dependence upon the air as a source of nutrients (Bargagli *et al.* 2002), they are excellent biomonitor candidates with the added benefits of having a low ecological footprint and cost.

One of the biggest challenges in determining the relative abundance of As species in solid matrices, such as lichens, is having a quantitative extraction method which can maintain the original chemical fractionation pattern (Kroukamp et al. 2016, Kroukamp et al. 2017). Extractions using simple solvents, such as deionized water (DIW) coupled with chromatographic techniques can be extremely helpful in terms of evaluating readily available As forms without compromising the individual species integrity (Kroukamp et al. 2019). However, these types of studies are not able to provide information about the non-water extractable forms, leading to low extraction recoveries in mass balance calculations. In contrast, conventional sequential extraction techniques are comprised of several extraction steps where each extraction step uses solvents of differing chemical properties resulting in higher mass balance yields (Tokahoğlu and Kartal 2004, Kroukamp et al. 2017).

In an effort to address the well-known issue of lack of selectivity of step-wise extractions (Banerjee 2003, Michalke 2003), this study combines the use of chromatography and sequential extractions to evaluate the chemical speciation of the most common (Koch et al. 1999, Machado et al. 2006) and toxicologically relevant forms of arsenic in a lichen air biomonitor. We propose the replacement of the water extraction step reported by Kroukamp *et al.* (2017) with an optimized extraction and chromatographic separation of five As species (Kroukamp *et al.* 2019), thereby greatly improving the analyte selectivity. This extraction is followed by the other extraction steps as discussed by Kroukamp *et al.* (2017) with the

exception that the final step uses HF in addition to HNO_3 and H_2O_2, to liberate any remaining arsenic bound within the crystal lattice of superficial silicate materials on the lichen thallus.

2. Materials and methods

2.1. Sample collection

Fresh lichen material, *Parmotrema austrosinense,* (Zahlbr.) Hale*,* was collected with plastic forceps from a rural location at the foot of the Waterberg Mountains (GPS co-ordinates -24.4880278 S, 27. 8137778 E), and the Johannesburg Botanical Gardens (JHB, GPS co-ordinates -26.159875 S, 27.99346 E) in South Africa after the rainy season (first week of March 2017) at each site (SAWS 2018). Collection took place 100 m away from any main roads, from all sides of the tree and 2 - 4 m above soil level to ensure that bias due to direct exposure to concentrated automobile byproducts, dominating winds and contamination by soil was prevented (Monaci *et al.* 2012, Kroukamp *et al.* 2017). Clinging foreign material (such as bark, insects, moss etc.) was removed from the lichens prior to storage, and the cleaned lichens were placed in acid-washed polypropylene bottles.

On the same day as the sample collection, the adhering side of the lichens received detailed cleaning under a lighted magnifying station, and gently tapped, removing any particles of foreign material which resulted from the cleaning. Samples were not washed since As from the atmosphere is mostly in the particulate form and washing would remove particles adhering to the lichen surface (Frati *et al.* 2005, Kroukamp *et al.* 2017). Ten grams of the pooled lichen material from each site was hand-shredded using nitrile-gloves and passed through a 1-4.699 mm Endecott sieve to improve the homogeneity of the bulk lichen material (Kroukamp *et al.* 2017). Lichens were air-dried overnight in a cool, dark place and ground the following day using a porcelain pestle and mortar. The resulting powder was sieved through an Endecott 420 µm sieve to ensure homogeneity.

2.2. Meteorological data collection

Average rainfall, maximum and minimum temperature and humidity were recorded by the South African Weather Service (SAWS 2018) for the three months prior to sampling (summer) to provide information on factors which could affect analyte concentrations at the time of sampling. Information was gathered from weather stations nearest to the sample sites, namely Lephalale Weather Station (GPS coordinates 23.6760 S, 27.7050 E, elevation 839 m) to represent a rural (Waterberg) site and Johannesburg Botanical Gardens Weather Station (GPS co-ordinates 26.1560 S, 27.9990 E, elevation 1624 m) to represent an urban (JHB) site.

2.3. Instrumentation and calibration standards

All analyses took place using a NexION® 300X ICP-MS (PerkinElmer Inc. Shelton, Connecticut, USA). For chromatographic applications, the ICP-MS was coupled to a Flexar™ HPLC (PerkinElmer Inc.). Data was originally acquired on Chromera® software (PerkinElmer Inc.), however due to limitations of this software, chromatograms were exported from Chromera and imported into Clarity 8.0™ chromatography software (DataApex Inc, Prague, Czech Republic) for processing. Instrument performance was evaluated daily, where checks included nebulizer gas flow, torch alignment, oxide (CeO/Ce<2.5%) and doubly charged (Ce^{++}/Ce<3%) optimizations. Since As is a monoisotopic element with a low background atmospheric concentration of 1-3 ng/m^3 (ATSDR 2006) and a theoretical ionization efficiency of only 52% (Klaue and Blum 1999), it was necessary to optimize additional instrument conditions such as RF power, torch alignment, nebulizer gas flow, plasma gas flow, auxiliary gas flow and torch sampling depth to improve the sensitivity of the instrument towards As (Supplementary Material: Table S1).

Calibration standards (0.1-20 ppb, n=6) of arsenite (As III) and arsenate (As V) were prepared from stock solutions (1000 mg/L As III and As V respectively, Inorganic Ventures, Virginia, USA). A stock solution of 10 mg/L arsenobetaine (AsB), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) were prepared from AsB (≥95% Fluka, Sigma Aldrich, Missouri, USA), monosodium acid methane arsonate (99.5% Supelco, Sigma Aldrich), and cacodylic acid (≥ 99.0%, Sigma Aldrich) respectively. All calibration standards and samples were prepared just before analysis. Initial method development had shown that there was no presence of an $^{75}ArCl^{+}$ interference on $^{75}As^{+}$, and consequently the lichen samples were analyzed in standard mode. Although collisional focusing can be a useful tool in improving analyte sensitivities (Tanner *et al.* 2002), it was not employed here since As concentrations in these specific samples were low, and the addition of a collision gas was found to drastically reduce the analyte signal to levels where sensitivity and accuracy were both severely compromised.

2.4. Total sample digestion

Powdered and sieved lichen material which had been sampled randomly from the bulk material described in Section 2.1 (0.1 g) was placed on weighing paper (Whatman, Sigma Aldrich), made anti-static with an anti-static gun (Zerostat Anti-static Instrument, Sigma Aldrich, USA) and weighed in triplicate on an analytical balance (XP205, Mettler Toledo, Ohio, USA). Thereafter, each sample was placed into acid-cleaned Teflon® digestion vessels and 7 mL HNO_3 (65%, Suprapur®, Merck, Darmstadt, Germany), 2 mL H_2O_2 (30%, Suprapur, Merck, Darmstadt, Germany) and 1 mL HF (40%, Suprapur, Merck, Darmstadt, Germany) were added to the sample. Prior to microwave digestion, the samples were degassed in a fume hood for 20 min as is recommended for microwave digestions involving peroxide and organic materials (Mindak *et al.* 2014). The digestion acids used were in accordance with the method proposed by Koz *et al.* (2010), so as to ensure that any metals bound within the lattice of insoluble crystals, such as silicate minerals, would be released into solution.

In contrast to the method proposed by Koz *et al.* (2010), we used a sample mass of 0.1 g since powdered plant material with masses above this had previously been found to greatly increase the likelihood of the microwave digestion vessels venting, resulting in analyte losses. The samples were digested in an analytical digestion microwave (Mars 6, CEM, Matthews, NC, USA) where a microwave program of 20 min ramp time to 180 °C, and a 20 min hold time was used. After digestion, vessels were air-cooled to ambient temperatures before slowly opening with the intention of gradually lowering the internal pressure of the vessel, thereby reducing the loss of volatile analytes. The digestate was quantitatively transferred using 3 washings of DIW, filtered through a quantitative filter paper (0.22 µm hardened ashless, Merck) and diluted to 50 mL in centrifuge tubes (Griener Bio-one, Kremsmünster, Austria) using DIW. Prior to analysis, the samples were further diluted five times and rhodium was used as an internal standard to account for minor differences in the sample matrix and instrument drift. For quality control and quality assurance, two certified reference materials, namely Tea leaves (INCT-TL-1, Institute of Nuclear Chemistry and Technology) and Lichen CRM (BCR Reference Material no 482, Trace elements in lichen (*Pseudevernia furfuracea*)) were prepared and analysed in the same manner as the samples. All results were translated back into µg/g of the dry lichen weight post-analysis.

2.5. Sequential extraction of arsenic from lichens with modified water extraction step

The sequential extraction used in this chapter has been summarized in Table S2 (Supplementary Material), and detailed descriptions are given in the sections which follow. Each method described was applied to 10 replicate samples from both the rural and urban sites to demonstrate the application of the proposed method in real samples from sites with different sources of As. Volumes of the extraction solvents were altered in order to keep the sample-mass-to-extraction-solvent volumes consistent with those reported in previous work (Kroukamp *et al.* 2017). All results were translated back into µg/g of dry lichen weight for comparison.

2.5.1. Water extractable fraction

As previously optimized (Kroukamp et al. 2019), ten replicates of pulverized lichen material (0.07 g) which were randomly sampled from the bulk material described in Section 2.1 were placed on weighing paper and weighed on an analytical balance after having been made anti-static. Samples were extracted using 7 mL deionized water for 24 h with an ultrasonic bath (Integral Systems, South Africa) at 57 °C in 15 mL acid-washed centrifuge tubes (Griener Bio-one, Austria) in accordance with the optimized extraction procedure described in Kroukamp *et al.* (2019). Each sample was centrifuged at 600 rpm for 10 min (Hettich Zentrifugen EBA 20, Germany), and the supernatant collected and filtered through a pre-wet (using 0.5 mL MeOH; ≥99.9%, HPLC Plus, Sigma-Aldrich) 0.45 µm polytetrafluoroethylene (PTFE) syringe filter (Membrane Solutions, Washington, USA).

Each sample pellet was then subjected to four additional washings using 0.5 mL DIW, where each washing was followed by agitation in an ultrasonic bath (1 min), centrifugation (10 min, 600 rpm), extraction and filtration of the supernatant as described above. The combined extract and washings were then analyzed directly using HPLC-ICP-MS with the parameters described in Table S1 (Supplementary Material). A blank was run after every sample during chromatographic analysis to ensure that there was no observable carryover between samples and that all As species from the previous injection had been eluted.

2.5.2. Reducible fraction

In order to keep the ratios of extractant-to-sample-mass the same as in previous work (Kroukamp *et al.* 2017), 7 mL of hydroxylamine hydrochloride (0.25 M, pH 2, ReagentPlus® 99%, Sigma Aldrich) was added to each of the residual sample pellets acquired from the previous extraction step, resuspended and extracted at room temperature for 5 h using a magnetic stirrer (Stuart Hotplate Stirrer, SB302, setting 7) and PTFE-coated micro-magnetic stirrer beads. The stirrer beads were removed, the samples centrifuged for 10 min, the supernatants collected and filtered through pre-wet PTFE syringe filters, and the samples diluted 10-fold with DIW for ICP-MS analysis.

2.5.3. Oxidizable fraction

To keep the sample mass-solvent ratios the same as in previous work (Kroukamp *et al.* 2017), the pellets from the previous step were quantitatively transferred into perfluoroalkoxy alkane vials (PFA, Savillex, Minnesota, USA) using 5.25 mL of H_2O_2 (30% Suprapur, Merck, Germany). The samples were allowed to react for the period of time it took to evaporate the H_2O_2 to near dryness on a hotplate at 95 °C. This step was followed by a second extraction using H_2O_2 (5.25 mL, 30 % Suprapur, Merck) which too was evaporated to near-dryness. Ammonium acetate (10.5 mL, 2.5 M, pH 3, ≥98%, Sigma Aldrich) was added to each sample and the samples stirred on a magnetic stirrer at room temperature for 1.5 h. The stirrer beads were removed, and the digest transferred to a 15 mL centrifuge tube. The samples were centrifuged for 10 min, the supernatant of each sample collected, filtered and diluted 15-fold with DIW prior to analysis by ICP-MS.

2.5.4. Non-bioavailable fraction

Using HNO_3 (0.49 mL, 65% Suprapur, Merck), H_2O_2 (0.14 mL, 30% Suprapur, Merck) and HF (0.07 mL, 40% Suprapur, Merck), the remaining pellet for each sample was quantitatively transferred back into its respective PFA vial to complete the remaining digestion. The lids for the vials were tightened and the samples allowed to reflux for 5 h at 95 °C. After digestion, the samples in solution were evaporated to near-dryness on the hotplate and then diluted with 1 mL HNO_3. The sample in solution was evaporated to near-dryness again, and this step of adding HNO_3 to the sample and evaporating was repeated twice more with the intention to vaporize the HF from the sample so that it would not interact with the glassware in the ICP-MS. Upon the final HNO_3 addition, the sample was diluted to 10 mL with DIW, filtered through a pre-wet PTFE filter and the internal standard added which created a small dilution of 1.01x prior to analysis by ICP-MS.

2.6. Species stability over 1 month

Although the long-term stability of bioavailable As species in the lichen matrix was not within the scope of this study, the short-term stability of the water-soluble fraction of As and the respective species was evaluated for the urban site. This was done by setting aside 5 g of dry and cleaned lichen material (shredded but not pulverized) in an acid-washed polypropylene vial in a cool, dark place for a period of a month. Starting on the day of collection, every week, 1 g of the bulk material was pulverized (as described earlier) and 0.07 g sub-samples of this material were taken in triplicate for processing. The samples were then extracted using water (Step 1) and chromatographically separated using the mobile phase and conditions described in Table S1 (Supplementary Material). Species percentages based upon the relationship between the individual species and the sum of the As species were thereafter compared.

2.7. Statistical analyses

Statistical analysis took place using the program SPSS 25.0. Independent T-test with Levenes Test for Equality of Variance and T-test for equality of means were run for inter-site comparisons. For comparisons within a site and across different species/ extractions, one-way ANOVA was used with suitable *post hoc* tests; Dunnett's T-3 test and Scheffes Analysis. Statistically significant differences were determined with significance set at 95% ($p<0.05$).

3. Results

3.1. Meteorological data and total metal analysis

Weather data was collected to evaluate the potential link between weather conditions and arsenic speciation in lichen samples and were based upon statistics collected from weather stations closest to the sites. The average daytime temperature was 4 °C higher in the rural site than in the urban site over the 3 months prior to sampling (Supplementary Material: Fig. S1 and Fig. S2). Night time temperatures were on average 5 °C higher in the rural site than in the urban site. In terms of precipitation and humidity, the rural site had on average 83 mm less rain and 5% lower humidity than the urban site. As such, it is likely that lichens collected from the rural site were under higher stress than lichens from the urban site and consequently, may not have been as metabolically active as lichens from the urban site (Nash and Gries 1995, Bergamaschi *et al.* 2007). The total concentration of As in the urban and rural sites were 1.85 ± 0.33 µg/g and 0.44 ±0.025 µg/g, where the accuracy of the analysis was demonstrated by recoveries of 91 and 118 % for the lichen and tea leaf CRM respectively.

3.2. Sequential extraction

3.2.1. Step 1: Water extraction

HPLC-ICP-MS studies were performed on extracts from Step 1 obtained using the instrumentation and standards outlined in Section 2.3 and summarized in Table S1 (Supplementary Material). The calibration correlation co-efficients for the calibration standards for all As species in the water extraction step were between 0.99800 - 0.99999 (Supplementary Material: Table S3). The 10 replicate extractions were highly repeatable, with peak area percentage relative standard deviations (%RSD) ranging between 1.46-5.94% (Supplementary Material: Table S3, Fig. S3 and Fig. S4). The retention times of the As species under study were consistent across the 10 replicates, with retention time %RSD between 0.36-0.93% across both sites and shows that the matrix is adequately homogenous since changes in the matrix could cause shifts in the chromatographic peak positions. Most of the As species under study were above the method detection limits (MDL, Supplementary Material: Table S3) with signal-to-noise ratios (S/N) exceeding 3 (Heyden and Boqué 2009).

Monomethylarsonic acid concentrations were below the MDL with a S/N of 1 for both sites, thus explaining the large %RSD of the chromatographic area of this peak in samples from the rural sites.

In previous work, the peak positions of AsB and As III had been confirmed to be baseline resolved in the lichen matrix through spiking (Kroukamp *et al.* 2019). Here, the poor resolution of AsB and As III in non-spiked samples is likely due to the presence of an additional, unidentified arseno-compound in the lichen samples eluting just after AsB. Likely candidates are arsenoribose, tetramethylarsonium ion (TETRA) (Hsieh and Jiang 2012), trimethylarsine oxide (TMAO) (Mrak et al. 2006), where these two species may co-elute with AsB. When comparing Fig. S3 to Fig. S4 (Supplementary Material), there is better separation between AsB and As III in the rural site than in the urban site which points to higher concentrations of the unidentified arseno-compound at the urban location. Nevertheless, AsB and As III were well-enough resolved to gain semi-quantitative information about these two analytes. Results for all other analytes under study (DMA, MMA, As V) can be regarded as being quantitative. The apparent high chromatographic baseline for both chromatograms (Supplementary Material: Fig. S3 and Fig. S4) is simply due to these being mass-normalized chromatograms, where the signal intensity had been divided by the mass for each data point to ensure that any observed differences in area (concentration) are not due to the differences in the weighed mass. Original baselines prior to mass normalization were around 200 counts per second (cps).

Concentrations of all As species in the water extracts of lichen samples were higher in the urban site than the rural site (Fig. 1), where DMA and AsB+U1 were statistically significantly higher ($p<0.05$). Concentrations of MMA are shown here only for reference. Since the S/N was 1 for this analyte for both sites, it is regarded as being below the MDL.

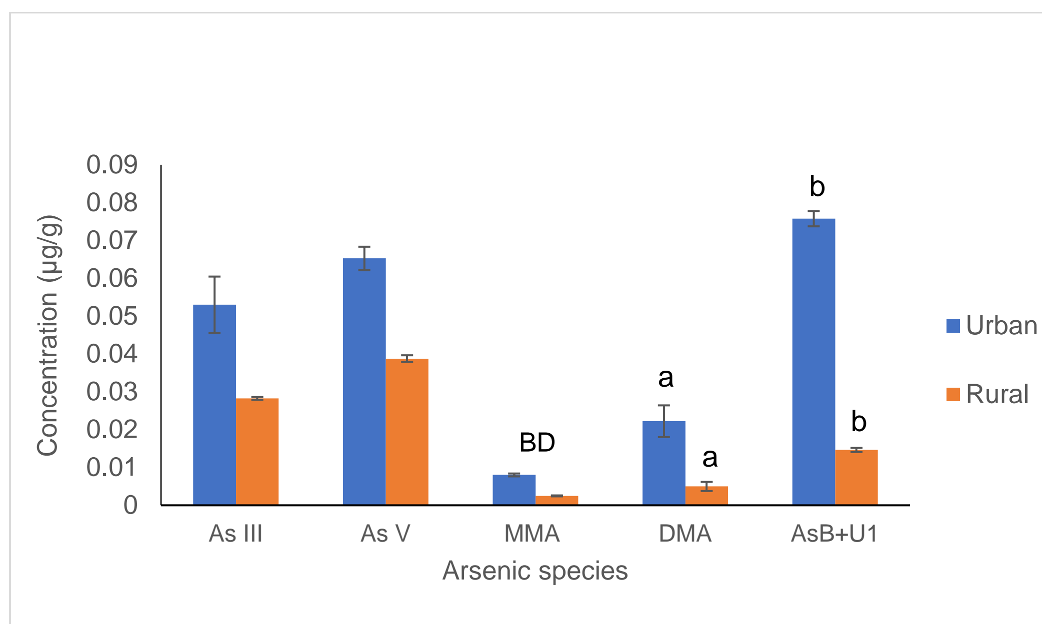


Fig. 1 Averaged concentrations (blank subtracted values) of the various As species from the water extraction of a bulk lichen material (Step 1) where U1 refers to the peak eluting on the tail end of AsB. Significantly different values ($p<0.05$) are indicated by corresponding letters above the bars. BD indicates values which are below the MDLs. Values are based upon the dry weight of the pulverized lichen where error bars show ± standard deviation (SD), n=10

When comparing the individual As species to the sum of As species, the order of relative abundance of As in the rural site was As V>As III>AsB+U1>DMA>MMA (Supplementary Material: Fig. S5). In contrast, for the urban site this was AsB+U1>As V>As III>DMA>MMA. This observation could either be due to differences in weather, where higher rainfall at the urban site (Supplementary Material: Fig. S2) in comparison to the rural site would facilitate the removal of the soluble inorganic As species deposited on the surface of the lichens or different sources of As at the two sites. Higher rainfall at

this site would also remove particulate As from the air, thereby reducing ambient concentrations. Furthermore, since lichens have faster metabolisms when they are wet (Nash and Gries 1995, Bergamaschi *et al.* 2007), high rainfall coupled with lower temperatures and higher humidity could increase the metabolism of inorganic As species which had been deposited on the surface of the lichens in the months or years prior to sampling, as lichens are known to store some metals for as long as 2-5 years (Walther *et al.* 1990).

3.2.2. Remaining sequential extraction steps

For the remaining sequential extraction steps, no chromatographic speciation was employed since the solvents used have reducing or oxidizing properties and would compromise the integrity of the species extracted in the solvent. Extracted As in lichens from both the urban and rural sites showed the same trend for the various extractions, where concentrations of As were found in the order of abundance of oxidizable>reducible>water extractable>residual (Fig. 2a). In both sites, As was predominantly bound to organic matter and sulphides (oxidizable), with the second highest concentrations found in carbonates and oxides (reducible), followed by those which are readily bioavailable (water-soluble), and with the least amount bound to residual insoluble minerals such as silicates. These findings contrast to those by Farinha *et al.* (2004) who had concluded that As in the foliose lichen *Parmelia sulcata*, was predominantly bound to refractory compounds, where such findings in their study are likely to point to a different source of As, such as windborne silicate-based dusts. In our study, concentrations of As in both the oxidizable and non-bioavailable/residual fractions from the rural site are statistically lower ($p<0.05$) than the urban impacted site, where other concentrations are higher in the urban site but not statistically significantly higher.

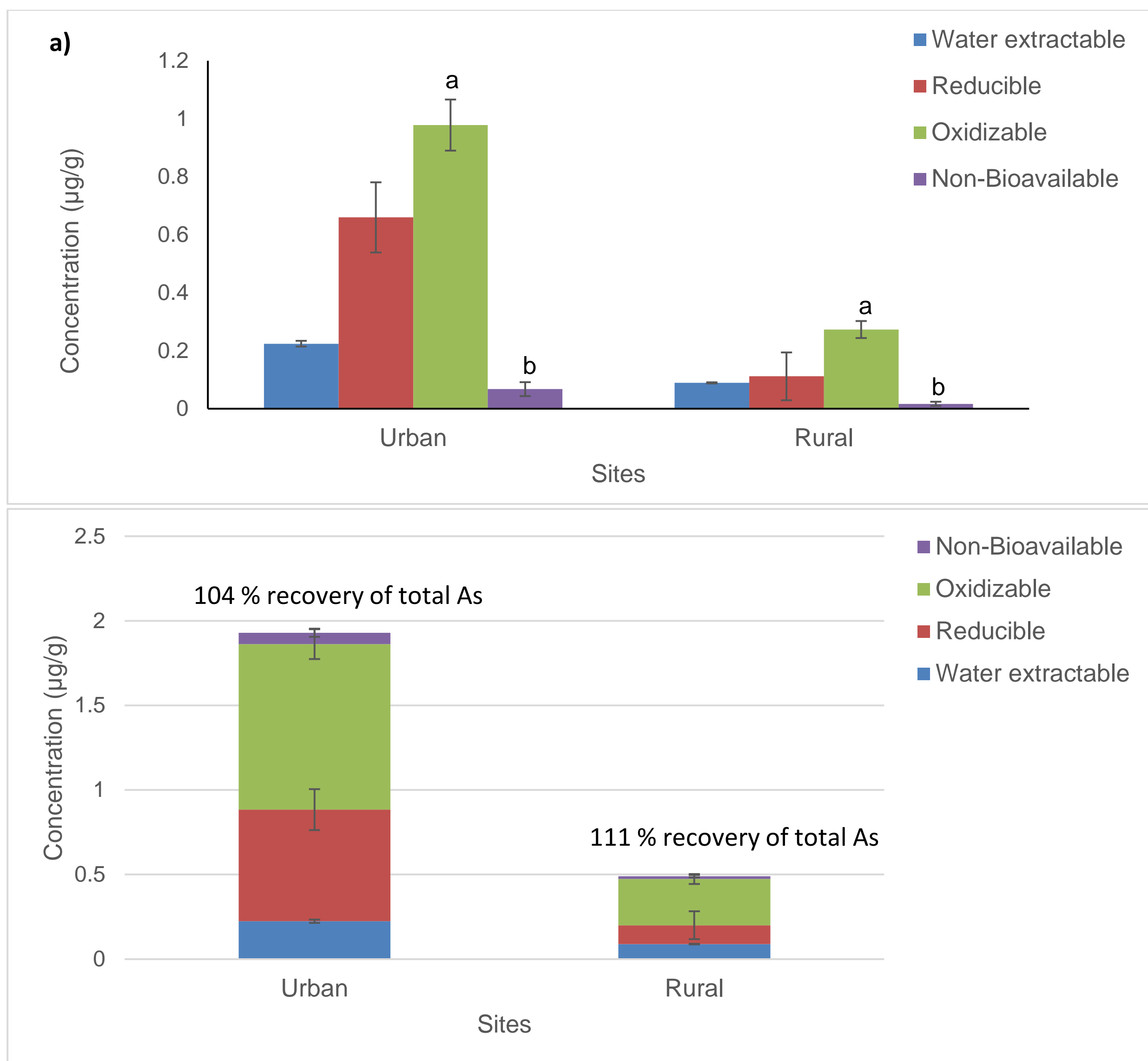


Fig. 2 Average concentrations (blank subtracted values) of As in the bulk lichen samples from urban and rural sites from the four-step extraction procedure as optimized in this study. **a)** Statistically significant differences ($p<0.05$) are indicated by corresponding letters above the bars. Values are based upon the dry weight of the sample where error bars show ±SD. **b)** showing percentage recovery of sum of extractions in comparison to the total As concentration as determined by microwave digestion

This information relates to bioavailability and is extremely useful in determining the ecological fate of atmospheric As and source apportionment. For example, since the largest portion of the As present in lichens is bound to sulphides, this could point to gold mining as a potential source of the As at both sites since arseno-sulphidic minerals are often associated with gold-bearing minerals (Villaescusa and Bollinger 2008). The rural site is 50 km away from the mining town of Thabazimbi which has a great deal of Platinum Group Metal (PGM) and ferrochrome mines in the surrounding areas due to rich deposits of these metals (Von Gruenewaldt and Merkel 1995, Clarke *et al.* 2009, Fisher *et al.* 2011). Although this may be a potential source of As at the rural site, the lower summative concentration of all As species at the rural site in comparison to the urban site shows that the impacts at the former site are not as localized as the urban site and are likely to be due to natural or far away sources. The urban site, in contrast, is 15 and 40 km away from two major gold mines respectively where the Witwatersrand Basin is still considered to be the world's largest gold resource (Naicker *et al.* 2003, South African Chamber of Mines 2014) and may account for some of the arsenic found in the lichen samples from this site.

The second highest abundance of arsenic was found in the reducible fraction, where this refers to As species which are bound to carbonates and oxides and would typically become available only under anoxic conditions. It is therefore extremely likely that upon exposure to a strongly reducing environment, such as atmospheric deposition onto anoxic soils and sediments, that the As present in the reducible fraction will become bioavailable in the form of As III. Since lichens are known to secrete oxalic acid which is a reducing agent (Haas and Purvis 2006), this may assist in making the reducible fraction of As available for lichen metabolism.

The water-soluble arsenic species were primarily in the inorganic form at the rural site and in the organic form, AsB+U1, at the urban site. This goes to show that the time of sampling and seasonal differences, in addition to emission sources, could play a significant role in the data gathered from sampling sites and its interpretation. Findings from our study strongly disagree with findings by Mrak *et al.* (2006) who suggested that lichens are not able to represent the chemical forms present in the environment. In contrast we have found that lichens are not only able to provide a wealth of information about the different chemical forms of As but can also provide information about the timing of pollution events based upon the relative chemical species and progression of methylation activities. Such information can also be extremely useful in determining source apportionment on the provision that there is existing knowledge of potential pollution activities within the area.

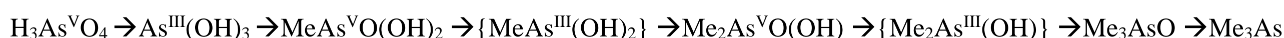
3.3. Comparison to other studies

Upon comparing different studies involving the sequential extraction of As from lichens, Farinha *et al.* (2004) found poor recoveries of As (2-9.43%) using the three-step sequential extraction of As from the foliose lichen *P. furfuracea*, where extraction solvents water, $CaCl_2$ and H_3PO_4 were used respectively. Mrak *et al.* (2006) achieved better recoveries of As (45%) by applying a five-step sequential extraction procedure which had been designed for use in soils to the epiphytic, foliose lichen, *Hypogymnia physodes*. Extraction solvents for this method were $(NH_4)_2SO_4$, $(NH)_4H_2PO_4$, NH_4-oxalate buffer, NH_4-oxalate buffer + ascorbic acid, and KOH for the different extraction steps, where As III, As V, MMA, DMA, AsB were all found. Trimethylarsine oxide, glycerol-ribose and phosphate-ribose were detected in accordance with method proposed by Wenzel *et al.* (2001). Total As concentrations in their study were 5.04 ±0.09 µg/g. In contrast to both studies, we found excellent recoveries of As using our method, with percentage recoveries of 104% and 111% for the urban and rural sites respectively (Fig. 2b, Supplementary Material: Table S4), where the SD between replicate analyses can account for the variance from 100% recovery. The key factor resulting in these high yields was the replacement of the conventional water extraction step with an optimized one (Kroukamp *et al.* 2019), followed by the sequential extraction which was not only able to provide a wealth of information about the chemical species of As present in the sample, but also assisted in source apportionment evaluations.

3.4. Species stability

The stability of the different As species in the readily-available (water-soluble) fraction was the only step considered for short-term stability studies since this is the perceived “bioavailable” fraction and is of greatest toxicological interest. For this study, the comparative shift of the different As species was of key interest. Therefore, the relative percentages of the individual As species in comparison to the sum of the five As species from each week were used for this comparative assessment (Fig. 3). In this way, differences in concentrations due to different subsets of the bulk lichen material between weeks could be accounted for. Fig. 3 shows that the relative abundance of the As species under study shifted over a short-term storage period of one month, shedding some light upon the mechanisms of detoxification of As by the foliose lichen, *P. austrosinense*. If these mechanisms are of interest, a more detailed study of these mechanisms can be recommended for future assessments.

The percentage of As III increased in comparison to the sum of the As species from the initial assessment (T0) to a week later (T1) where this is likely due to metabolism (Fig. 3). Since As V needs to be reduced to As III prior to methylation (Cullen 2014), it is highly likely that the increase of As III from T0 to T1 is part of this strategy, and is facilitated by the secretion of oxalic acid, which is a reducing agent, by the lichen (Haas and Purvis 2006). After two weeks from the initial assessment (T2), As III concentrations decreased in comparison to both T0 and T1. By three weeks after this initial assessment (T3), As III was below the MDL (Supplementary Material: Table S3). This increase in As III at T1, followed by a decrease in As III at T2 and T3, and the correlating increase in AsB and the unidentified organo-As compound (U1) by week T3 supports the pathway for As detoxification as proposed by Challenger in which As III is methylated to AsB and other organo-arseno compounds (Challenger 1945, Cullen 2014). The Challenger pathway can be briefly described as follows (Challenger 1945, Cullen et al. 1995):



$$H_3As^{V}O_4 \rightarrow As^{III}(OH)_3 \rightarrow MeAs^{V}O(OH)_2 \rightarrow \{MeAs^{III}(OH)_2\} \rightarrow Me_2As^{V}O(OH) \rightarrow \{Me_2As^{III}(OH)\} \rightarrow Me_3AsO \rightarrow Me_3As$$

The percentage of As V in comparison to the sum of the five As species decreased slightly in week T1 which correlates with the increasing As III at this time. Thereafter it increased week by week for T2 and T3 respectively. The increase in As V is counter to what we would expect in terms of the methylation of As V, since As V is generally reduced to As III and then methylated (Cullen 2014). Cullen (2014) proposed that increasing As V concentrations over time, as had been observed by some researchers in bacteria cultures, would imply parallel detoxification processes, where some species of As may be converted into As V through oxidative processes, whereas others may detoxify the more toxic arsenic species, such as As III, into AsB and other less-hazardous arseno-compounds. In contrast, we propose that for lichens the presence of oxalic acid (Haas and Purvis 2006) could reduce and make readily available As which was previously bound to carbonates and oxides thereby explaining the increase in As V in the water-soluble fraction. Over time, the resulting As V could be further reduced to As III. To support this hypothesis, future studies would need to follow the water extraction of As with a sequential extraction to determine if the concentration of the As bound in the reducible fraction decreases with the increasing As V concentration over time, which was outside the scope of this study. The percentage of DMA in the sample remained relatively unaffected over T0-T3. Monomethylarsonic acid was not evaluated as it was below detection ($S/N<3$) for all extractions. Arsenobetaine and the unknown As species (U1) increased week on week implying that this and possibly the species which eluted just after AsB were the metabolic byproducts of As detoxification mechanisms (Challenger 1945, Cullen 2014).

Although it may be argued that the metabolism of As by a lichen may go against the ideal of a biomonitor, this may in fact provide a great deal of useful information regarding the magnitude and timing of pollution events. Some time-based studies have investigated As metabolism by algae and fungi (Cullen *et al.* 1995, Cullen 2014). Since lichens result from the symbiotic relationship between algae and fungi, this information may be useful in explaining which biological component is responsible for the respective As species. In the fungus *Apotrichum humicola*, As V was found to be reduced to As III within 2 days after exposure, thereafter it was converted to TMAO with small amounts of MMA and DMA over the course of 30 days (Cullen *et al.* 1995, Cullen 2014). In contrast, an algae, *Polyphysa peniculus,* metabolized As V to As III and DMA, where MMA and TMAO were found to be absent in this study (Cullen 1994, Cullen 2014). Our study agrees with findings by Cullen *et al.* (1995) and Cullen (2014) which had shown that if As III is found to be present in the sample, the impact is likely to be fairly recent (a few weeks). The presence of As V may in part be due to soluble As V attached to particulate matter in the air but may also have resulted from the metabolism of As bound to carbonates and oxides from older exposures which have been mobilized through their interaction with oxalic acid as hypothesized in this study. The presence of AsB and an unknown As species (U1) in lichens is most likely a detoxification byproduct of more harmful As species (As III and As V) present in air, where the higher the concentration of AsB, the less recent the exposure event.

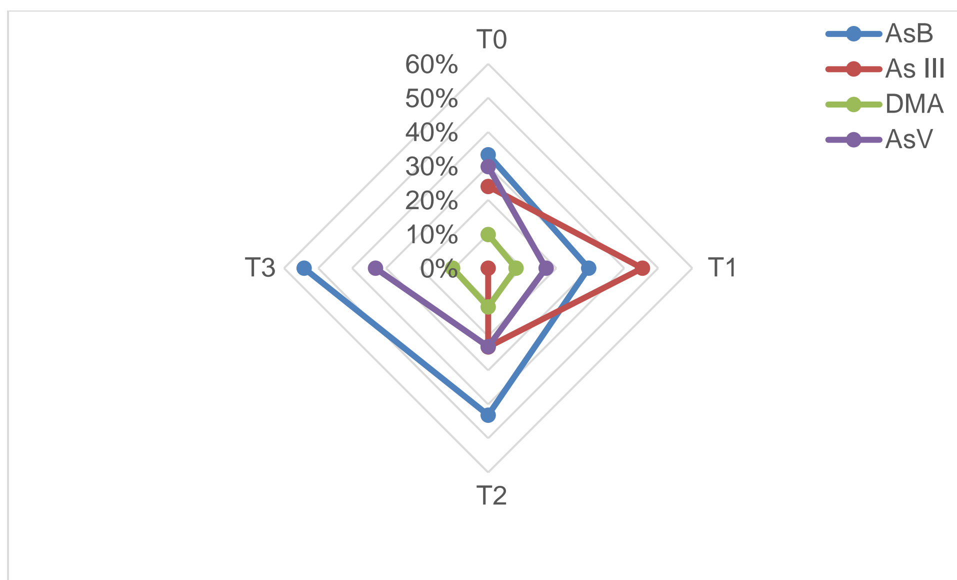


Fig. 3 Stability of various As species in the water extraction step over a period of a month as determined by HPLC-ICP-MS. Results are shown as percentage of species under investigation in comparison to the sum of the five arsenic species. Where T0 represents the initial evaluation, and T1, T2, and T3 are one week after, two weeks after and three weeks after the initial assessment.

4. Conclusions

This study has shown that the four-step sequential extraction of a lichen material, involving the ultrasonic extraction and chromatographic separation of As from the first step of the extraction, followed by a traditional sequential extraction procedure greatly improved the selectivity and recoveries of As. The application of the previously developed method to extract and chromatographically separate five As species from one another in a lichen matrix (Kroukamp *et al*. 2019) was found to be highly reproducible with low %RSD between samples and elutions. The sum of As species from the four extraction steps resulted in excellent mass balance recoveries (104% and 111% in lichens from the urban and rural sites respectively), and this information, combined with meteorological data provides useful information regarding source apportionment, temporal impacts and bioavailability, proving fitness for purpose of this method in the evaluation of As species and non-water soluble fractions in air using a lichen biomonitor. This study also provided some insight into the short-term stability and metabolism of As species in processed and stored samples of lichen biomonitor, *P. austrosinense*, and proposed a possible explanation for the counter-intuitive increase in As V observed by some researchers.

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Paper 5- Supplementary Information

Species distribution patterns of arsenic in a lichen biomonitor

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Supplementary material

Table S1: Experimental conditions for the ICP-MS and HPLC

Instrument	Condition	Name or Value
ICP-MS	Model	NexION® (PerkinElmer, 300X)
	RF Power	1500 W
	Plasma/Coolant gas flow	17 L/min
	Auxiliary Gas flow	1.4 L/min
	Torch Sampling Depth	- 1.0 mm from standard set point
	Injector	Quartz, 2.0 mm
	Spray Chamber	Quartz cyclonic spray chamber with central baffle
	Nebulizer	Quartz Meinhard® Nebulizer
	Mode of analysis	Standard mode
HPLC	Model	Flexar™ HPLC
	Autosampler Loop size	250 µL
	Autosampler Injection Mode	Partial loop injection
	Anion Exchange Column	Hamilton PRP X-100 (5 µm, 4.6 x 150 mm, Sigma Aldrich)
	Mobile Phase	17.5 mmol NH_4NO_3 in 1% MeOH

Table S2: Summary of optimized parameters for extracting As from pulverized lichen, *P. austrosinense*. Step 1 of the extraction was optimized in Kroukamp *et al.* (2019), whereas steps 2 and 3 follow previous work (Kroukamp *et al.* 2017). Step 4 was adapted to follow the extraction proposed by Koz *et al.* (2010)

Extraction step	**Parameter**	**Description/unit**
STEP 1: *Water-extractable fraction*-Soluble/Exchangeable As	Mass of powdered lichen material	0.07 g
	Extraction solvent	DIW
	Volume of extraction solvent	7 mL
	Method of extraction	Ultrasonic bath
	Temperature of extraction	57 °C
	Length of time of extraction	24 hr
	Dilution prior to analysis	None
STEP 2: *Reducible Fraction*-As bound to carbonates and oxides which are reducible	Extraction solvent	$HONH_2{\cdot}HCL$ (0.25 M, pH 2)
	Volume of extraction solvent	7 mL
	Method of extraction	Magnetic stirring
	Temperature of extraction	Room temperature
	Time of extraction	5 hr
	Dilution prior to analysis	10x
STEP 3: Part 1- *Oxidizable fraction*-As bound to organic matter and sulphides	Extraction solvents	2 x H_2O_2
	Volume of extraction solvent	5.25 mL for both additions of H_2O_2
	Method of extraction	Hotplate
	Temperature of extraction	95 °C
	Length of time of extraction	until samples were nearly dry
	Dilution prior to analysis	See **Step 3: Part 2**
STEP 3: Part 2- *Oxidizable fraction*-As bound to organic matter and sulphides	Extraction solvents	$C_2H_7NO_2$
	Volume of extraction solvent	10.5 mL
	Method of extraction	Magnetic stirring
	Temperature of extraction	Room temperature
	Length of time of extraction	1.5 hr
	Dilution prior to analysis	15x
STEP 4: *Non-bioavailable fraction*-residual As	Extraction solvents	HNO_3, H_2O_2, HF
	Volume of extraction solvent	0.49 mL HNO_3, 0.14 mL H_2O_2, 0.07 mL HF
	Method of extraction	Hotplate
	Temperature of extraction	95 °C
	Length of time of extraction	5 hr
	Dilution prior to analysis	1.01x (slight dilution due to addition of internal standard)

Table S3 Water extraction results for the extraction of 10 replicate analyses of fresh lichen material collected from an urban and rural impacted site using HPLC-ICP-MS, where * indicates compounds which are below detection (BD)

As species	Parameter	Urban site	Rural site
AsB+U1	Calibration correlation co-efficient (r^2)	0.99999	
	Retention time RSD (%)	0.51	
	Area RSD (%)	2.44	2.40
	Average S/N	46	10
	MDL (μg/g)	9.65E-05	
As III	Calibration correlation coefficient (r^2)	0.99800	
	Retention time RSD (%)	1.84	
	Area RSD (%)	5.94	3.18
	Average S/N	11	7
	MDL (μg/g)	1.88E-04	
DMA	Calibration correlation coefficient (r^2)	0.99999	
	Retention time RSD (%)	0.36	
	Area RSD (%)	1.66	3.25
	Average S/N	9	3
	MDL (μg/g)	8.61E-05	
MMA*	Calibration correlation coefficient (r^2)	0.99960	
	Retention time RSD (%)	0.93	
	Area RSD (%)	4.02	47.86
	Average S/N	1	1
	MDL (μg/g)	2.70E-04	
As V	Calibration correlation coefficient (r^2)	0.99990	
	Retention time RSD (%)	0.39	
	Area RSD (%)	3.20	1.46
	Average S/N	4	3
	MDL (μg/g)	8.40E-04	

Table S4 Concentrations of As in lichen, *P. austrosinense,* from an urban (n=10) and rural (n=10) site from the proposed 4-step extraction procedure where the water extractable content is the sum of species as determined by HPLC-ICP-MS in the water extraction step. Results are shown in µg/g (mean±SD). Here, MDL denotes the 'method detection limit'

Site	Water extractable	Reducible	Oxidizable	Non-bioavailable	Total As	Summative extraction efficiency (%)
Urban Site	2.24E-01±9.85E-03	6.60E-01±1.21E-01	9.78E-01±8.81E-02	6.74E-02±2.40E-02	1.85E+00±3.33E-01	104%
Rural Site	8.91E-02±1.91E-03	1.12E-01±8.24E-02	2.73E-01±2.93E-02	1.68E-02±7.24E-03	4.42E-01±2.55E-02	111%
Calculated MDL	5.67E-04	5.99E-04	1.02E-02	5.67E-04	3.89E-04	

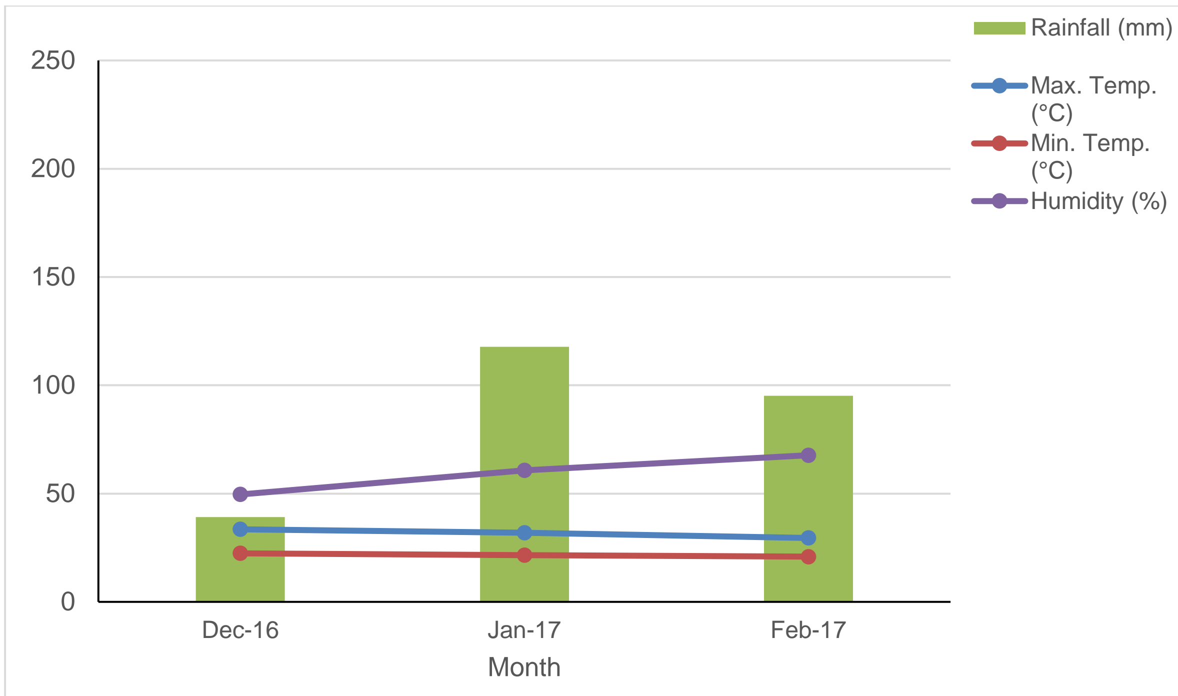


Fig. S1 Weather statistics for the rural site. Average rainfall expressed in mm, the minimum and maximum temperatures in °C and the relative humidity is expressed as percentage. Readings were collected from Lephalale Weather Station (GPS co-ordinates: 23.6760S, 27.7050 E, 839 m elevation)

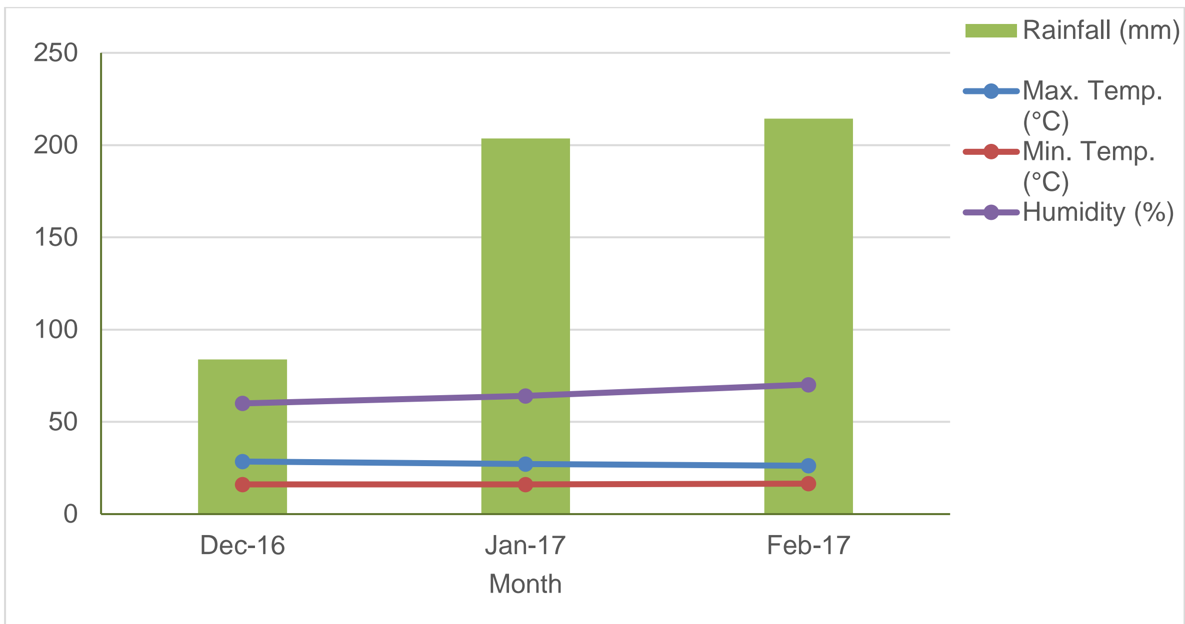


Fig. S2 Weather statistics for the urban site. Average rainfall expressed in mm, the minimum and maximum temperatures in °C and relative humidity is expressed as percentage. Readings were collected from Johannesburg Botanical Garden Weather Station (GPS co-ordinates: 26.1560 S, 27.9990 E, 1624 m elevation)

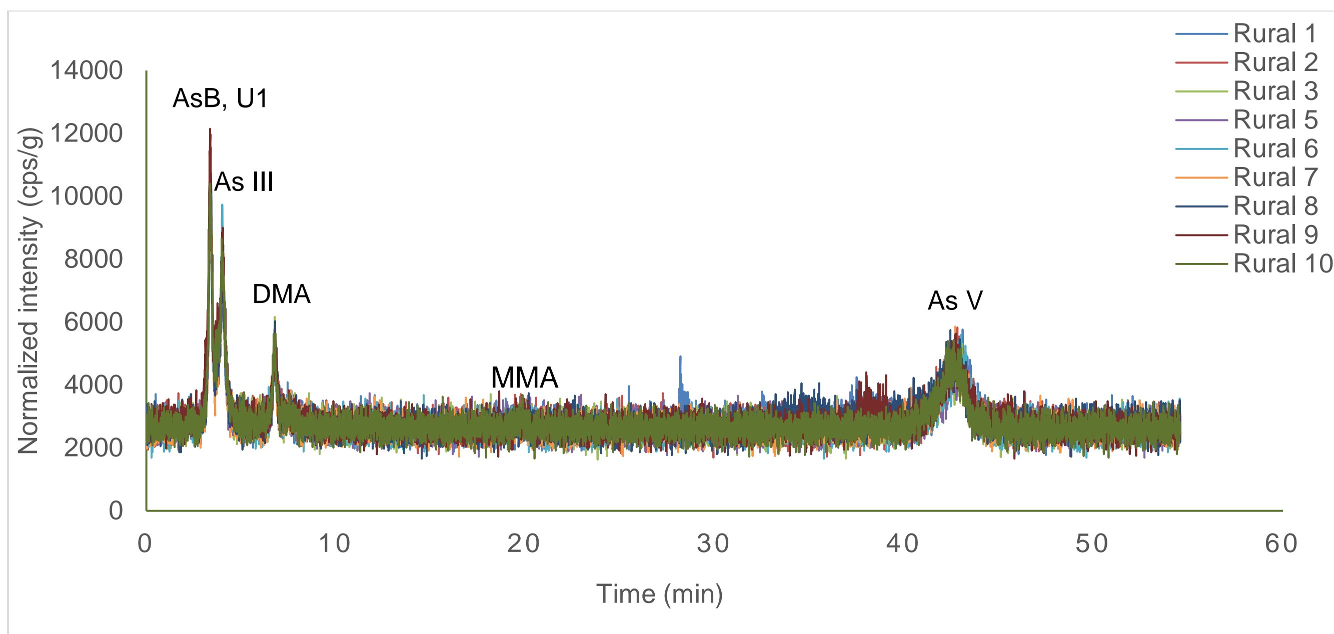


Fig. S3 Mass normalized intensities (cps/g) of 10 replicate analyses of freshly-collected lichen from a rural site (Waterberg) extracted with water (Step 1) in an ultrasonic bath for 24 h and analyzed using HPLC-ICP-MS. Five arsenic species (AsB+U1, As III, DMA, MMA and As V), as well an unknown arsenic species (U1), are shown

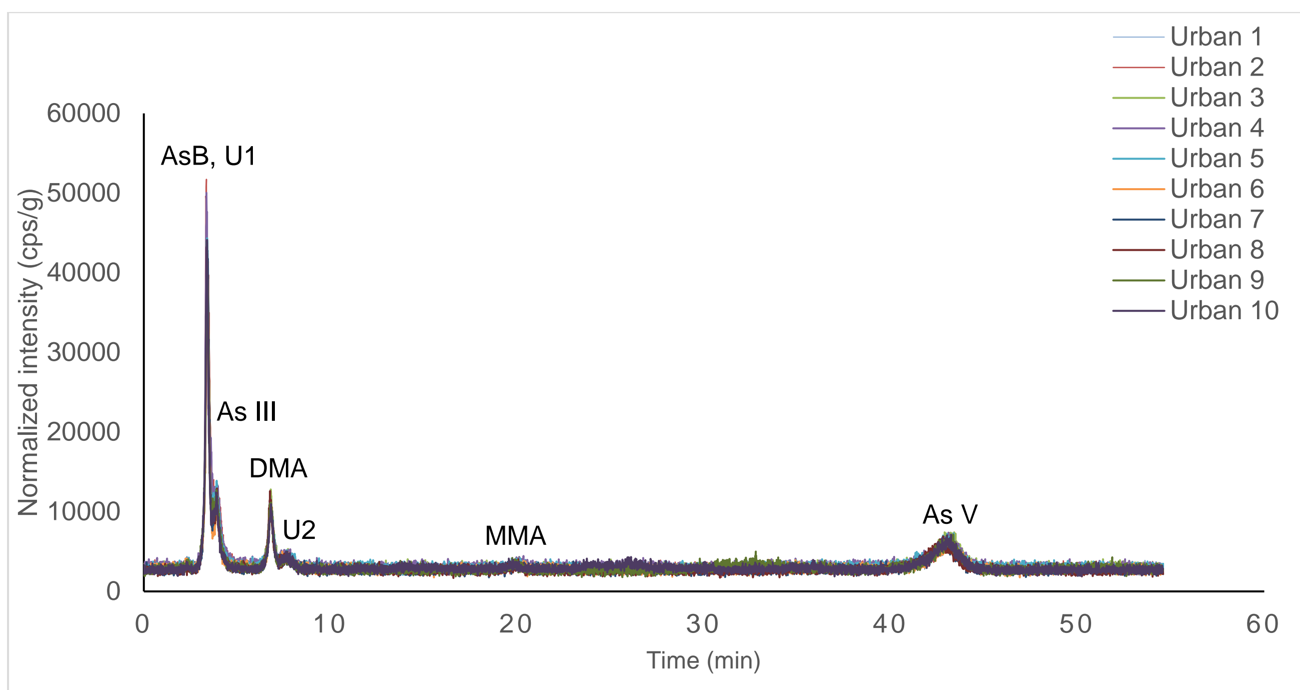


Fig. S4 Mass normalized intensities (cps/g) of 10 replicate analysis of freshly-collected lichen from an urban site (JHB) extracted with water (Step 1) in an ultrasonic bath for 24 h and analyzed using HPLC-ICP-MS. Five arsenic species (AsB+U1, As III, DMA, MMA and As V), as well as two unknown arsenic species (U1, U2), are shown

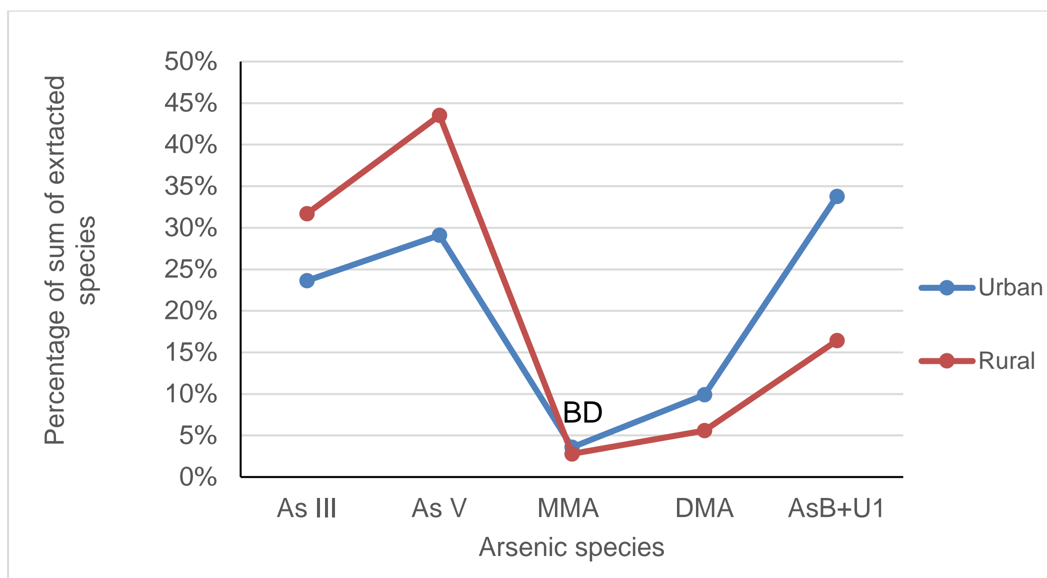


Fig. S5 Relative percentage of each As species in relation to the sum of the five As species (As III, As V, MMA, DMA and AsB+U1) under study, where 'BD' indicates 'below detection'

References:

Kroukamp, E.M., Godeto, T.W., Forbes, P.B.C., 2017. Comparison of sample preparation procedures on metal(loid) fractionation patterns in lichens. *Environmental Monitoring and Assessment*. 189, 451.

Kroukamp, E.M., Godeto, T.W., Forbes, P.B.C., 2019. Optimized extraction of inorganic arsenic species from a foliose lichen biomonitor. *Manuscript submitted for publication.*

Koz, B., Celik, N. Cevik, U., 2010. Biomonitoring of heavy metals by epiphytic lichen species in Black Sea region of Turkey. *Ecological Indicators.* 10, 762-765.

Chapter 5 Overall Conclusions and Future Work

5.1 Overall conclusions

The analysis of chemical species of metal(loid)s in air which elicit a toxicological response in humans is of great interest to both the public and the scientific community, where information obtained can form the foundation of guidelines and national air pollution regulations. Gathering of this information over large tracts of land can be challenging if conventional methods involving the use of air filters are employed, however the use of biomonitors, such as lichens can provide a wealth of information about the presence and abundance of airborne pollutants. Their low cost and ease of sampling allows for comprehensive evaluations to be made regarding air pollution over large areas, including those which are remote or inaccessible.

Despite the abundance of studies of metals and metalloids in lichens, there are still many areas which require research, where their quantification is no easy task. The work outlined in this thesis was able to address some of these issues and highlights important considerations when employing lichen biomonitors in such assessments.

The lichen, *P. austrosinense* is pantropical, and extends into adjacent regions including Africa. It was evaluated at two sites in South Africa with differing anthropogenic impacts for the presence and abundance of a number of metal(loid)s, and was found to be a suitable biomonitor for routine air pollution monitoring since differences in the metal(loid) concentrations could be observed. Following this initial assessment, sixteen metals and metalloids were evaluated in lichens from three previously unstudied locations in South Africa with different sources of airborne metal(loid)s, including a Global Atmospheric Watch baseline station for which there is no known data related to the concentrations of metals in lichens at this site. The results obtained from this study were compared to the two known published reports on South African lichens as biomonitors of air pollution by metals, where comparisons could be drawn for Cr, Cu, Mn, Ni and Pb. The importance of using the same lichen species and sampling during the same season was emphasized, where these factors are capable of introducing an unknown bias into comparative assessments. This study further demonstrated the usefulness of the foliose lichen, *P. austrosinense*, as a global biomonitor of air pollution for a number of different metal(loid) analytes, where clear differences between metal concentration between sites could be observed. Lichen samples from the urban site (Johannesburg) were found to have higher concentrations of most metal(loid)s, where this was thought to be predominantly due to high density of traffic and industrial facilities. Lichens from the rural site (Waterberg) were believed to have gained metals predominantly from the surrounding geology and mining activities which would increase the amount of airborne, windblown soils at this site. The GAW site was found to be a suitable reference site having low concentrations of most metals except for Ni which was thought to be predominantly due to natural sources such as sea spray and possibly some industrial activities in the adjacent bay. The inclusion of meteorological and geological data in this study greatly improved the understanding of factors which may affect the concentrations of metal(loid)s in the lichen thallus, and their inclusion is highly recommended in studies of this type.

A variety of different sample preparation strategies were compared for total metal(loid) and speciation evaluations. The novel application of a sequential extraction scheme to a lichen matrix was found to be extremely useful in the quantification of metal(loid)s in lichens resulting from atmospheric deposition. Since there is little consensus in the literature regarding which sample preparation strategies should be employed, this study will serve as a guideline for future analyses of this type. A 10 g portion of lichen material shredded and passed through a mesh of >1 and <4.699 mm was found to adequately represent a homogenous sample population. Although the cryogenic freezing of the lichen material followed by pulverization was found to be the preferred method of sample preparation for determining the total metal(loid) concentrations, this was not the case for determining metal(loid) fractionation patterns in lichens. Consequently, the historical use of liquid nitrogen as an appropriate method to freeze plant samples prior to pulverization, extraction and speciation, in

some studies, should be re-evaluated for its suitability in this application. Although the use of sequential extractions for metal(loid) assessments may result in the co-extraction of some chemical species, this technique was shown to provide a good indication of metal fractionation patterns of certain elements in the lichen matrix. An observed change in the species distributions over a storage period of one month implied that if the original chemical species of an analyte in a sample is of interest, that the sample should be processed and analysed as soon as possible to avoid biotransformation. The findings from this study indicated that the methods used in sample preparation, preservation and storage greatly affect the concentration of metals and their relative chemical species distribution, and therefore should be carefully considered when choosing a method with the best analytical accuracy.

Through the optimized extraction of arsenic from the lichen matrix and development of a chromatographic separation method, the baseline resolution of arsenobetaine and arsenite from one another using an isocratic method on a PRP X-100 anion exchange column, and utilising a relatively inexpensive and eco-friendly mobile phase was possible. Due to the complex nature of the lichen matrix, based upon literature (Koch et al. 1999, Machado et al. 2014), a presumably organo-arsenic species was found to elute just after arsenobetaine thereby affecting the baseline resolution of AsB and As III in the lichen matrix. Despite this encounter, the peaks were still resolved well enough to gain semi-quantitative information and as such, the characterization of five of the most toxicologically relevant arsenic species using a single analytical column was possible. The application of the developed method to lichens from two different sampling sites (rural and urban) found higher total As concentrations at the urban site (6.43 ± 0.25 µg/g) in comparison to the rural site (1.87 ± 0.05 µg/g). The summative concentration of the different As species in the lichen matrix were found to be 1.22 ± 0.5 µg/g at the urban site with an order of species abundance of As V>As III>AsB+U1>DMA>MMA, and 0.74 ± 0.06 µg/g at the rural site, where the order of species abundance was As V>AsB+U1>As III>DMA>MMA. Differences in concentration and the order of species abundances were able to demonstrate potential sources of arsenic pollution in the air at both sites.

The use of the optimized extraction and chromatographic method, which had been developed in Paper 4 in the water extraction step, was found to greatly add to our understanding of source apportionment. Combined with the stepwise extraction procedure, the last study in this thesis resulted in some of the highest reported mass balance recoveries of arsenic species from a lichen matrix in comparison to the total arsenic in the sample (104 and 112% recovery of the total As concentration). Moreover, the developed method was highly reproducible and when combined with meteorological data and an evaluation of short-term stability, enabled conclusions to be drawn regarding the timing of pollution events and potential sources. Another interesting finding from this work was a new proposed mechanism for As metabolism by lichens which accounts for the increase in As V over time as has been observed by some researchers, although this hypothesis will need to be confirmed through further study.

In conclusion, the interpretation of concentration values of multiple elements in lichen biomonitors can greatly benefit from a knowledge of the meteorological parameters affecting the levels of the elements. The use of metal(loid) speciation in lichen biomonitor studies can provide a wealth of information about source apportionment and the timing of pollution events, where an understanding of the metabolism of the analyte of interest by the lichen species is essential.

5.2 Future outlook

It is evident that speciation of metal(loid)s in lichens may elicit source information, the timing of pollution events, and the extent of human and environmental exposure to chemical forms of toxicological significance. Although there has been great progress in the field of speciation studies in lichen biomonitors, there is still a need to create more sustainable and ecologically sound extractions and chromatographic separations. South Africa would greatly benefit from the implementation of a lichen biomonitoring program to assist in the evaluation of pollution of air by metal(loid)s in remote parts of the country. A challenge which may be encountered in the future is that naturally occurring lichens which are less tolerant to pollution may become fewer in number in highly polluted areas, making the implementation of *in situ* lichen evaluations difficult.

Considerations by lichenologists and allied scientists to regularly monitor and identify vulnerable or endangered species may help ensure their sustainable use against climatic or pollution related uncertainties. Moreover, as the global climatic conditions change, some lichen species which are less tolerant to changes in temperature may disappear from areas in which they have previously been found. In such cases, the implementation of transplanted lichen biomonitors which are more tolerant to pollution may be necessary for short-term evaluations.

As this study has shown, the standardization of approaches using lichens as biomonitors of air pollution would be highly beneficial and would allow for more meaningful and accurate comparisons to be drawn between studies. Through not standardizing sample collection, processing and analytical techniques it is near-impossible to tell if observations between studies are statistically significant or due to the subjectivity of the analyst and the techniques being employed. As such, an increase in the number of studies evaluating these key issues would help generate reliable analytical data and maximize the beneficial use of lichens as pollution biomonitors.

Studies which include the analysis of organic and inorganic pollutants in the same species of lichen, collected from the same site and over the same period of time could also greatly assist with source apportionment. For example, in an agriculturally impacted area, the characterization of organic pollutants would be able to provide information about what types of chemicals are being used at a specific site and their source. Interrelating this with the concentrations of metal(loid)s would in some cases be able to either confirm or reject these findings and further support hypotheses regarding the pollutant source.

Furthermore, it would be highly recommended to expand the number of studies looking into the biotransformation of the chemical species of analytes of interest by lichens so as to increase our understanding of the source and magnitude of impacts. This would fill the knowledge gap regarding species-specific biotransformation mechanisms. The combination of real-time monitoring data using air filters with biomonitoring would also prove to be useful in providing complimentary information about short and long term air pollution patterns at different sites.

Appendices

Appendix A

The certificates for the certified reference materials employed over the course of this thesis are shown below.

National Research Council Canada — Conseil national de recherches Canada

Certified Reference Material

DOLT-4

Dogfish Liver Certified Reference Material for Trace Metals

This reference material is primarily intended for use in the calibration of procedures and the development of methods for the analysis of marine fauna and materials with a similar matrix.

Elements for which certified values have been established for this dogfish (*Squalus acanthias*) liver CRM, along with their expanded uncertainty ($U_{CRM} = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO Guide [1] and k=2 is the coverage factor) are listed in Table 1. It is intended that U_{CRM} encompasses every aspect that reasonably contributes to the uncertainty of the certified mass fraction [2]. Values are based on dry mass.

Table 1. Certified Values for DOLT-4

Element	Mass Fraction (mg/kg)
Arsenic (d,e,h)	9.66 ± 0.62
Cadmium (d,e,i,p)	24.3 ± 0.8
Copper (d,e,i,p)	31.2 ± 1.1
Iron (d,i)	1833 ± 75
Lead (d,e,p)	0.16 ± 0.04
Mercury (c,d,p)	2.58 ± 0.22
Nickel (d,e,i,p)	0.97 ± 0.11
Selenium (e,h)	8.3 ± 1.3
Silver (d,e,p)	0.93 ± 0.07
Zinc (d,i,p)	116 ± 6
CH_3Hg (as Hg)(g,s,t)	1.33 ± 0.12

Coding

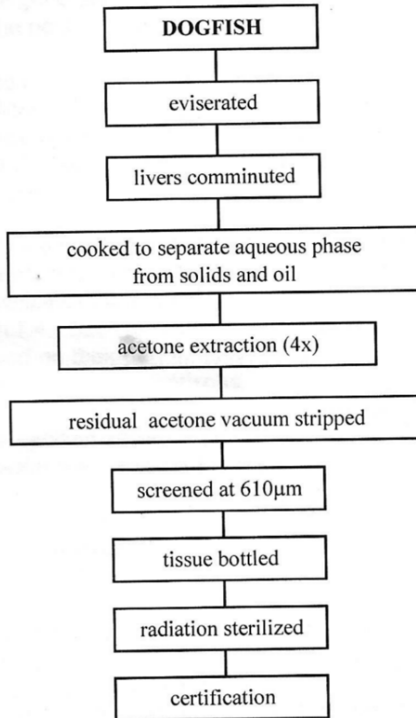
The coding refers only to the instrumental method of determination of the measurand.

c - Cold vapour atomic absorption spectrometry.
d - Inductively coupled plasma mass spectrometry.
e - Electrothermal vaporization atomic absorption spectrometry (ETAAS).
g - Solid phase microextraction (SPME) isotope dilution gas chromatography mass spectrometry.
h - Hydride generation atomic absorption spectrometry.
i - Inductively coupled plasma atomic emission spectrometry.
p - Isotope dilution inductively coupled plasma mass spectrometry (ID-ICPMS).
s - SPME isotope dilution gas chromatography ICPMS.
t - Ethylation cold vapor atomic fluorescence spectrometry.

NRC · CNRC

Preparation of DOLT-4

This reference material was processed at the Guelph Food Technology Center, Guelph Ontario. The preparation sequence is illustrated below.



The material was sterilized by gamma irradiation (miminimum dose of 25 kGy) at the Canadian Irradiation Centre, Laval, Québec

Sampling

A sample mass of 250 mg of material (dry mass basis) is the minimum sample intake for which the established uncertainty is valid.

Instructions for Drying

Moisture content should be determined using a separate sub-sample. DOLT-4 can be dried to contant mass by:
(1) drying at reduced pressure (e.g., 50 mm Hg) at room temperature in a vacuum desiccator over magnesium perchlorate for 24 hours;

(2) vacuum drying (about 0.5 mm Hg) at room temperature for 24 hours.

Information Values

Table 2 presents information values for elements which could not be certified because of insufficient information to accurately assess uncertainties.

Table 2. Information Values for DOLT-4

Element	Mass Fraction, (mg/kg)
Na	6800
Mg	1500
Al	200
K	9800
Ca	680
V	0.6
Cr	1.4
Co	0.25
Sr	5.5
Mo	1
Sn	0.17

Storage and Handling

This material should be kept in the original bottle tightly closed and stored in a cool location, away from any significant radiation sources such as ultraviolet lamps and sunlight. The contents should be well mixed by rotation and shaking prior to use, and the bottle tightly closed immediately after sampling.

Calculation of Certified Values

DOLT-4 was provided as an unknown sample to a group of laboratories participating in an annual intercomparison for trace metals in marine samples coordinated by NRCC [3]. Data generated by NRCC were also included in the pool of intercomparison results.

Laboratories were requested to provide triplicate results using an analytical method of choice based on total digestion of the sample. DOLT-3 was provided as a quality control sample.

Data were returned to NRCC for evaluation. Results from a select sub-group of participants were used for the certification of DOLT-4. Such laboratories were selected based on their performance history in previous intercomparisons.

The certified values were calculated from the unweighted means of the results. Data were first examined for outliers using the Dixon and Grubb's Tests. Testing of variances was conducted using the Cochran and Bartletts Tests.

Included in the overall uncertainty estimate are uncertainties in the batch characterisation (u_{char}) and uncertainties related to possible between-bottle variation (u_{hom}). Expressed as standard uncertainties these components can be combined as:

$$u^2_{c(CRM)} = u^2_{char} + u^2_{hom}$$

Based on NRC's experience with similar materials, uncertainty components for long and short term stability were considered negligible and are thus not included in the uncertainty budget.

Results for the various uncertainty components used to calculate the certified values are summarized in Table 3.

Table 3. Statistical Data for DOLT-4

Element	data sets	u_{char}, (mg/kg)	u_{hom}, (mg/kg)
As	10	0.22	0.21
Cd	12	0.25	0.31
Cu	10	0.31	0.46
Fe	10	22	30
Pb	8	0.016	0.013
Hg	8	0.014	0.11
Ni	9	0.024	0.049
Se	9	0.18	0.63
Ag	8	0.017	0.028
Zn	11	2	2
CH_3Hg	3	0.016	0.057

Expiration of Certificate

A predecessor CRM, DOLT-2, has been periodically analyzed for more than nine years and found to be both physically and chemically stable over this time interval. We expect similar characteristics from DOLT-4. The stability of this CRM will continue to be monitored and any significant irregularity will be posted on our web site.

The certified values for DOLT-4 are considered valid until April 2014, provided the CRM is handled and stored in accordance with instructions herein.

References

[1] Guide to the Expression of Uncertainty in Measurement, ISBN 92-67-10188-9, 1st ed. ISO, Geneva, Switzerland (1993).

[2] ISO Guide 35:2006, Reference materials — General and statistical principles for certification Geneva, Switzerland (2006)

[3] S. Willie, Twentieth Intercomparison for Trace Elements in Marine Sediments and Biological Tissues, NRC No. 50099, October 2007.

Acknowledgements

The following staff members of the National Research Council Canada, participated in the certification: P. Maxwell, C. Brophy, L. Yang and S. Willie.

The cooperation of I. Britt and A. Mannen of the Guelph Food Technology Centre, Guelph, ON, Canada in the preparation of this material is gratefully acknowledged.

The following laboratories participated in the certification of DOLT-4:

ALS Environmental
Vancouver, B.C. V5L 1K5

Australian Institute of Marine Sciences
Queensland, 4810, Australia

Australian Nuclear Science and Technology Organizastion, Environmental Science Program
Menai, N.S.W., Australia

Battelle Pacific Northwest
Sequim Bay Road, Sequim, WA 98382

Massachusetts Water Resources Authority
Central Laboratory, Winthrop, MA 02152

NOAA, National Ocean Service,
Hollings Marine Laboratory, Charleston, SC 29412

San Francisco Public Utilities Commission
Southeast Laboratory, Sn Francisco, CA,

Texas A. & M.
College of Veterinary Medicine
Trace Element Research Laboratory
College Station, TX 77843-4458

Texas Parks and Wildlife
Environmental Contaminants Laboratory
San Marcos, TX 78666

U.S. Customs Laboratory
Savannah, GA 31408

USGS-WRD
3039 Amwiler Road, Atlanta, GA 30360-2824

Canada

Updates

Users of this material should ensure that the certificate in their possession is current. Please consult our web site at http://www.nrc-cnrc.gc.ca for any new information.

As additional data become available, the certified values may be updated and reliable values assigned to additional measureands.

Certificate issued May 2008.

The results presented in this certificate are traceable to the SI through gravimetrically prepared standards of established purity and international measurement intercomparisons. As such, they serve as suitable reference materials for laboratory quality assurance programs, as outlined in ISO/IEC 17025. NRCC CRM's are registered at the Bureau International des Poids et Mesures (BIPM) in Appendix C of the Comité International des Poids et Mesures database listing Calibration and Measurement Capabilities accepted by signatories to the Mutual Recognition Arrangement of the Metre Convention.

Comments, information and inquiries should be addressed to:

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Également disponible en français sur demande.

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POLISH CERTIFIED REFERENCE MATERIAL
FOR MULTIELEMENT TRACE ANALYSIS

TEA LEAVES (INCT-TL-1)

The material was prepared from black tea, usually packed in tea bags. Tea leaves collected and processed in Argentina were ground and sieved. All precautions were taken to avoid contamination of the material with metals. The next step was homogenization by mixing in a plastic drum. After preliminary homogeneity checking by X-ray fluorescence, the material was distributed into PP bottles and final homogeneity testing was performed by neutron activation analysis determining Br, Ce, Co, Cr, Cs, Fe, K, La, Mn, Na, Rb, Sc and Sm in subsamples (Fisher's test). Good homogeneity of INCT-TL-1 at least for sample weight of 5 mg was confirmed. Long-term stability has been assured by sterilization of the whole lot of the material by electron beam radiation and is being monitored during storage. Certification of the candidate reference material was done on the basis of world-wide interlaboratory comparison exercise in which 109 laboratories from 19 countries using various analytical methods, participated. Data evaluation was performed using the software package ACQS-1. Several criteria were employed to decide whether the overall mean can be given the status of recommended (certified) or information value. The validity of certified values was confirmed for several elements using definitive methods developed in this Laboratory.

INCT-TL-1 - certified values

Major elements		Trace elements					
Element	Concentration wt, %	Element	Concentration mg/kg (ppm)	Element	Concentration mg/kg (ppm)	Element	Concentration mg/kg (ppm)
Al	0.229±0.028	**As**	0.106±0.021	**Eu**	0.050±0.009	**Sm**	0.177±0.022
Ca	0.582±0.052	**Ba**	43.2±3.9	**Hg**	0.005±0.0007	**Sr**	20.8±1.7
K	1.70±0.12	**Br**	12.3±1.0	**La**	1.00±0.07	**Tb**	0.027±0.002
Mg	0.224±0.017	**Cd**	0.030±0.004	**Lu**	0.017±0.002	**Th**	0.034±0.005
Mn	0.157±0.011	**Cl**	573±48	**Na**	24.7±3.2	**Tl**	0.063±0.005
S	0.247±0.025	**Co**	0.387±0.042	**Ni**	6.12±0.52	**V**	1.97±0.37
		Cr	1.91±0.22	**Pb**	1.78±0.24	**Yb**	0.118±0.013
		Cs	3.61±0.37	**Rb**	81.5±6.5	**Zn**	34.7±2.7
		Cu	20.4±1.5	**Sc**	0.266±0.024		

INCT-TL-1 - information values

Trace elements							
Element	Concentration mg/kg (ppm)	Element	Concentration mg/kg (ppm)	Element	Concentration mg/kg (ppm)	Element	Concentration mg/kg (ppm)
B	26	**Nd**	0.810	**Se**	0.076	**Tm**	0.017
Fe	432	**P**	1810	**Ta**	0.008		
Hf	0.028	**Sb**	0.050	**Ti**	30		

The material was prepared and certified by the staff of the Dept. of Anal.Chem., of INCT under the supervision of prof.dr R.Dybczyński.

EUROPEAN COMMISSION

JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements

irmm Institute for Reference Materials and Measurements

CERTIFIED REFERENCE MATERIAL
BCR® – 482

CERTIFICATE OF ANALYSIS

LICHEN			
	Mass fraction based on dry mass		Number of accepted sets of data p
	Certified value [1] [mg/kg]	Uncertainty [2] [mg/kg]	
Al	1103	24	9
As	0.85	0.07	6
Cd	0.56	0.02	8
Cr	4.12	0.15	7
Cu	7.03	0.19	10
Hg	0.48	0.02	8
Ni	2.47	0.07	8
Pb	40.9	1.4	10
Zn	100.6	2.2	13

1) The certified value was calculated from the unweighted mean of the means of p accepted datasets. The certified value is traceable to the SI.

2) Half-width of the 95 % confidence interval of the mean defined in 1).

This certificate is valid for one year after purchase.

Sales date:

The minimum amount of sample to be used is 250 mg.

DESCRIPTION OF THE SAMPLE

The material consists of a lichen powder in a glass bottle containing about 15 g of powder. Additional information on the preparation, the certified and indicative values is given in the certification report.

NOTE

This material has been certified by BCR (Community Bureau of Reference, the former reference materials programme of the European Commission). The certificate has been revised under the responsibility of IRMM.

Brussels, December 1995
Revised: May 2007

Signed:

Prof. Dr. Hendrik Emons
Unit for Reference Materials
EC-JRC-IRMM
Retieseweg 111
2440 Geel, Belgium

ANALYTICAL METHOD USED FOR CERTIFICATION

- Cold vapour atomic absorption spectrometry
- Cold vapour atomic fluorescence spectrometry
- Direct current plasma atomic emission spectrometry
- Differential pulse anodic stripping voltammetryu
- Electrothermal atomic absorption spectrometry
- Hydride generation atomic absorption spectrometry
- Inductively coupled plasma/isotope dilution mass spectrometry
- Inductively coupled plasma emission spectrometry
- Inductively coupled plasma mass spectrometry
- Instrumental neutron activation analysis
- Isotope dilution mass spectrometry
- Neutron activation analysis with radiochemical separation

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- University of Gent, Gent (BE)

SAFETY INFORMATION

The usual laboratory safety precautions apply.

INSTRUCTIONS FOR USE

It is intended for the verification or validation of an analytical procedure for the determination of the analytes or a certain part of them. This material is not intended for use as a calibrant. The sample can be used as it is from the bottle. Before the bottle is opened, it should be shaken manually so that the material is re-homogenised. The correction to dry mass should be made on a separate portion of 100 mg which should be dried in an oven at 102 °C for 3-4 h until constant mass is attained.

STORAGE

Upon arrival the material shall be stored at 18 °C in the dark.
However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

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NOTE

A technical report on the production of BCR-482 is available on the internet (http://www.irmm.jrc.be). A paper copy can be obtained from IRMM on request.

Appendix B

A book chapter was produced as a result of the work done for this thesis in the role of co-author responsible for the authorship of the sections of the book chapter dealing with inorganic analytes.

Book chapter:

Patricia B.C. Forbes, Leandri van der Wat and Eve M. Kroukamp, Chapter 3: Biomonitors, in Comprehensive Analytical Chemistry vol. 70: Monitoring of Air Pollutants: Sampling, Sample Preparation and Analytical Techniques, Patricia Forbes (Ed.), (2015), 53-108, Elsevier, Netherlands. ISBN: 978-0-444-63553-2 http://dx.doi.org/10.1016/bs.coac.2015.09.003

Chapter 3

Biomonitors

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1. INTRODUCTION

Biomonitors are living organisms used to obtain quantitative and qualitative information about certain aspects of the environment, as they are able to assimilate contaminants over an extended period of time and are therefore reflective of environmental conditions [1]. For this reason, various biomonitors are useful in monitoring air pollution in a given region. They are commonly used in studies where the high cost of technical equipment, which would measure the air pollution profile over an extended period of time for a range of contaminants, cannot be afforded. Biomonitors provide an integrated pollution profile as a result of their ability to accumulate a range of air pollutants [2]. Most air pollution biomonitoring studies have used plants such as fungi, lichens, mosses, pine needles, tree bark and leaves [3,4]. This is because plants have been shown to bioaccumulate air pollutants through a number of different mechanisms such as entrapment of particulates, gas exchange through stomata and surface ion exchange. Moreover, plants are capable of accumulating these pollutants in concentrations which far exceed their basic metabolic requirements.

One of the earliest biomonitoring studies was on the decline of lichens in Paris and its surrounding areas as a result of air pollution [5]. Numerous subsequent studies have shown the effect of air pollution on various plants [6–8]. Applications such as these are known as *bioindicator* studies, where plants and other organisms are assessed for changes in physiology, abundance and behaviour [1]. Typically the visual health of an organism is evaluated across a geographical region by noting morphological changes, as shown in Figure 1, for example, where a study on the photosynthetic activity of lichens exposed to diesel exhaust fumes was investigated [9].

On the other hand, *biomonitoring* studies provide quantitative information obtained from measurements of accumulated compounds, reflecting the environmental profile from which the organism was sampled. As instrumentation capable of the quantification of pollutants became more accessible, an

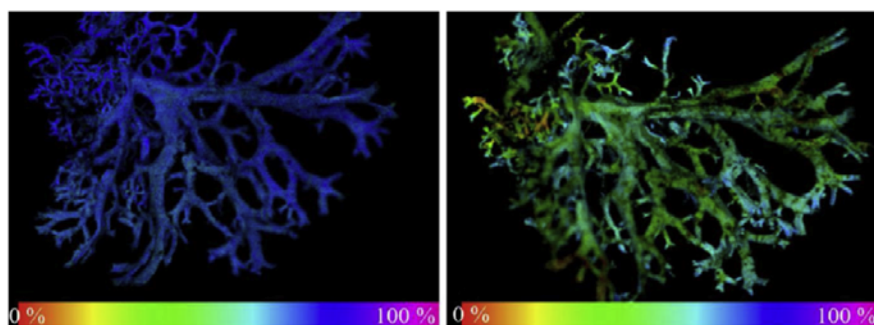

FIGURE 1 False colour imaging of *Pseudevernia furfuracea* lichen before (left) and after (right) exposure to diesel exhaust, confirming the loss of photosynthetic activity (dark blue (dark grey in print versions) indicates high photosynthetic activity) [9]. *Image courtesy of* the Journal of Environmental Protection.

increase in the number of biomonitoring studies linking environmental concentrations to concentrations in biological populations has occurred [1]. A study by Blasco et al. combined the use of lichens as biomonitors and bioindicators to assess the air quality in the Pyrenees, which included both the mapping of the lichen biodiversity as well as an investigation into the polycyclic aromatic hydrocarbon (PAH) content of lichens [10]. Typically, biomonitors are either removed from their original location and analysed directly (passive biomonitors) or transplanted from a lesser impacted to a contaminated site for a specific period of time (transplant/active biomonitors) [11].

Mosses were used to measure fluorine emitted from industrial sources in the 1950s [12] and since the 1960s, mosses have been used to chart heavy metal deposition in Scandinavia [13]. Lichens have been used as bioindicators, for sulphur dioxide, nitrogen dioxide, ozone and hydrofluoric acid [14–18].

Target analytes in biomonitoring studies have included organic compounds such as hydrocarbons as they accumulate within organisms because they are lipophilic. PAHs and their halogenated derivatives, which are potentially hazardous to living organisms [19], have been studied using lichens, moss, pine needles and bark. Other organic analytes of interest include phenols [20], polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs). Inorganic air pollutants have been extensively studied using biomonitors, including isotopes [21], metals [1] (such as Fe, Zn, Pb, Cu, Mn, Co, Cd, Ni, Cr and Hg), lanthanides and rare earth elements [22], ammonia and nitrogen. In order to study these air pollutants, different types of organisms have been used, including the bark of various tree species, lichens, pine needles, moss, tobacco, wheat, barley, maize, grass, leaves of trees, leaf cuticles, wild chard and various shrubs.

When using biomonitors for air pollution monitoring, the selection of an appropriate biomonitor is vital. The chosen species should be widely dispersed throughout the sampling area [22], which may allow for national and international pollution monitoring initiatives [23]. Target organisms should be chosen based on their abundance, bioaccumulation potential and ecological relevance to the specific air pollution study. Different biomonitors may also be representative of the system for different time periods, and so the desired monitoring period and pollutant retention should be established prior to sampling. Mosses, for example, exhibit concentrations which are representative of longer accumulation periods than can be found in leaves [4]. Species selection is also important because studies have shown that different plant species within a group may experience selectivity towards specific contaminants [4,24]. In addition, if the relationship between concentrations of contaminants within the monitor and the environmental exposure levels are poorly defined, the information gained is purely qualitative [1]. As a result, a clear link between the amount of contaminant from different sources to which a biomonitor organism is exposed, and the concentration which is bioconcentrated needs to be determined [25]. Tree bark and leaves, for example,

may be exposed to a variety of pollutant sources such as soil, air and groundwater whereas others, such as lichens, rely almost entirely upon atmospheric sources. It is also for this reason that higher order organisms are not as suitable as plants for air biomonitoring purposes, due to the wide range of sources to which they are exposed.

The accumulation of airborne metals and organic pollutants by biomonitor organisms is expected to be related to a number of physical and chemical factors of the pollutants, such as water solubility, hydrophobicity, vapour pressure, particle size and metal oxidation state. Environmental aspects such as temperature, as well as biomonitor species, also affect uptake rates [4]. Early work in the field of biomonitors focused on the establishment of dose–response relationships, in terms of correlations between elemental levels in lichen biomonitors and sampled air particulate matter (such as Ref. [26]). Hanssen et al. [27], Ross [28], Berg et al. [29], as well as Berg and Steinnes [24], all demonstrated the correlation between metal concentrations in moss and wet deposition. Later on, studies by Augusto et al. [30] compared the concentrations of PAHs accumulated by lichens, pine needles, soil and direct air sampling. The correlation of the air samples to both pine needles and lichen PAH data suggested that both biomonitors were effective at providing an understanding of atmospheric PAH levels.

This chapter focuses on the application of various organisms as biomonitors in air pollution monitoring, with emphasis on the more commonly utilised biomonitor species; namely lichens, moss, conifer needles and tree bark. The extraction of target analytes from within the organism's matrix as well as common analytical techniques employed to determine the identities and quantities of analytes extracted from the biomonitors are outlined. Sampling considerations, as well as general analytical challenges, are also discussed. Biomonitoring has been applied in numerous air pollution studies, and some examples of these are included in this chapter.

2. LICHENS

2.1 Introduction

Lichens are the product of a symbiotic relationship between algae (phycobiont) and a fungus (mycobiant) [31]. They are perennial, represent uniform morphology over their life span, and are slow growing [32]. Annual radial growth of 0.1–1.0 mm has been reported for crustose lichens, 1.0–2.5 mm for foliose lichens and 2.0–6.0 mm for fruticose lichens, respectively [33]. This growth rate varies between species and also between geographical locations for one species, and has been correlated to rainfall [33]. Prevalent in a variety of biomes, lichens are found in extremes of temperature, from the arctic tundra to the rainforests. Lichens grow on a range of substrata, both natural, such as rock, trees, soil, wood and leaves, as well as anthropogenic, including rubber,

plastic, glass, stonework, concrete, plaster, ceramic, tiles and bricks [33]. The microclimate has been found to significantly impact on lichen colonisation and biodiversity, particularly factors such as precipitation, light intensity, humidity, wind speed and air quality [33].

The lack of a cuticle in lichens allows them to gain most of their nutrients from the air [32], and since this movement is unhindered, the hyper-accumulation of metals and organic pollutants is possible. Certain species of lichens which persist on rocks are able to supplement their nutritional requirements by releasing chemicals onto the rock substrate, thereby accelerating breakdown of the rock surface [34]. Lichens which persist on trees, however, simply use trees as a substrate and are not believed to acquire any nutrients from the tree or bark [35].

Some lichens are particularly sensitive to pollution where high atmospheric pollutant concentrations may result in the reduction or complete absence of a particular lichen species in an environment. An example of this was found in a study by Dongarrà and Varrica [36] where lichens (*Parmelia* spp.), which were found in abundance in other areas, were absent within close proximity to a volcano. Such traits are desirable in bioindicator studies. Other types of lichens are more robust; withstanding high levels of atmospheric pollutants (e.g. *Parmelia sulcata*) and some may possess detoxification mechanisms capable of degrading or excreting pollutants [34]. In situations where pollutant loading is high, however, lichens may experience reduced growth and altered morphology [37].

It was Hawksworth and Rose [38] as well as Rogers [39] who first applied lichens as biomonitors with respect to the uptake of sulphur dioxide from the atmosphere. Sloof et al. [40] compared the concentration of pollutants in lichens to the atmospheric concentrations of suspended and deposited particulate matter. Elemental analyses of Zn, Co and Sc were performed on lichens by Sloof and Wolterbeek [41], which correlated well with determined atmospheric concentrations. Migaszewski et al. [20] showed that lichens were also suitable for the study of atmospheric PAHs, followed by Guidotti et al. [42] who performed a lichen transplant study, targeting PAHs. Augusto et al. studied the PCDD/F profile for the first time in lichens sampled in Portugal [43]. For a full review on lichens as biomonitors for organic air pollutants, the reader is referred to a paper by van der Wat and Forbes [44]. Lichens have also been used to study ammonia and nitrogen deposition [45,46]. Over the years, lichen biomonitoring research has been conducted across a wide spectrum of inorganic analytes including Hg, most transition metals, radionuclides, fluoride, S, N and acid rain, as a result of bioaccumulation in the lichen thallus [2,31,47–50].

Lichens found in nature exist in different growth forms, namely foliose (leaflike, Figure 2), crustose (form a crust), squamulose (flattened-pebble appearance) and fruticose (branching tubes).

Proposed mechanisms for accumulation of air pollutants by lichens include the trapping of particles on the surface, diffusion of gases across the cell

FIGURE 2 A foliose lichen suitable for air pollution biomonitoring.

membrane or ion exchange. Factors such as surface water (rain) have been found to facilitate the movement of metals into the lichen thalli via ion-exchange processes [51]. Extracellular uptake of metal ions is determined by ligands in the walls of the fungus component of the lichen, whereas intracellular uptake is subject to the nature of the ion, the cell membrane permeability and the affinity of extracellular ligands for cations [52]. As a result of the lipophilic nature of the surface of lichens, the uptake of hydrophobic compounds, such as semi-volatile organic compounds (SVOCs), is easily accommodated by the surface, where the compounds' incorporation into cells is metabolically controlled [53,54]. Organic air pollutants are typically lipophilic, thus uptake tends towards dry deposition [55,56].

2.2 Lichens as Biomonitors of Inorganic Air Pollutants

Metal concentrations in lichens have been shown to correlate well with atmospheric concentrations [3]. It was thought that mosses are more likely to accumulate metals than lichens due to their growth structure and porosity [3]; however, contradictory evidence later found that lichens possessed higher concentrations of heavy metals than mosses [57]. Foliose lichens have primarily been used as biomonitors [1,3,22,31,32,36,57], due to their ease of removal from the substrate. They also present a large surface area and are widely distributed in a variety of different habitats. Crustose [58], fruticose [59] and squamulous [58] lichens have also been used in air pollution monitoring, although to a lesser extent. Crustose lichens are often difficult to remove from the substrate without contamination of the sample taking place. Certain types of *Parmelia* species growing on rock surfaces have been used to assess air pollution [36], although uptake of minerals from the rock substrate may impact on the results [34]. Contamination from soil and

groundwater are also potential limitations in such studies. It should be noted that *Diploschistes muscorum* is capable of sequestering metals as oxalate crystals or lichen acid complexes [34].

Species differences have been found to affect the bioconcentration potentials for certain elements, as was shown in a study by Mendil et al. [59]. In this study Fe, Zn and Cr were found to accumulate highest in *Physcia adscendes*, Mn and Cu in *Flavoparmelia caperata*, Ni and Cd in *Xanthoria parietina* and Pb in *Ramalina polymorpha*. Species differences have also been observed for the monitoring of nitrogen where *P. sulcata* was found to accumulate the lowest amount of nitrogen, and higher concentrations were observed in both *X. parietina* and *Physcia* spp. [21]. Active biomonitoring using lichen transplants has the advantage of allowing their use in areas where they may be naturally absent and prevent the influence of substrate concentrations on *in situ* samples [60].

In terms of the analysis of inorganic pollutants in lichens, a wide range of techniques has been employed, such as inductively coupled plasma—mass spectrometry (ICP-MS), inductively coupled plasma—optical emission spectrometry (ICP-OES), X-ray absorption spectroscopy, flame atomic absorption spectroscopy and instrumental neutron activation analysis. Some of the most common inorganic analytes which have been assessed in lichen biomonitoring studies, and the range of concentrations thus determined, are shown in Table 1.

2.3 Lichens as Biomonitors of Organic Air Pollutants

Lichens were first applied as biomonitors to the investigation of inorganic pollutants, but in time their application to the monitoring of organic air pollutants such as PAHs [42], phenols [20], PCDD/Fs [63] and PCBs [64—66] was realised, for which various lichen species have been employed [67—70]. Table 2 shows examples of the organic analytes studied using lichens, as well as the corresponding sample preparation techniques, methods of analysis and concentrations thus determined.

As with mosses, lichens are believed to accumulate organic air pollutants by both dry and wet deposition, in a passive fashion [40]. Lipid metabolites released onto the lichen surface are suspected to behave in a similar way to the cuticle in plants [71]. Bauer et al. [72] and Baur et al. [73] showed that diffusion rates of heavier persistent organic pollutants (POPs) are lower than those of the lower molecular weight POPs, therefore heavier POPs are most often associated with particles that remain on the surface of lichens. Augusto et al. [74] also showed that the concentrations of PCDD/Fs remain stable after periods of wet conditions (rain or fog, for example), indicating that some POPs are incorporated into the lichen thallus and are not easily rinsed off. The trend has not been established for all classes of organic air pollutants, but a similar trend is expected [74,75].

TABLE 1 Examples of Inorganic Air Pollutants Determined Using Lichens as Biomonitors

Lichen Species	Location	Analytes	Sample Mass (g)	Extraction Method	Detection Method	Concentration Detected	References
Parmelia sulcata	The Netherlands	Al, As, Br, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, Hf, Hg, I, K, La, Lu, Mg, Mn, Na, Ni, Pb, Pb, Sb, Sc, Se, Sr, Th, Tl, U, V, W, Yb, Zn	0.030–0.1 g	N/A	INAA	Range (μg g^{-1}): Lu 0.009–Fe 30,000	[1]
Hypogymnia physodes	Finland	Cr, Ni	0.2 L fresh volume	Wet digestion	ICP-OES	Range (μg g^{-1}): Cr 4–5, Ni 3.3–3.8	[3]
H. physodes	Finland	Cr, Pb, Ni, Cd, Cu, Fe, Zn	Whole thalli	Wet digestion	ICP-OES	Average (μg g^{-1}): Cd 0.69, Cr 2.1, Cu 7.1, Fe 540, Ni 2.5, Pb 17, Zn 84	[57]
H. physodes	Slovenia	Ag, As, Ba, Br, Ce, Cd, Co, Cr, Cs, Fe, Ga, Hf, Hg, K, La, Mo, Na, Rb, Sb, Sc, Se, Sr, Sm, Tb, Th, U, W, Zn	0.1–0.2 g	N/A	INAA	Range (μg g^{-1}): Tb 0.001–K 8644	[22]
Parmelia sp.	Italy	Au, As, Ba, Br, Ca, Co, Cr, Cs, Fe, Hf, K, Mo, Na, Rb, Sb, Sb, Sc, Th, U, Zn, Cu, Pb, Ni, Mn, Sr, V, P, Mg, Ti, Al, Y	Not specified	N/A	INAA, ICP	Range (ppm): Au 0.05–Ca 14,667	[36]
Parmelia caperata	Italy	Cr, Cu, Fe, Mn, Zn, Al, Cd, Ni, Pb, Hg	Not specified	Pressurised digestion system	FAAS and GFAAS	Range (μg g^{-1}): Cr 0.1–Al 277	[32]

Parmotrema austrosinense	South Africa	Cr, Mn, Co, Ni, Zn, Sn, Pb	0.25 g	Microwave	FAAS	Average ($\mu g\ g^{-1}$): Pb 41.5–181.1, Mn 97.1	[31]
Ramalina polymorpha, Xanthoria parietina, Physcia stellaris, Flavoparmelia caperata, Physcia adscendens	Turkey	Fe, Mn, Zn, Pb, Ni, Cr, Cu, Cd	1 g	Microwave	FAAS, GFAAS	Range ($\mu g\ g^{-1}$): Fe 103.8–455.5, Mn 31.6–170.5, Zn 10.4–77.6, Pb 0.9–6.5, Ni 1.0–10,1, Cr ND-3.8, Cu ND-25.6, Cd ND-1.5	[59]
P. sulcata	Ghana	Sb, Mn, Cu, V, Al, Co, Hg, As, Cd, Th	0.2 g	N/A	INAA	Range (ppm): Co 0.12–Mn 1097	[61]
Cladonia furcata, Hypocenomyce scalaris, Lepraria incana	Poland	Mn, Pb	0.005–0.020 g	Ultrasound	FAAS	Range ($\mu mol\ g^{-1}$): Zn 1.28–17.16, Pb ND-2.66	[58]
X. parietina	Turkey	Al, As, Cd, Co, Cu, Fe, Hg, K, Mn, Ni, Pb, S, Ti, Tl, V, Zn	1 g	Wet digestion	ICP-MS	Range ($\mu g\ g^{-1}$): Tl 0.02–Fe 13,550	[62]
P. sulcata, P. adscendens, Physcia tenella, X. parietina	Germany	N isotopes	0.003–0.004 g	N/A	Element analyser coupled with isotope ratio MS	Range (%): 28–41	[21]

ND, not detected; FAAS, flame atomic absorption spectroscopy; ICP-OES, inductively coupled plasma-optical emission spectrometry; ICP-MS, inductively coupled plasma-mass spectrometry; INAA, instrumental neutron activation analysis; GFAAS, Graphite furnace atomic absorption spectroscopy.

TABLE 2 Various Lichen Biomonitoring Studies for the Analysis of Organic Air Pollutants

Lichen Species	Location	Analytes	Sample Mass (g)	Extraction Method	Analysis Method	Concentration Detected	References
Pseudevernia furfuracea (L.) *Zopf*	Dolomites, Italy	PAHs	0.6–0.8	Automated Soxhlet extraction	GC-MS	758.0 ng g^{-1}	[76]
Parmotrema hypoleucinum	Portugal	PAHs	2	Soxhlet	HPLC-FLD-DAD/V-UV	95.5–873.8 ng g^{-1}	[30]
P. hypoleucinum (Steiner) Hale	Portugal	PAHs	2	Soxhlet	HPLC-FLD-DAD/V-UV	93.56–599.24 ng g^{-1}	[69]
P. hypoleucinum (Steiner) Hale	Portugal	PAHs	2	Soxhlet	HPLC-FLD-DAD/V-UV	58–536 ng g^{-1}	[77]
Hypogymnia physodes	Poland	PAHs	Not reported	Soxtec liquid–solid extraction	GC-MS	3–1887 ppb	[20]
Parmelia sulcata	Spain	PAHs	0.2	DSASE	GC-MS	352–1654 ng g^{-1}	[78]
Evernia prunastri	Pyrenees, Spain	PAHs	0.2	DSASE	GC-MS	692–6420 ng g^{-1}	[10]
P. sulcata	Somport tunnel, Spain	PAHs	0.2	DSASE	GC-MS	0.91–1.92 μg g^{-1}	[68]
P. sulcata, E. prunastri, Ramalina farinacea, P. furfuracea, Usnea sp. and *Lobaria pulmonaria*	Pyrenees, Spain	PAHs	0.2	DSASE	GC-MS	238–6240 ng g^{-1}	[79]
Xanthoria parietina	Zaragoza city, Spain	PAHs	0.2	DSASE	GC-MS	+/– 330 ng g^{-1}	[80]

Rinodina sophodes (Ach.) Massal	Kanpur city, India	PAHs	2	Soxhlet	HPLC-V-UV	0.189–0.494 $\mu g\ g^{-1}$	[81]
Phaeophyscia hispidula	Himalayas, India	PAHs	2	Soxhlet	HPLC-V-UV	3.38–25.0 $\mu g\ g^{-1}$	[82]
Pyxine subcinerea	India	PAHs	2	Soxhlet	HPLC-V-UV	1.25–187.3 $\mu g\ g^{-1}$	[67]
Dermatocarpon vellereum Zschacke	Himalayas, India	PAHs	2	Soxhlet	HPLC-V-UV	0.136–4.96 $\mu g\ g^{-1}$	[83]
P. furfuracea	Italy	PAHs	2	USAE	GC-MS	56–159 $\mu g\ kg^{-1}$	[42]
P. furfuracea	Italy	PAHs	2	USAE	GC-MS	168.0–395.0 $ng\ g^{-1}$	[84]
Xanthoria Parientina	Portugal	PCDD/Fs	Not reported	Solvent extraction	GC-HRMS	427.74 $ng\ kg^{-1}$	[43]
X. Parientina	Portugal	PCDD/Fs	Not reported	Soxhlet	HRGC/HRMS	197.5–1218.7 $ng\ kg^{-1}$ dw	[63]
X. Parientina and *Ramalina canariensis Steiner*	Portugal	PCDD/Fs	Not reported	Soxhlet	HRGC/HRMS	170.8–1058.6 $ng\ kg^{-1}$	[70]
X. Parientina	France	PCDD/Fs, PCBs	Not reported	Not reported	GC-HRMS	Only toxic equivalents reported	[85]
Pyxine coralligera	Venezuela	PAHs	2	USAE	HPLC-FLD-DAD	0.24–9.08 $\mu g\ g^{-1}$	[86]
Cladonia alpestris	Sweden	PCBs, DDT, DDE and chlorinated hydrocarbons	Not reported	Soxhlet	GC-ECD	–	[65]

GC-MS, gas chromatography-mass spectrometry; HPLC-FLD-DAD/V-UV, high performance liquid chromatography fluorescence detector-diode array detector/visible ultraviolet detector; HPLC-V-UV, high performance liquid chromatography visible ultraviolet detector; GC-HRMS, gas chromatography - high-resolution mass spectrometry; HRGC/HRMS, high resolution gas chromatography/high resolution mass spectrometry; GC-ECD, gas chromatography-electron capture detector.

One of the limitations of using lichens as biomonitors is that for a long time, there existed no studies reporting the translation of the concentrations of organic analytes found in lichens into atmospheric equivalent concentration values. However, a study by Augusto et al. [74] investigated PAH concentrations in lichens and compared the results to those from an active outdoor air sampler. This study showed a good correlation for the sum of the 16 U.S. Environmental Protection Agency (EPA) PAHs, benzo[*a*]pyrene and the higher molecular weight PAHs. Seasonal fluctuations in the PAH profile were also observed in this study, suggesting that the season in which lichens are sampled should be kept consistent [74].

According to Jensen [87], who studied pine needles in Scandinavia and compared the results to an ongoing moss and lichen study, lichens were not able to accumulate high levels of the more volatile SVOCs, such as hexachlorocylohexane (HCH). HCH levels were seen to have large, erratic variations amongst lichen samples, compared to pine needles that showed good reproducibility. It was therefore concluded that lichens are not suitable for the analysis of HCH, as a result of the large variations after rainfall, suggesting that lichens are not capable of accumulating these specific analytes sufficiently [75,87].

In contrast, a study by Augusto et al. [30] showed that lichens accumulate PAHs more effectively than soil and pine needles. It was similarly demonstrated in a study by Schrlau et al. [70] that lichens accumulate PAHs more effectively than pine needles and mosses. Migaszewski et al. [20] investigated PAHs in lichens and sampled the host bark in addition to lichens, concluding that the lichen contained higher levels of PAHs, compared to its host bark. The concentrations were also found to be consistent throughout various lichen samples (of the same species), despite growing on different tree species. These studies have provided convincing evidence of the potential use of lichens as biomonitors for the study of atmospheric pollution, particularly in terrain that is difficult to access with bulky, expensive sampling equipment.

Interspecies differences in lichens have been investigated for both PCDD/Fs [88] as well as PAHs [79], where differences in the profile of both classes of pollutants were observed, which highlights the importance of appropriate choice of lichen species, as well as consistent sampling to minimise variation. Three different lichen classes have been studied to identify which lichens absorb organic air pollutants most effectively. The lichen class absorbing low molecular weight POPs most effectively is reported to be fruticose lichens as a result of their high surface area to volume ratio, whereas foliose lichens appear to favour the absorption of the higher molecular weight organic pollutants [69,70,79,88]. However, in general, despite the lichen class studied, similar POP profiles have been reported; where two- and four-ring PAHs are primarily found in PAH studies [10,20,30,42,68,76,82] with phenanthrene, naphthalene, fluoranthene and benzo[*a*]anthracene usually being the

PAHs found at the highest concentrations [30,79]. PCDD/F profiles often are dominated by TeCDD/PeCDDs and PeCDFs [43,63,88].

3. MOSSES

3.1 Introduction

Mosses are flowerless, nonvascular plants that form part of the phylum Bryophyta. They are often found in damp, shady locations and grow in dense mats. Mosses can be divided into two predominant categories: *Pleurocarpous* mosses which are prostrate, highly branched and matted [89] and *Acrocarpous* mosses which are tufted, upright, slightly branched and are more tolerant to dryness and air pollution than the *Pleurocarpous* spp. [89]. The four main growth types of mosses are sheets, cushions, haircaps and rockcaps. Mosses have a wide distribution and can be found from sea level to some of the highest altitudes occupied by plants [90]. They grow on a variety of different substrates including trees, rocks, buildings and in wetlands.

Similar to lichens, Bryophytes do not possess a root system or waxy cuticle and have poorly developed vascular bundles [4]. Mosses are therefore reliant upon the air as a major source of nutrients, although some Bryophytes may also acquire metals from the substrate upon which they are growing through rising capillary water [24]. Mosses possess a large surface area to mass ratio [4] and their lack of a cuticle layer allows minerals, water and pollutants to easily penetrate the plant cells, and the cationic exchange capacity of mosses is high [91]. Moreover, the absence of well-developed vascular bundles allows mosses to absorb higher concentrations of metals than other vascular plants [92]. As outlined by Spagnuolo et al. [25], the major factors affecting accumulation of metal contaminants by mosses are the amount and type of metal deposition as well as the form of metal (soluble or insoluble). In addition, meteorological conditions are of importance [93]. For inorganic air pollutants, the cation-exchange properties of mosses are important in terms of analyte uptake, whilst for organic analytes, the capacity of mosses to absorb large organic molecules is key [94]. The Antarctic mosses have been used as biomonitors to assess POPs and PAHs [95]. An observed decrease in concentrations of these pollutants was observed over a period of 8 months after a fire at the Brazilian Antarctic station, indicating detoxification mechanisms of mosses.

3.2 Mosses as Biomonitors of Air Pollutants

Mosses from the *Pleurocarpous* species are used most frequently in air biomonitoring studies because of their easy isolation from the substrate, and therefore contamination by soil particles is minimal [4,96]. Due to their slow growth and large surface area, accumulated metal concentrations are often high, increasing the ease of chemical analysis and reducing the effect of small

amounts of contamination which may occur [24]. In terms of sampling, ground growing mosses are generally collected in areas between canopies and therefore do not reflect deposits which may occur below the canopy of trees.

In terms of metal accumulation, mosses have been shown to reflect temporal trends of atmospheric deposition over longer periods than leaves, whilst for organic air pollutants, mosses such as *Leptodon smithii* have been found to accumulate higher concentrations of atmospheric metals than holm oak leaves but lower concentrations of low molecular weight PAHs [4]. In a study by Kansanen and Venetvaara [3], mosses were shown to have a clear relationship between deposition and uptake, exhibited good reproducibility and were found to accumulate higher concentrations of metals in comparison to lichens, although this was disputed by Lippo et al. [57]. The discrepancies in these findings could be due to a number of factors such as cleaning procedures and the part of the plant used for analysis as Lippo et al. [57] used the youngest segments of the moss for analysis whereas Kansanen and Venetvaara [3] used a mixture of young and old segments. It is not always possible to find mosses growing in industrial areas, which are often the areas of interest for air pollution studies [97]. Moreover, certain species of mosses may be absent in dry areas and therefore may not be suitable for global sampling campaigns [98]. The porous structure of mosses is also particularly difficult to clean of detritus therefore they cannot be used to accurately determine bioconcentrated pollutants such as metals [25].

Species differences in mosses have been observed in biomonitoring studies, where *Tortula muralis* was found to accumulate higher concentrations of Fe, Cu and Cd than *Tortula intermedia*, and the latter was found to accumulate higher concentrations of Mn and Zn [59]. *Barbula unguiculata* from this study was also found to exhibit a degree of selectivity for certain target elements, specifically Pb, Ni and Cr. Species differences have also been observed in studies by Berg and Steinnes [24] where *Hylocomium splendens* accumulated higher concentrations of Cr, Fe, Co, Ni, Cu, Ga, Nb, Mo, Sb, Eu, Gd, Tb, Dy, Er, Tm, Lu, W, Tl, Pb and Th than *Pleurozium schreberi*, which exhibited selective accumulation for V, Mn, Rb and Cd. Uyar et al. [91] assessed a number of different species and found favourable accumulation in *Hypnum lacunosum* for Fe and Cr, *B. glaerosum* for Pb, *Hypnum cupressiforme* for Ni and *S. purum* for As, Cu and Co. Species differences have even been observed in N isotope studies where concentrations were found to be lowest in *P. schreberi* and the highest in *H. cuppresiforme*. Such selectivity exhibited by species is an important consideration in moss species selection. These differences also imply that interspecies calibrations cannot be effectively used in biomonitoring initiatives.

A range of inorganic and organic air pollutants have been determined in moss substrates from as early as 1950 [94]. In some cases both inorganic and organic analytes have been targeted in the same study, such as by Gerdol et al. [99], where PAHs and metals were determined in urban and rural sites in

Northern Italy. It is possible to use mosses that are naturally available in the study area, or to transplant them from ‘clean areas’ or background sites to sampling sites of interest. This overcomes the restrictions of the lack of natural occurrence and also allows for well-defined exposure times [94]. Moss biomonitors have been used to determine the fate and atmospheric transport of air pollutants, such as the study by Liu et al. [100], which provided insights into the role of high mountains in South China in the atmospheric transport of PAHs. Mosses such as *Polytrichum formosum* have been used as biomonitors for Pb source apportionment studies using isotope ratios [101]. Mosses have also been employed in national surveys, such as in Hungary, where heavy metals in moss growing at 116 sites were determined [102]; as well as in long-term studies, such as that of Poikolainen et al. [103], which investigated the temporal variation in heavy metal content of mosses from a sample site over the period 1985–2000 [103]. Examples of some of the most commonly assessed inorganic and organic pollutants in mosses are included in Table 3, which also includes the analytical methods employed and the range of concentrations found.

4. CONIFER NEEDLES

4.1 Introduction

As a result of the waxy layer on conifer needles, they are able to accumulate gaseous and particulate-bound pollutants [111]. It is believed that pine needles accumulate organic air pollutants by an uptake process via their stomata or via diffusion through the wax layer [112]. The accumulation ability of pine needles is important since they take up metals at concentrations much higher than the required essential levels. As a result of their large surface area, they are able to trap airborne elements, which are further improved by their high cation-exchange capacity [113]. Conifers are evergreen; therefore they can accumulate air pollutants for several years. In addition, needles ranging from 1 to 3 years can be found on the same branch and the age of pine needles can be easily determined [114].

The first study using pine needles as biomonitors was targeted towards trace elements (Hg, Cd, Cu, Zn, Fe and Co) in Italy [115]. In later years, various SVOCs including chlorobenzenes (1,2,3,4-tetra-, penta-, hexachlorobenzene (HCB)), HCH isomers (α-, β-, γ-, δ-, ε-HCH), DDX (*p,p′*-DDT, -DDE, -DDD), PCB congeners and PAHs [116] were analysed in pine needles. PAHs have also been investigated using pine needles in other studies [117].

Due to the prevalence of pine species across the world, they are a good choice of biomonitor since they allow a global understanding of pollutant levels in pine needles to be determined, as well as to establish pollutant transport patterns between regions. They have therefore been employed in intercontinental studies, such as in a comparison of atmospheric PAH levels

TABLE 3 Examples of Biomonitoring Studies Focusing on Inorganic and Organic Air Pollutants in Mosses

Moss Species	Location	Analytes	Sample Mass (g)	Extraction Method	Detection Method	Concentration Detected	References
Hylocomium splendens and Pleurozium schreberi	Finland	Cr, Ni	2 L fresh moss	Wet digestion	FAAS	Range ($\mu g\ g^{-1}$): Cr 1–6, Ni 3–6	[3]
H. splendens	Norway	Li, Be, B Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Y, Mo, Cd, Sb, Te, Cs, Ba, La, Hg, Tl, Pb, Bi, Th, U	0.5	Wet digestion in pressurised vessels	ICP-MS	Range ($\mu g\ g^{-1}$): Be 0.02–Ca 3100	[104]
H. splendens	Norway	Na, Mg, Al, Cl, K, Ca, Sc, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Br, Rb, Sr, Zr, Mo, Ag, Sb, I, Cs, Ba, La, Sm, Eu, Tb, Yb, Hf, Ta, W, Au, Th, U	0.5	N/A	INAA	Range (ppm): Sc 0.03–Fe 72,100	[105]
H. splendens, P. schreberi	Finland	Cr, Pb, Ni, Cd, Cu, Fe, V, Zn	Green parts	Wet digestion	ICP-OES	Range ($\mu g\ g^{-1}$): Cd 0.38–Fe 380	[57]
Hypnum cupressiforme	Turkey	Pb, Cd, Zn, Cr, Mn, Fe	2	Hotplate wet digestion	FAAS	Range ($\mu g\ g^{-1}$): Cd 6.87–Fe 16,500	[106]

H. splendens, P. schreberi	Norway	Li, Be, Mg, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Y, Zr, Nb, Mo, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Tl, Pb, Th, U	0.5	Pressurised hotplate wet digestion	ICP-MS	Range ($\mu g\ g^{-1}$): Tm 0.000095–Ca 3700	[24]
Polytrichum formosum	Germany	Pb isotopes	0.15–0.2	Microwave	ICP-MS	Range of ratios: 1.131–1.154	[101]
Pleurocarpous mosses	Turkey	Fe, Pb, Ni, Cr, As, Cu, Co	1	Hotplate wet digestion	GFAA[a]	Range ($\mu g\ g^{-1}$): Ni 0.11–Fe 4166.5	[91]
Tortula muralis, Barbula acuta, Barbula unguiculata, Tortula ruralis, Bryum capilare, Tortula intermedia	Turkey	Fe, Mn, Zn, Pb, Ni, Cr, Cu, Cd	1	Microwave digestion	FAAS and GFAA	Range ($\mu g\ g^{-1}$): Cu 1.6–Fe 468.1	[59]
H. cupressiforme	Spain	V, Cr, Ni, Cu, Zn, As, Cd, Hg, Pb and total nitrogen	Not specified	Microwave digestion	ICP-MS	Range ($\mu g\ g^{-1}$): Hg 0.03–Zn 1087	[93]
H. cupressiforme	Spain	PAHs and NPAHs	0.2	Ultrasound	APGC-Q-TOFMS[b]	Sum of PAHs ($ng\ g^{-1}$) 188–1733, sum of NPAHs ($ng\ g^{-1}$) 41–315	[107]

Continued

TABLE 3 Examples of Biomonitoring Studies Focusing on Inorganic and Organic Air Pollutants in Mosses—cont'd

Moss Species	Location	Analytes	Sample Mass (g)	Extraction Method	Detection Method	Concentration Detected	References
H. cupressiforme	Italy	Cd, Cu, Zn, As, Pb, Ni, V, Pt, Pd, Cr, Mn, Al, Fe	0.25	Microwave	ICP-MS	Concentrations not reported. Bioconcentration in order of: Al > Fe > Mn > Zn > Pb > Cu > V > Cr > Ni > As > Cd > Pb > Hg	[97]
H. cupressiforme	Romania	PCBs, PBDE, OCPs, DDT and metabolites, HCH isomers	3	ASE system[c]	ECNI-GC-MS[d]	Range (ng g^{-1}): sum HCH 8.9–133, sum DDT 5.8–95.3, sum Chlordanes: ND-0.4	[108]
H. cupressiforme, P. schreberi, Pseudoscleropodium purum	Germany	N isotopes	0.003–0.004	N/A	Elemental analyser coupled with isotope ratio mass spectrometer	Range: 18–49%	[21]
Leptodon smithii	Italy	Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Zn and 16 PAHs	Not specified	Microwave digestion	FAAS. GFAA, GC-MSD[e]	Total PAHs (ng g^{-1}): 72.33–380.98	[4]

Sanionia uncinata, Warnstorfia sarmentosa	Antarctica	PCBs, HCB, PAHs	3 g	Soxhlet extraction	GC-ECD[f]	Sum PCBs (ng g^{-1}): ND-1.64, DDTs: ND-1.93, HCHs: ND-2.91, sum PAHs: 126–955	[95]
Brachythecium rutabulum	Spain	PAHs	10–30 mm long moss shoots	Ultrasonic bath	GC-MS	Not specified	[109]
T. muralis	Italy	PAHs, N, Cd, Co, Cr, Cu, Fe, Ni, Pb, V, Zn	5–0.5 g	Ultrasound, wet digestion	GC-MS, elemental analyser, ICP-MS	Metals: range (μg g^{-1}): Cd 0.35–Fe 15,605	[99]
Sphagnum	The Netherlands	PAHs	20 g	Soxhlet	LC-fluorescence detection	Range (ng g^{-1}): 15 (benzo(k) fluoranthene, rural site)–13,000 (fluoranthene, impacted site)	[94]
H. cupressiforme	Hungary	PAHs	5 g	Ultrasound	HPLC-fluorescence detection	Total PAHs (μg kg^{-1}): 0.1567–10.45 × 10^4	[110]
H. cupressiforme	Hungary	Cd, Cr, Cu, Fe, Ni, Pb, V and Zn	0.2 g	ASE	ICP-AES	Mean (mg g^{-1}): Cd 0.9–Fe 2070	[102]
H. splendens and P. schreberi	Finland	Cd, Cr, Cu, Fe, Ni, Pb, V and Zn (and As and Hg for some samples)	~2 g	Wet digestion	ICP-AES; CV-AFS[g] (Hg); GFAA (As)	Range (μg g^{-1}): Cd 0.01–Fe 3150	[103]

Continued

TABLE 3 Examples of Biomonitoring Studies Focusing on Inorganic and Organic Air Pollutants in Mosses—cont'd

Moss Species	Location	Analytes	Sample Mass (g)	Extraction Method	Detection Method	Concentration Detected	References
Hypnum plumaeformae	China	PAHs	5 g	ASE	GC-MSD	Sum PAHs (ng g^{-1}): 310–1340	[100]

ICP-AES, inductively coupled plasma-atomic emission spectroscopy; FAAS, flame atomic absorption spectroscopy; ICP-OES, inductively coupled plasma-optical emission spectrometry; ICP-MS, inductively coupled plasma-mass spectrometry; INAA, instrumental neutron activation analysis; GC-MS, gas chromatography-mass spectrometry.
[a]*Graphite furnace atomic absorption.*
[b]*Atmospheric pressure gas chromatography–quadrupole time-of-flight mass spectrometry.*
[c]*Accelerated solvent extraction.*
[d]*Electron capture negative ion gas chromatography–mass spectrometry.*
[e]*Gas chromatography–mass selective detector.*
[f]*Gas chromatography–electron capture detector.*
[g]*Cold vapour–atomic fluorescence spectroscopy.*

between Korea, Mexico and the United States [118]. Not only are the needles used as biomonitors, but also often the bark of pine trees is studied in addition to the needles as a comparison [119]. Mosses have also been studied in conjunction with pine needles for comparative purposes to determine persistent organic air pollutants at local and regional scales [120].

In terms of trace elements, where the elements exceed the essential (background) concentration it is indicative of additional uptake either via the soil or via atmospheric wet and dry deposition [121]. Using enrichment factors, elemental source apportionment is possible if soil or bedrock concentrations are determined concurrently with the pine needle concentrations [122], as in a study by Lehndorff et al. [121].

4.2 Conifer Needles as Biomonitors of Organic Air Pollutants

Pine needles have been assessed as a tool for long-term trend determinations of atmospheric levels of organic pollutants, specifically PAHs, PCBs and organochlorine pesticides (OCPs) [114], where it was found that the needles provided very similar information to high-volume air sampling.

Pine needles have been used to study POPs, as it has been shown that they accumulate these pollutants [70]. However, it was observed that lichens accumulated higher concentrations of POPs, compared to the pine needles. This was also observed by Migaszewski et al. [20] in Poland, who found that the lichen *Hypogymnia physodes* bioaccumulated higher levels of organic analytes than pine needles. This is most likely due to the fact that lichens have longer (and mostly undefined) exposure periods than that of pine needles [77]. Pine needles are also known to accumulate gas-phase POPs preferentially over particle-bound POPs [70], possibly due to the smaller surface area of the needles.

As a result of a DDT spraying program conducted in the Southern regions of East Germany during 1984, a study was conducted by Eriksson et al. [123] to monitor the extent of pesticide pollution, which showed that pine needles are appropriate biomonitors for DDT and its derivatives. Around the same time, Stratchen et al. [124] studied organochlorine compounds in pine needles across West Germany, Denmark, Sweden and Norway. Successfully targeted compounds included HCB, pentachlorophenol, HCH isomers (α-HCH and γ-HCH (lindane)), DDT and DDE. The study found that there was no difference amongst pine needles sampled from trees of different ages. This has not been the case for studies of other air pollutants, such as that by Bargagli et al. [115] which used pine needles to monitor Hg, as well as a study by Lehndorff et al. [117] who found that fresh and old needles varied in total PAH concentration. The study by Lehndorff et al. [117] also identified large seasonal variations, where the older pine needles showed large increases in total PAH concentration during the winter months. A study conducted in Portugal [125] investigated the PAH profile across several geographic regions in

Portugal; in cities, industrial areas, rural areas and remote locations. The PAH concentrations found in the needles ranged from 96 ng g^{-1} dry weight (dw) (remote sites), 118 ng g^{-1} dw (rural sites), 337 ng g^{-1} dw (urban sites) to 866 ng g^{-1} dw (industrial areas).

Pine needles were used to study the PAH profile in Portugal, Greece and Spain, where Ratola et al. [126] found PAH levels to be similar in all countries (279, 301, 294 ng g^{-1} dw, respectively) with a general dominance of three- and four-ring PAHs. This study also used the diagnostic ratios of phenanthrene/anthracene and fluoranthene/pyrene that indicated mixed petrogenic and pyrogenic sources of PAHs, demonstrating the applicability of using pine needles as biomonitors in this regard. A recent study by Silva et al. [127] investigated brominated flame-retardants (BFRs), PCBs, OCPs, PAHs and one class of contaminant of emerging concern, the synthetic musk fragrances (SMCs) in a multicomponent study using pine needles. Various determined concentrations across sites ranged 0.45–0.87 ng g^{-1} dw (BFRs), 0.35–1.01 ng g^{-1} dw (PCBs), 0.26–12.2 ng g^{-1} dw (HCBs), 245.7–967.8 ng g^{-1} dw (PAHs) and around 277.5 ng g^{-1} dw (SMCs). A selection of various studies using pine needles as monitors for organic air pollutants are summarised in Table 4.

4.3 Conifer Needles as Biomonitors of Inorganic Air Pollutants

The use of vascular plant leaves such as conifer needles has successfully been applied to the biomonitoring of heavy metals [132], such as a study by Bargagli et al. [133] which found elevated levels of mercury in older pine needles and a similarity between the concentration in pine needles and lichens was observed. It was suggested that pine needles are accumulators of mercury, and that the release of metals from pine needles may play an important role in the mercury cycle [133].

In a study by Ceburnis and Steinnes [134], correlations between the concentration of heavy metals in both needles and mosses suggested that mosses were more appropriate as biomonitors for metals due to higher elemental concentrations. Their study therefore recommends the use of mosses when a multi-component analysis is to be made, but does not disregard the use of pine needles when moss is not suitable or available for use as a biomonitor. It has also been suggested that pine needles are only feasible as biomonitors for trace elements when sampled close to the pollution source under investigation [134].

Lehndorff et al. [135] sampled pine needles in an urban area in Cologne, Germany, for the biomonitoring of atmospheric Fe, Pb, Cd, Zn, Cr, Cu, Ni and sulphur, and correlated the results to PAH data. The concentrations found for Fe, Pb and Cd were 132, 1.1 and 0.06 mg g^{-1}, respectively, showing accumulation above essential nutrient levels. Sulphur concentrations were found to be between 868 and 2076 mg g^{-1}, and the elevated levels of Fe, Pb and Zn were suggested to be indicative of high traffic volumes, as supported by the PAH results [135]. A study by Dmuchowski and Bytnerowicz [136] mapped

TABLE 4 Various Studies Using Pine Needles to Monitor Organic Air Pollutants

Pine Species	Location	Analytes	Sample Mass (g)	Extraction Method	Analysis Method	Total Concentration Detected	References
Pinus pinea L.	Portugal	PAHs	10	Soxhlet/USAE	GC-MS	21.86–339.28 ng g^{-1}	[126]
P. pinea L. needles and bark	Portugal	PAHs	2	USAE/MAE	GC-MS	213–1773 ng g^{-1}	[119]
P. pinea L.	Portugal	Pesticides	5	USAE	GC-MS	18.76–56.05 ng g^{-1}	[128]
Wet needles (mixed species)	Korea, USA, Mexico	PAHs	10	Column	GC-MSD	31–563 ng g^{-1}	[118]
Pinus sylvestris L.	Germany	PAHs	25	ASE/Soxhlet	GC-MS	Up to 55 ng g^{-1}	[129]
Pinus pinaster	Portugal	PCBs, BFRs, HCBs, PAHs, SMCs	5	USAE	GC-MS ion trap	0.26 (HCBs)–967.8 ng g^{-1} (PAHs)	[127]
Pinus nigra	Germany	PAHs	10	ASE	GC-MS	51–410 ng g^{-1}	[117]
Pinus thunbergii Parl., *Pinus densiflora Sieb et Zucc*	Japan	PCDD/Fs, PCBS, PCNs	Not reported	Soxhlet	HRGC-HRMS	0.17 (TCDDs)–2100 pg g^{-1} (PCNs)	[130]
P. pinaster, P. pinea	Portugal	PAHs	1/5	MA-HS-SPME[a]	GC-MS	111–1320 ng g^{-1}	[131]

GC-MS, gas chromatography-mass spectrometry.
[a] *Microwave-assisted headspace solid-phase microextraction.*

the distribution of sulphur, Zn, Cd, Pb, Cu, and As across Poland using Scots pine needles. Deposition zones were therefore identified and were used to identify sources of air pollution, as well as the directions in which pollutants were being dispersed over the sampling years [136].

A consideration that needs to be borne in mind when using pine needles to monitor inorganic air pollutants is whether the fraction related to compounds deposited on the needle surface (and not incorporated into the organism) is desired in the final extract. This is necessary, since a study by Wyttenbach et al. [132] found that the fraction washed off can contribute as much as 60% to the total concentration of an element. This has implications on how the results of a study are interpreted and its associated uncertainties. Examples of studies involving conifer needles for inorganic air pollutant biomonitoring are provided in Table 5.

5. TREE BARK

5.1 Introduction

The accumulation of pollutants by tree bark is mostly due to the ability of the tree to absorb pollutants from soil, where water serves as a solvent for a number of ionic materials [139]. Tree bark has, however, been used in air biomonitoring studies because bioconcentrated fractions have been found to correlate with atmospheric values for 46 elements [98]. Trees are also easily identified, widely dispersed and easily sampled [98]. Pine bark in particular has shown to be effective for the assessment of spatial and temporal trends in air pollution studies because it is very porous and inert, and pine trees are widely distributed [119,140], as discussed in Section 4.

5.2 Tree Bark as a Biomonitor of Air Pollutants

The high lipid content and large surface area of tree bark contribute to hyperaccumulation of organic pollutants [141] such as POPs [119], where the estimated residence time of PCBs and PCDD/Fs in tree bark is >10 years [142]. In a study by Türkan et al. [106] a reduction in pollution was observed at increasing distances from the source, however, these differences were not as marked as what was observed for the moss, *H. cupressiforme*. In contrast, a study by Tarcau et al. [108] found that DDT and its metabolites as well as HCH isomers were in higher concentrations in tree bark samples than in moss. Species-pollutant selectivity and differences in sample collection and sample preparation may contribute to these differences. In terms of PAH biomonitoring, Ratola et al. [119] found that pine needles were more effective bioaccumulators of PAHs than tree bark, with concentrations up to 17 times higher in the needles than in the bark. Inorganic analytes commonly assessed in bark biomonitoring studies are SO_2 and heavy metals, where the SO_2 concentrations largely depend upon the pH of the bark [143]. Tree bark is used

TABLE 5 Various Studies Using Pine Needles as Biomonitors for Trace Metal Pollution Monitoring

Pine Species	Location	Analytes	Sample Mass (g)	Extraction Method	Analysis Method	Total Concentration Detected	References
Pinus halepensis L.	Jordan	Pb, Cd, Cu and Zn	2	Ashed in furnace, dissolved in $HNO_3 + HCl$ (1:1)	ICP-AES	0.12–262 μg g^{-1}	[137]
Pinus Eldarica Medw.	Iran	Pb, Zn, Cu, Ni, Cr	1	Microwave digestion in concentrated HNO_3	FAAS	0.39–62.3 ppm	[138]
Picea abies	Lithuania	As, Cd, Cr, Mn, Pb, V, Zn	1.5	Digested in hot concentrated HNO_3 for 3 days	AAS	0.018–684 μg g^{-1}	[134]
Pinus nigra	Germany	Ba, Ca, Cd, Fe, Mo, Na, Pb, Sb, Ti, V, Zr		Hot aqua regia digestion	HR-ICP-MS	Ca (1.5–12.1 g kg^{-1}), Fe (32–350 mg kg^{-1}), Na (2–676 mg kg^{-1}), Ba, Ti and Pb (0.1–4.5 mg kg^{-1}). Mo and Sb (20–1000 mg kg^{-1}), Cd and Zr (500–300 mg kg^{-1})	[121]

ICP-AES, inductively coupled plasma-atomic emission spectroscopy; FAAS, flame atomic absorption spectroscopy; AAS, atomic absorption spectroscopy; HR-ICP-MS, high resolution-inductively coupled plasma-mass spectrometry.

less commonly in the biomonitoring of air than other biomonitors such as mosses and lichens [1,57,106]. This is because the potential sources of pollutants are not limited to the air. In comparison to lichens and mosses, tree bark poorly reflects regional differences of inorganic atmospheric pollutant concentrations [57]. Bark has also been shown to accumulate lower concentrations of heavy metals than mosses or lichens [3]. An additional challenge in using bark as a biomonitor of air pollution is the lack of standardisation of techniques [106], therefore future studies employing the use of bark will need to address these challenges. Table 6 shows some examples of studies which have used tree bark in the assessment of inorganic and air organic pollutants.

6. OTHER PLANTS

A number of other plant species have been employed as biomonitors of air pollution, although to a somewhat lesser extent than the species discussed in the previous sections of this chapter. The leaves of various species of trees and shrubs have been used to determine levels of organic pollutants, where PAHs have been studied in *Ficus benghalensis* leaves [145]; in leaf cuticles and leaf tissue [146]; in leaves of *Quercus ilex* L. [147] and in maize [148], for example. In addition, Barriada-Pereira et al. [149] determined pesticide levels in various plants.

These matrices have similarly been used for inorganic air pollutants, such as heavy metals [150]. Celik et al. [151] utilised the leaves of *Robinio pseudo-acacia* L. to monitor airborne Fe, Zn, Pb, Cu, Mn and Cd; whilst Calzoni et al. [152] employed the perennial shrub, *Rosa rugosa*. Studies have also investigated the impacts of inorganic air pollutants on the growth and viability of agricultural crops, an example being the effects of sulphur dioxide pollution on wheat [153].

A concern with these biomonitors is that the levels of pollutants determined therein may not solely reflect the contribution arising from exposure to atmospheric concentrations, but also from uptake from the soil by the root system (a portion of which may have arisen from deposition of the pollutant from the air), and from groundwater and irrigation water. This limitation needs to be taken into consideration when interpreting the results from such studies [98].

7. SAMPLING CONSIDERATIONS

When using biomonitors, a number of factors should be carefully considered in order to minimise variations caused by species differences, specificity of the organism towards the target analytes and meteorological conditions.

1. A sufficient amount of the organism must be present in the ecosystem under study to contribute towards a representative sample amount (unless a transplant study is planned).

TABLE 6 Examples of Biomonitoring Studies of Inorganic and Organic Analytes Using Tree Bark

Common Name	Plant Species	Location	Analytes	Sample Mass	Extraction Method	Detection Method	Concentration Detected	References
Pine	Not mentioned	Finland	Cr, Ni	0.2 L Fresh sample volume	Wet digestion	FAAS	Range ($\mu g\ g^{-1}$): Cr 0.5–3.5, Ni 1.1–2.2	[3]
Turkish pine	*Pinus brutia*	Turkey	Pb, Cd, Zn, Cr, Mn, Fe	2 g	Hotplate wet digestion	FAAS	Range ($\mu g\ g^{-1}$): Cd 2.25–Fe 1850	[106]
Scots pine	*Pinus sylvestris* L.	Finland	Cr, Pb, Ni, Cd, Cu, Fe, V, Zn	Outermost 3 mm	Wet digestion	ICP-OES	Range ($\mu g\ g^{-1}$): Cd 0.31–Fe 110	[57]
Pine tree	*P. sylvestris*	Germany, Norway, Russia, Poland	Benzo[*a*]pyrene, fluoranthene, pyrene, α-hexachloro-cyclohexane (α-HCH), DDT, Al, As, B, Ca, Cd, Ce, Cr, Cu, Fe, Hg, Mo, NH_4^+, Ni, NO_3^-, PO_4^{3-}, Pb, Sr, SO_4^{2-}, Ti, V, W, Zr, Zn	0.5 g for Heavy metals, 5 g for PAHs	Microwave and solvent extraction device	IC, HPLC-MS, GC-ECD, ICP-OES, ICP-MS,	Range ($\mu g\ g^{-1}$): Hg 0.001–Ca 27,000, benzo(*a*)pyrene 0.0004–0.0210, fluoranthene 0.0074–0.1332, pyrene 0.005–0.083, α-HCH 0.008–0.120, DDT 0.019–0.304	[140]

Continued

TABLE 6 Examples of Biomonitoring Studies of Inorganic and Organic Analytes Using Tree Bark—cont'd

Common Name	Plant Species	Location	Analytes	Sample Mass	Extraction Method	Detection Method	Concentration Detected	References
Olive tree	*Olea europaea*	Portugal	46 trace elements	<0.001 g	N/A	INAA and PIXE	–	[98]
Indian liliac, mango, sacred fig, cashew, cassod tree, gum arabic tree, sea almond tree	*Azadirachta indica, Mangifera indica, Ficus religiosa L., Anacardium occidentale L. Cassia siamea lam., Acacia senegal. Terminalia catappa* used for validation of method	Nigeria	Cd, Cr, Cu, Fe, Ni, Pb, V, Zn	1 g	Ashing and wet digestion	FAAS	Range ($\mu g\ g^{-1}$): Ni 5.0–Fe 695	[144]
Cluster pine, stone pine	*Pinus pinaster and Pinus pinea*	Portugal	PAHs	1,2 and 5 g	Microwave and ultrasound	GC-MS	Sum PAHs ($ng\ g^{-1}$): 22–1773	[119]
Not specified	*Tili, Pinus, Fagus sylvatica*	France and Germany	PCDD/Fs and PCBs and trace metals As, Sb, Cd, Co, Sn, Ni, Cr, Pb, Fe	5–30 g	ASE	GC-ECD, ICP-OES and ICP-MS	GC-EDC ($ng\ g^{-1}$): PCBs 4–140 HRGC-MS ($ng\ g^{-1}$): PCBs 0.006–41.01 metals (ppm): As 1.7–Fe > 10,000 ppm	[142]

Oak, cherry, hornbeam, alderwood, ashwood	Not mentioned	Romania	DDT and metabolites, and HCH isomers	1.5 g	ASE system	GC-MS, GC-ECNI/MS	Range (ng g^{-1}): sum HCH 12.4–131.4, sum DDT 510.8–442.6, sum chlordanes: ND-0.6	[108]
Black alder, silver birch, Scots pine, sweet cherry, plum, English oak, sessile oak, willow, small-leaved lime, large-leaved lime, Norway maple, Sycamore, European hornbeam, common hazel, European ash, apple, Canadian poplar, checker tree	*Alnus glutinosa, Betula pendula, P. sylvestris, Prunus avium, Prunus domestica, Quercus robur, Quercus petraea, Salix* spp., *Tilia cordata, Tilia platyphyllos, Acer platanoides, Acer pseudoplatanus, Carpinus betulus, Corylus avellana, Fraxinus excelsior, Malus domestica, Populus canadensis, Sorbus torminalis*	Germany	N isotopes	0.003–0.004 g	N/A	Element analyser coupled with isotope ratio mass spectrometer	Range (%): 1–21	[21]

GC-ECD, gas chromatography-electron capture detector; FAAS, flame atomic absorption spectroscopy; ICP-OES, inductively coupled plasma-optical emission spectrometry; ICP-MS, inductively coupled plasma-mass spectrometry; INAA, instrumental neutron activation analysis; PIXE, particle-induced x-ray emission; GC-ECNI/MS, gas chromatography-electron capture negative ion mass spectrometry; GC-MS, gas chromatography-mass spectrometry.

2. The biomonitor must be widely distributed throughout the desired sampling region.
3. The biomonitor should be easy to identify or alternatively access to an expert who is able to correctly identify the organism is required.
4. Good tolerance of the organism towards the target pollutants.
5. The population of the organism should be well established so that future sampling campaigns can be undertaken to establish trends over a period of years and so that the biodiversity of the sampling region is not negatively impacted.
6. There should be evidence of a correlation between the concentration of the target pollutants in the atmosphere and the concentration of the pollutants in the tissue of the chosen organism.
7. A similar bioaccumulation factor (the biomagnification of the analyte concentration in the tissue of the biomonitor compared to the ambient levels as established in point 6) should be maintained for samples collected at different sites by keeping the species, substrate from which it is sampled, meteorological conditions and sample handling parameters as consistent as possible.

It is also necessary to establish, prior to analysis, whether the presence of the target pollutants in the tissues is as a result of atmospheric pollution only (thus showing specificity towards the uptake mechanism) or if the pollutants are accumulated by other means, introducing a bias [154]. For this reason, lichens and mosses are popular choices, since it is believed that the only contribution of pollutants to their tissue is as a result of atmospheric sources (via dry and wet deposition) [155].

Augusto et al. [74] performed a study on PAHs in lichens and found seasonal variations (highest PAH values during the dry winter) and demonstrated that meteorological parameters such as temperature, air pressure, humidity as well as wind speed affected the PAH concentrations in both lichens and sampled air. A study by Jensen [87] showed erratic variations in the SVOC concentrations in both lichens and mosses, and linked these results to weather conditions; where HCH concentrations were higher after rainy spells compared to dry, hot weather. The concentrations of HCHs remained nearly constant in the pine needles, which were analysed alongside the moss and lichen samples, suggesting that the hydration state of lichens and mosses may affect the accumulation of organic air pollutants therein [75]. Degradation of photosensitive analytes, which occurs more significantly during the summer months, has also been noted in biomonitoring studies [156], and humidity and air pressure may contribute towards the absorption and retention of organic air pollutants [77].

A study by Wolterbeek and Bode [157] proposed that when biomonitors are to be utilised in trace metal studies, the pH, NH_4^+, NO_3^- and SO_4^{2-} levels within the biomonitors are also relevant, as the retention of metals, such as Ca and Hg are affected by acidic precipitation, which in turn is influenced by micro- and

macro-climatic factors. It has also been proposed by Tretiach and Carpanelli that when considering lichens and their assimilation of pollutants, it might be more meaningful to represent the assimilation rates based on the chlorophyll content rather than on the principle of dry weight [158]. They recommended that the concentration of chlorophyll in samples should always be reported.

7.1 Sampling of Biomonitors for Organic Analytes

A standardised approach has not yet been formulated with respect to sampling biomonitors used to target organic analytes. However, recommended best practise which minimises uncertainties in the results generated are included in this section.

The geographical location (co-ordinates) where sampling takes place should be recorded, along with the sampling altitude, since atmospheric pressure has been shown to influence results [74]. The location of nearby air pollutant emission sources should be noted. Sampling should be conducted during a dry spell, in order to minimise any variations introduced by rain wash-off. The weather (wind direction, temperature, humidity and air pressure) during sampling should be noted. Acquiring longer-term meteorological data for the sampling sites (such as annual rainfall and temperature) may also assist with data interpretation. The species of the substrate from which the biomonitors are to be removed must be identified, as well as the total number of individual substrates from which biomonitors were sampled.

No smoking should be permitted near the sampling site in order to prevent sample contamination. Gloves should be worn, and sterilised stainless steel tweezers or a sterilised blade is recommended for the removal of the biomonitors for organic air pollutant monitoring. For lichens, sampling is typically performed at a height of at least 50 cm above the ground, on the branches and trunk of trees [68]. In the case of pine needles, whole needles are removed, typically from the bottom and outer branches of the trees. Moss samples are collected away from trees (on dead tree stumps and over the ground only) and stored in amber jars. If bark is the biomonitor of choice, it is to be sampled from a height >150 cm above the ground, on the trunk, removing only the most external layer [159]. Samples may be stored in brown paper bags, but amber vials are more commonly used to reduce contamination and analyte loss, and to prevent photodegradation of the analytes. Wrapping the samples in foil prior to placing them into plastic bags is an alternative [160].

The samples should be kept cool to reduce any losses, and manually cleaned of all dirt, bark, leaves or stones once in the laboratory, although most organic air pollutant biomonitoring studies do not mention a washing step. Lichens are typically oven dried at 35 °C for 3–4 days to remove all moisture [68], before being ground with an agate mortar and pestle, and stored in the freezer until use. Pine needles are either cut into 1-cm-long pieces or left whole and stored (wrapped in foil and placed in a plastic bag) in the freezer

without drying. The water content of the needles may be established by drying ~5 g of needles at 80 °C until a constant weight is obtained [131]. Moss samples may be dried at 40 °C for 2–3 days prior to being ground in a blender, and stored in glass jars in the freezer [107].

7.2 Sampling of Biomonitors for Inorganic Analytes

The basic best practice principles of sampling biomonitors for inorganic air pollutants are similar to those for organic target analytes, as described in Section 7.1, therefore only the differences in methods are presented here. Samples should be taken using acid-washed plastic [161], nylon, ceramic or Teflon tweezers [36] in order to prevent metal contamination thereof, and powder-free latex, nitrile or vinyl gloves should be worn.

Epiphytic lichens should be collected 1–2 m above the ground [3], and sampling should be done all around the tree to limit the effect of prevailing wind conditions (unless this factor is to be studied). Lichens of a similar diameter should be sampled to reduce variations in age of the lichens, although it is recommended that the outermost 3–4 mm of the foliose lichen thallus be detached for analysis if the aim of the study is to assess concentrations which have been accumulated over the period of a year [32], however, these restrictions may lead to reduced sample mass. Epiphytic moss samples should be collected at 1–2 m above the ground and should not be older than 2–3 years [4].

Samples should be stored in acid-washed polyethylene or polypropylene bottles or paper bags and stabilised as soon as possible. Samples should be cleaned of extraneous material prior to storage to ensure that these materials do not contaminate the biological monitor. This cleaning step should take place as soon as possible after sampling so as to limit changes which may occur due to microbial activity and surface dust deposition. There has been little standardisation with respect to sample pre-extraction preparation. Some authors recommend washing the samples with water to remove surface dust deposits [3], however washing may result in the dissolution of ionic compounds. Ultrasonic baths have been used to clean foliose lichens [162]. Lichens are not washed, however, in the determinations of metal concentrations in adhering particles [22] or isotopes [21], but if the bioconcentrated portion is of interest, then the samples should be washed [109].

Mosses are particularly difficult to clean effectively due to their porous nature [25]. Techniques such as nitrogen air jets and ultrasonic probes have been used to remedy this, however, it was found that nitrogen air jets are too aggressive (at 4–8 bars) and could not effectively remove extraneous materials at lower pressures [25]. The results from such pressurised jet systems were also found to be irreproducible and unpredictable. Attempts to remove particulate matter from mosses by using an ultrasonic probe were found to be an unviable option since it required the presence of a solution, which leached analyte from the sample [25].

It has been recommended in a number of studies that samples be ground and homogenised [163], but this may increase the risk of contamination [161]. Drying or freezing steps aid homogenisation and stabilise the sample in terms of microbial activity, thereby preventing changes to the analyte concentration which microbes may induce through their metabolic processes. Commonly used techniques are air-drying [32,36], oven-drying [164], freezing to between −20 and −80 °C [101], freeze-drying [165] and cryogenic freezing with liquid nitrogen [22,166]. Pre-ashing of samples has been used [3]; however, the use of this technique may liberate volatile elements such as Bi, Cd, S, Sb and Se [163].

8. ANALYTE EXTRACTION METHODS

The most commonly employed techniques utilised to extract air pollutants from biomonitors are discussed in this section.

8.1 Soxhlet Extraction

Soxhlet has been used for over a century to leach analytes of interest from their solid matrices into a solvent of choice. The Soxhlet apparatus allows the continual dripping of condensed extracting solvent onto the sample, held in a porous thimble, encouraging extraction of the analytes into the solvent [167,168]. A siphon aspirates the solvent dripping into the thimble when an overflow volume level is exceeded, and the liquid is poured back into the distillation flask. This process allows the transfer equilibrium to be continually disturbed, improving the extraction efficiency when compared to static leaching techniques [168].

Soxhlet extraction has historically been used to extract organic analytes from lichens including PAHs and other SVOCs [30,43,67,82,169] (Table 7). The method has also been applied with success to the extraction of SVOCs from bark and pine needles [30,126,159,170,171].

Advantages of using Soxhlet extraction include the utilisation of either large or small sample volumes, the omission of a filtration step, the lack of matrix dependence and ease of use. The technique has been automated in commercial systems, which are faster and use less solvent [167]. A serious disadvantage of using the Soxhlet methodology for sample preparation is the large volumes of wasted solvent that are expensive to purchase and dispose of, many of which are harmful to the environment. Another disadvantage is the long extraction times (2–24 h) which decreases sample throughput and utilises more energy [76,88]. In addition, boiling solvent over the period of a few hours may degrade thermolabile species [168]. The introduction of newer techniques such as microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) are increasing in popularity, however, the use of Soxhlet for routine analysis remains relevant as a result of the relative

TABLE 7 The Different Soxhlet Extraction Conditions Applied in Various Biomonitor Studies Targeted Towards Organic Air Pollutants

Biomonitor	Analytes	Sample Mass (g)	Solvent Scheme	Solvent Volume (mL)	Extraction Time (h)	References
Lichen	PCDD/Fs	10	Methylene chloride:hexane (1:1)	Not reported	18–24	[43,88]
Lichen	PAHs	2	Dichloromethane	100	16	[67,82,83,172]
Lichen	PAHs	2	Acetonitrile	200	24	[30,88,173]
Lichen	PAHs	0.2	Dichloromethane	250	6	[80]
Lichen	PAHs	0.6–0.8	Hexane:acetone (1:1)	150	2	[76]
Lichen	PCDD/Fs	Not reported	Toluene	200	24	[63,88]
Lichen	Chlorinated hydrocarbons	Not reported	Hexane	Not reported	8	[65]
Pine needles	PCDD/Fs, PCBs, PCNs	Not reported	Toluene and dichloromethane:methanol (1:1)	300 each	7 each	[130]
Pine needles	OCPs	Not reported	Hexane:acetone (1:1)	80	20	[171]

affordability of the method as well as its robustness, therefore, it is often used as a reference technique when new extraction methods are developed.

8.2 Microwave-Assisted Extraction

The principle of MAE is that the analytes of interest adsorbed or absorbed by the substrate are partitioned into the extraction solvent by the application of microwave energy (in the range of 0.3–300 GHz). Microwaves heat the contents of the vessel simultaneously and nearly instantaneously, without heating the vessel, reducing extraction times significantly.

The extraction efficiency is heavily dependent on the choice of extraction solvent, since the solvent should readily absorb microwave energy [174]. As a result, non-polar solvents produce poor extraction efficiencies, unless they are used in conjunction with solvents that have large dipole moments [175]. The solvent should selectively and efficiently extract the analytes without overheating, which would lead to degradation. The water content of samples should also be controlled to minimise losses due to heating [176], thus it is recommended that the sample is air dried, oven dried at low temperatures, or freeze dried, as applicable.

MAE can be considered as either pressurised MAE, where the vessels are closed, with the pressure and temperature being controlled (by elevating the pressure), or as focused MAE, where the extraction vessel is kept open, and the extraction takes place at atmospheric pressure and the boiling point of the solvent [177,178].

8.2.1 Extraction of Organic Analytes

The earliest use of microwave energy to assist in the extraction of organic analytes dates back to the 1980s, where household equipment was used [179,180]. Specialised laboratory equipment has since been developed. Guo and Lee demonstrated in their study on PAHs in soils that higher temperatures during extraction increases the diffusion rates of the solvent which then permeate the solid matrix more effectively. The partition coefficient is also affected such that the analytes are released from the solid more readily at higher temperatures [174].

Typically, a cleanup and concentration step is necessary after MAE before the extract can be analysed. The filtration and cleanup procedure would also ideally serve to remove interferences and thus reduce matrix effects [181]. A study by Ratola et al. [119] compared the use of MAE and ultrasound-assisted extraction (USAE) to determine the PAH content of pine needles in Portugal. The study utilised a modified domestic microwave oven and dichloromethane:hexane (1:1, v/v) for the MAE extraction. The recovery of PAHs using MAE varied widely between 10% and 120%, with the recoveries of PAHs using USAE reported as being very similar although it was observed that MAE had better recoveries for the heavier molecular weight PAHs compared

to USAE. A study on PAHs in spruce needles and pollen [182] found MAE to be superior over USAE when n-hexane:acetone (3:2, v/v) was used, as shown in Table 8.

In general, when compared to traditional Soxhlet extraction, MAE offers a reduction in extraction time (between 5 and 20 min compared to >6 h for Soxhlet), reduced solvent consumption (10−50 mL in MAE compared to 250−500 mL in Soxhlet) as well as enhanced reproducibility and reduced solvent wastage [175,183].

A new microwave-assisted headspace solid-phase microextraction (MA-HS-SPME) technique was developed by Ratola et al. [131] for the analysis of PAHs in pine needles. The technique tested both polydimethylsiloxane and divinylbenzene fibres, and the optimised extraction technique produced clean extracts with no need for further cleanup steps. In particular, improved recoveries of higher molecular weight PAHs were observed, compared to previously developed USAE and MAE techniques [126,131].

8.2.2 *Extraction of Inorganic Analytes*

Microwave-assisted digestion is one of the most commonly used sample preparation techniques for the analysis of total metal concentrations from a range of matrices, as it is fast and effective. Samples are digested under high temperatures and pressures with minimum loss of sample and with substantially less acid than what is required with conventional methods. The analytically pure acids used depend upon the analytes and matrix of interest. Acid mixtures which are commonly employed in air biomonitor digestions are HNO_3 and H_2O_2 [113]; HNO_3, H_2O_2 and HF [164,184]; HCl and HNO_3 [185]; and HCl, HNO_3 and $HClO_4$ [186] with further examples provided in Table 9. Sample digestion runs should include spiked samples and method blanks so as to ensure that analyte recoveries are optimal and to check for sample contamination. The filtration of samples after digestion is necessary, where the composition of the filter and its pore size are important considerations.

Despite the fact that less acid is required in microwave digestion techniques than in conventional methods, high concentrations and limited dilution allowances in a number of techniques require that the acids are removed or reduced prior to analysis. Samples containing HF may be evaporated in an open vessel on a hotplate and then resuspended in a weak acid solution; however, the loss of volatile analytes may be incurred. Another option is the use of boric acid which complexes to F^- ions [187].

8.3 Ultrasound-Assisted Solvent Extraction

8.3.1 *Introduction*

USAE has gained popularity in the last few years as an attractive alternative to microwave digestion techniques [25,189]. Benefits of USAE over other techniques, such as Soxhlet extraction, accelerated solvent extraction and

TABLE 8 Examples of Microwave-Assisted Extraction Techniques for the Analysis of Organic Air Pollutants in Biomonitors

Biomonitor	Analytes	Sample Mass (g)	Solvent Scheme	Solvent Volume (mL)	Extraction Time	References
Pine needles	PAHs	2	Hexane:dichloromethane (1:1)	90	30 min at 513 W	[119]
Pine needles	PAHs, SMCs	2	Water and ethanol	50 water, 0.9 ethanol	60 min at 560 W	[131]
Pollen and spruce needles	PAHs	5	Hexane:acetone (3:2, v/v)	50	20 min	[182]

TABLE 9 Examples of the Use of Microwave Digestions for the Analysis of Inorganic Air Pollutants in Biomonitors

Biomonitor	Acids Used	Sample Mass (g)	References
Moss	4 mL HNO_3 (65%), 1 mL H_2O_2 (30%)	0.15–0.2	[101]
Lichen	Mixture of HNO_3, H_2O_2 and HF	0.05–0.1	[161]
Lichen	HNO_3	0.15	[166]
Garlic and Indian mustard	1.5 mL HNO_3 and 1.5 mL of H_2O_2	0.2	[188]
Lichen	3 mL HNO_3, 3 mL H_2O_2, 0.8 mL HF and 2 mL deionised water	0.25	[31]
Lichen and mosses	6 mL HNO_3 (65%), 2 mL H_2O_2 (30%) and 2 mL H_2O	1	[59]
Pine needles and bark	90 mL hexane:dichloromethane	1, 2, 5	[119]
Moss	Not specified	Not specified	[93]
Moss	2.5 mL H_2O_2 (30%), 5 mL HNO_3 (65%)	0.25	[97]
Tree leaves and mosses	HNO_3 and HF (2:1, v/v)	Not specified	[4]

supercritical fluid extraction, are that it is comparatively simple, efficient, cheap, is available in most laboratories and does not require high temperatures, pressures or concentrated harmful chemicals [190]. USAE techniques are commonly used in biomonitoring applications due to the fact that smaller sample sizes can be accommodated, less solvent is used, and the extractions are relatively fast compared to traditional liquid extraction techniques [42,80].

Ultrasonic techniques rely on the principle that once a solid sample in solution is subjected to ultrasonic energy, bubbles are formed and implode [189]. This causes high localised temperatures and pressures [189] which result in the extraction of compounds or elements from the sample matrix [25]. The major parameters affecting USAE are particle size, sample mass to extractant ratio [80,162,191], extractant concentration, sonication time and the use and choice of surfactants [191]. Surfactants are sometimes used to ensure that the sample is distributed homogenously and that small particles remain in suspension. Tween 85 and Triton X-100 were found to be ideal surfactants for use in biomonitoring studies, as opposed to surfactants such as glycerol that resulted in high backgrounds and poor reproducibility [191].

USAE can be used for a wide range of biomonitoring applications; from extraction of surface material, cleaning of samples [25] and the extraction of metallic species [188]. USAE requires fewer analytical steps than a number of other extraction techniques and is therefore less susceptible to contamination [162]. Furthermore, ultrasonic methods can also be used to accelerate sequential extraction steps that may be used in the partitioning of metals [190], and to facilitate the suspension and homogenous distribution of materials in slurry extracts [191]. Ultrasonic extraction is also applicable to the extraction of organic compounds from matrices to which they are weakly bound [190], as long as the heat is maintained at a temperature below which analytes are susceptible to degradation or volatilisation (for example, below 35 °C for PAH extraction from lichens [68]).

Sonication by an ultrasonic probe is up to 100 times more efficient than ultrasonic baths [162]. This is because the reproducibility and repeatability of ultrasonic baths is negatively influenced by non-uniformity of ultrasound energy distribution throughout the bath, as well as a loss of power with time [192]. The use of ultrasonic probes for the cleaning of moss samples was found to be ineffective, however, since the analyte of interest was leached from the sample into the solution [25]. Ultrasonic baths, on the other hand, are much gentler methods and are thus often preferred for speciation analysis [193] and may be a suitable alternative to probes for the removal of dust from the surface of samples such as moss.

8.3.2 Extraction of Inorganic Analytes

Compared to microwave digestions, USAE is used less frequently and requires a substantial amount of method development compared to the currently available microwave digestion methods. Table 10 provides examples of the use

TABLE 10 Examples of Biomonitoring Studies Which Have Used Ultrasonic Methods for Inorganic Analytes

Use	Species	Analytes	Optimal Extraction Medium	Sample Mass (g)	Optimal Sample Size	Optimal Temperature (°C)/Sonication Amplitude (%)/Extraction Time	References
Total extraction of metals	*Parmelia sulcata*, IAEA lichen 336, NIST SRM 2976	Na, K, Ca, Mg, Fe, Al, Cr, Mn, Co, Ni, Cu, Zn, Ge, As, Se, Rb, Sr, Zr, Ag, Cd, In, Sb, Cs, Ba, Pb, Bi	5 mL of 1% HNO_3	0.1	200–300 mesh	20–40%	[162]
Extraction of selenium from plants for selenium speciation	*Allium sativum* and *Brassica juncea*	Se species	0.1 M HCl, 25 mM ammonium acetate buffer (pH 5.6) and protease	0.2	Not specified	3 min	[188]
Total extraction of metals	Tobacco leaves	Al, As, Cd, Ni, Pb	10 mL of 15–20% HNO_3	0.2	<35 μm	60–80 °C	[189]
Suspension of extraction slurries	*Cupressus lawsoniana*, *Cupressus sempervirens*, *Quercus ilex*	Cd, Cu, Pb, Ni	5% HNO_3 with either Tween (0.5%) or Triton X (1%)	1–15 mg mL^{-1}	<32 μm		[191]

of ultrasonic extraction techniques in a number of studies for a variety of inorganic air biomonitoring applications.

8.3.3 Extraction of Organic Analytes

The USAE technique has grown in popularity because it is a fast method that uses less solvent and less energy, with satisfactory results. Furthermore, a sonication-assisted extraction procedure has been detailed by the EPA (Method 3550B) and USAE was also found to be superior over Soxhlet and pressurised liquid extraction in a study by Ratola et al. [126] that focused on PAHs in pine needles. Examples of experimental conditions when using USAE on various biomonitor matrices are given in Table 11. Concern has been raised about the possibility that high ultrasound frequencies may increase the degradation of SVOC analytes. A study by Manariotis et al. showed that phenanthrene, naphthalene and pyrene were degraded at frequencies above 582 kHz, particularly when the extractions were performed for long periods of time (>30 min) [194]. This should be carefully considered when developing an experimental method utilising USAE.

Whereas Guidotti et al. [42,84] used cyclohexane as the solvent for USAE of PAHs from lichens (sample mass 2 g), combining only two extractions of 30 mL each, Domeño et al. [80] extracted a 0.2 g lichen sample using portions of 15 mL dichloromethane in each of four subsequent extractions. A study, in which a novel dynamic ultrasonic-assisted extraction technique for lichens was developed by Domeño et al. [80], found that hexane extracted PAHs most efficiently and the new dynamic sonication-assisted solvent extraction (DSASE) technique has since been popular in numerous PAH studies using lichens [68,78,79], which utilises a continuous flow of solvent, resulting in reduced total solvent volumes, minimised extraction time and minimal sample handling compared to traditional USAE techniques, with a reduced possibility of analyte degradation.

8.4 QuEChERS

8.4.1 Introduction

This sample extraction technique was developed to be quick, easy, cheap, effective, rugged and safe. QuEChERS, as it was therefore named, was developed by Anastassiades et al. [195] and quickly found popularity as an effective sample preparation technique for pesticide analysis in foodstuffs [196]. The method serves as an alternative to traditional liquid–liquid and solid-phase extraction (SPE) techniques, reducing the number of steps as well as the total extraction time and solvent consumption [197].

The QuEChERS methodology involves two steps. The first step is the extraction of homogenised samples using an organic solvent of choice with a solution of extraction salts, often made up of magnesium sulphate (absorbs water and increases ionic strength), sodium chloride (improves partitioning to organic

TABLE 11 Experimental Conditions Used When Applying USAE to Biomonitors for the Study of Organic Air Pollutants

Biomonitor	Analytes	Sample Mass (g)	Solvent Scheme	Solvent Volume (mL)	Extraction Time (min)	References
Parmelia sulcata	PAHs	0.2	Hexane	2	10	[78]
Evernia prunastri	PAHs	0.2	Hexane	2	10	[10]
P. sulcata	PAHs	0.2	Hexane	2	10	[68]
P. sulcata, E. prunastri, Ramalina farinacea, Pseudevernia furfuracea, Usnea sp. and *Lobaria pulmonaria*	PAHs	0.2	Hexane	2	10	[79]
Xanthoria parietina	PAHs	0.2	Hexane	2	10	[80]
P. furfuracea	PAHs	2	Cyclohexane	30	30	[42]
P. furfuracea	PAHs	2	Cyclohexane	30	30	[84]
Pyxine coralligera	PAHs	2	Cyclohexane: dichloromethane (4:1 v/v)	30	30	[86]
Pinus pinea L.	PAHs	10	Hexane: dichloromethane (1:1)	30	10 (×3)	[126]
P. pinea L.	PAHs	2	Hexane: dichloromethane (1:1)	30	10 (×3)	[119]
P. pinea L.	Pesticides	5	Acetonitrile	30	10 (×3)	[128]
Pinus pinaster	PCBs, BFRs, HCBs	5	Hexane: dichloromethane (1:1)	100	10 (×3)	[127]
P. pinaster	PAHs, SMCs	5	Hexane: dichloromethane (1:1)	100	30	

phase) and buffer salts (trisodium citrate dihydrate and disodium hydrogen citrate sesquihydrate), followed by a centrifugation step. The addition of the extraction salts (Na_2SO_4 or $MgSO_4$) is an exothermic process and therefore increases the temperature of the reaction vessel, improving the extraction efficiency [195]. The second step then involves the cleanup of the supernatant using dispersive SPE (dSPE) which differs from SPE since it does not use a fixed solid bed. The addition of internal standards, buffers or acids can be done in the first step in order to improve the extraction efficiencies and track recoveries. It has been noted that water should be added to the extract, so that the extraction efficiency is improved as a result of the partitioning of polar compounds between the organic and aqueous phases [198,199]. The dSPE step involves the use of a drying salt (magnesium sulphate) as well as SPE sorbent packing materials such as primary−secondary amine (PSA), often supplemented with graphitised carbon black (GCB) or C_{18} material to clean the extracts and remove moisture. The dSPE step also removes sugars, fats, organic acids, proteins, pigments and sterols; thereby simplifying what would be many consecutive cleanup steps into one cleanup procedure. Instrumental analysis using either gas or liquid chromatography (GC or LC) follows, with or without the addition of a pre-concentration step after the cleanup.

Analysis of pesticides in many matrices has been undertaken, and QuEChERS has become a popular choice for extracting PAHs from foodstuffs [200−202]. Although no studies have been published on the use of QuEChERS to extract organic air pollutants from biomonitors, it certainly has potential to do so. Sadowska-Rociek and co-workers have evaluated the QuEChERS methodology for the determination of PAHs in fresh herbs [203]. The study found that acetonitrile gave recoveries between 71.6% and 116.9%. The sample extracts were cleaned up using PSA, GCB and $MgSO_4$, followed by GC-MS analysis. Included in the methodology was a 15 min shaking step, which deviates from the original QuEChERS methodology of extractions between 1 and 5 min [198], but remains, however, a considerably faster extraction technique compared to Soxhlet, USAE and some MAE methods.

In a QuEChERS study by Albinet et al. [198] on particle-bound PAHs in ambient air, analytes were extracted from particles collected onto filters with dichloromethane, acetonitrile or acetonitrile:water (5:2, v/v). The extraction with acetonitrile was the solvent of choice due to instrumental compatibilities; the different solvent schemes showed negligible differences in extraction efficiencies. Although the work by Albinet et al. [198] was not based on biomonitoring, the successful extraction of PAHs suggests that with the optimisation of extraction solvent, agitation time and cleanup methodology, analytes of interest could be extracted from biomonitors using the QuEChERS approach.

8.5 Sample Cleanup

Regardless of the extraction technique used to prepare the biomonitors for analysis, a cleanup step is necessary to remove lipids, proteins, pigments,

chlorophyll, carotenoids and other compounds that may cause matrix effects for organic analyte determinations or cause damage to expensive analytical instrumentation. Clean extracts are imperative to producing reliable qualitative and quantitative results, particularly of compounds present at low levels, as in the case of air pollutants.

The most popular cleanup technique for organic analytes in biological samples remains classical liquid adsorption chromatography [176] in which the prepared extracts are passed through adsorbent columns or solid-phase cartridges (Figure 3). The EPA has approved cleanup methods using silica gel, alumina and florisil (EPA methods 3630C, 3610B and 3620B, respectively) for organic extracts of solid environmental samples, including biomonitors. Ideally, the column material should allow for the selective separation of the target analytes from the matrix background. Additional parameters requiring optimisation are sorbent mesh size and amount (this can vary from small SPE cartridges to large columns filled with activated silica gel). If the biomonitor has a high lipid content, saponification may be necessary using an alkaline solution or a sulphuric acid wash prior to cleanup using a lipid-specific column that would separate the analytes from the lipids in the sample.

Sometimes combinations of adsorbent phases are required in order to efficiently remove unwanted compounds from the extract. In a tobacco study targeted towards OCPs, the cleanup step involved a florisil column cleanup step, followed by silica column chromatography [204]. In a similar manner, florisil cartridges with 5% deactivated alumina were used to purify extracts from grass and various plant species for the study of OCPs [149,171]. It has also been reported that PCBs, PCDD/Fs and polychlorinated naphthalenes (PCNs) were selectively eluted from a series of multilayer silica columns in a study of pine needles [205] and plant extracts containing DDT were passed through a florisil column, after being treated with sulphuric acid [206]. The cleanup of plant extracts for PAH-targeted analysis has also been performed

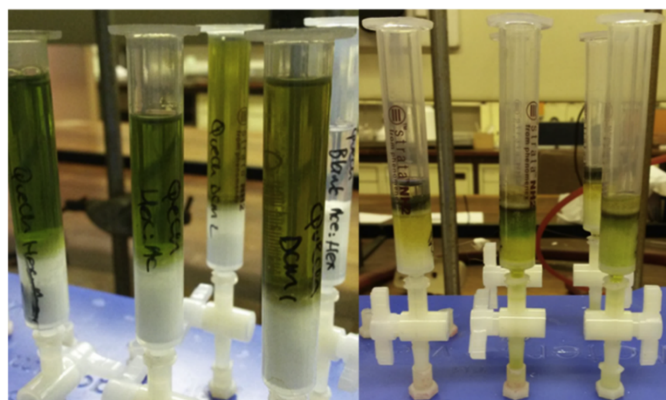

FIGURE 3 Solid-phase extraction of lichen extracts.

using florisil columns [207]. In order to clean up tree leaf extracts for the analysis of OCPs, Barriada-Pereira et al. [208] found that a carbon sorbent cleaned the extracts most selectively and efficiently. In a study on pine needles, Ratola et al. [126] determined that an alumina cartridge cleanup provided the best recoveries (between 72% and 100%) and limits of detection as low as 0.22 ng g^{-1} dw. A gel permeation cleanup procedure has also been developed for PAHs extracted from pine needles [129].

Silica column chromatography has been employed extensively to lichen extracts, with the use of different solvent schemes by different groups [42,70,82]. A direct comparison between the results of these studies is a challenge as a result of the different affinities solvents have for the different PAHs [80], leading to biased elutions. The mass of sorbent is also not clearly defined in most studies, which influences the extent to which the analytes are recovered.

More recently, SPE has been used to clean up lichen extracts targeted towards PAHs with mainly normal phase $-NH_2$ columns being used, after a study by Blasco et al. [78] found that these columns, combined with an elution solvent of hexane:dichloromethane (65:35) yielded the best recoveries. SPE remains a favourable choice in comparison to traditional column chromatography since it uses less solvent, reducing the likelihood of loss of SVOCs since the blow down and pre-concentration steps are shortened.

In terms of the QuEChERS technique, a cleanup step has been worked into the methodology, however, this cleanup step needs to be optimised for each application. In the study on emission samples by Albinet et al., simple filtration by the use of a polyethylene terephthalate syringe filter was considered the ‘cleanup’ step [198]. Sadowska-Rociek et al. [209] tested various sorbent types for the cleanup procedure for tea extracts, including C_{18}, PSA, strong anion exchange (SAX), $-NH_2$ and GCB. PSA is used to remove sugars, fatty acids and sterols, GCB is used to remove pigments (in particular, chlorophyll), C_{18} is used to remove non-polar compounds such as lipids, NH_2 is a weak anion exchanger and thus extracts acids and charged compounds, and finally SAX is a strong anion exchanger, typically used to extract organic acids. Sadowska-Rociek et al. found that SAX was the most appropriate sorbent for the tea samples, whilst the use of GCB resulted in losses of PAHs, in particular the high molecular weight PAHs [209]. This could be as a result of intercalation between the graphite structure and the planar structure of PAHs, thus caution should be exercised when it comes to the use of GCB, depending on the target analytes.

Size exclusion chromatography is primarily used to remove lipids and larger interfering compounds (typically larger than 500 Å). The gels most commonly used are Bio-Beads S-X3 [176]. The main advantage of using gel permeation chromatography over other techniques is that it is entirely non-destructive, unlike some of the liquid adsorption chromatography techniques that use sulphuric acid or alkaline solutions to remove lipids. As with the

liquid adsorption chromatography cleanup technique, the EPA has established a gel permeation chromatography cleanup method for organic extracts obtained from environmental samples (EPA Method 3640A GPC Cleanup). Hubert et al. [129] used size exclusion chromatography to clean up pine needles, mosses and tree leaves. An S-X3 Bio-Beads column was used for the separation of PAHs in spruce needle extracts, using chloroform as the mobile phase [210]; for the cleanup of pasture vegetation extracts targeted towards the analysis of PAHs [211]; as well as the analysis of PCDD/Fs in lichens [43,88]. Often size exclusion techniques are followed by liquid adsorption chromatography, since not all the interferences are removed as a result of the complex matrix of biomonitors.

9. ANALYTICAL TECHNIQUES

After target analytes have been extracted from biomonitors and the extract cleaned up, if necessary, it may then be pre-concentrated by evaporation procedures prior to analysis. An important consideration in biomonitoring studies is the use of internal standards (including deuterated standards of the analytes of interest), which may be added to the sample at various stages of the analysis in order to determine extraction recoveries and analytical efficiencies. Certified reference materials for some target analytes and some biomonitor species are commercially available, and these are valuable in method development and validation. The analytical techniques most commonly utilised in biomonitor studies have been included in many of the tables provided in this chapter. They are briefly discussed here, however, more detail on these techniques is provided in the chapters of Section III of this book.

9.1 Organic Analytes

GC and LC are common techniques used to analyse biomonitor extracts for organic air pollutants derived from a range of sample preparation methods. As an example, the EPA PAH-related methods all recommend the use of either GC or LC in the analysis of sample extracts from a range of matrices including ambient air and foodstuffs [212].

When analysing lichen extracts for PCDD/Fs and PAHs, the main analytical techniques used are high performance liquid chromatography (HPLC) (using reverse phase C_{18} columns) and GC, using a variety of column configurations with column lengths ranging from 15 to 60 m. GC-MS and LC with fluorescence detection both have their advantages. If a particular PAH is of concern, such as benzo[*a*]pyrene or anthracene, the sensitivity and selectivity of using LC with fluorescence detection will be far greater at trace levels, due to the highly fluorescent characteristics of these PAHs [212]. On the other hand, benzo[*ghi*]perylene has poor fluorescence sensitivity and is therefore better suited to analysis using GC-MS [212]. Sacrificing peak separation, particularly of the benzo fluoranthenes, as well as benzo[*a*]anthracene and

chrysene, for the sake of faster run times should be avoided to ensure an accurate representation of the PAH profile, owing to the different toxicities of PAH compounds. In general when deciding which method should be applied to a sample, a number of criteria guide the decision including:

- The type of matrix and analytes of interest.
  If the matrix is complex with a high level of interferences, GC-MS is a more appropriate technique due to the enhanced separation achievable using GC techniques [213]. Depending on how efficient the cleanup procedure is, it is likely that the resolving power of GC-MS would be advantageous over LC techniques, as long as the target analytes are amenable to GC analysis.
- The required sensitivity.
  Both GC-MS and HPLC coupled to a fluorescence detector (FLD) are known to be very sensitive, for example, detection limits as low as 0.1 ng g^{-1} dw for HPLC-FLD [214]. Other LC detectors such as the diode-array detector may not have the desired sensitivity for the analytes concerned. Comprehensive two dimensional GC—time-of-flight mass spectrometry (GCxGC-TOFMS) for environmental sample analysis allows full scan data at very fast scan rates, as well as enhanced peak capacity and reduced noise, leading to improved sensitivity [215—217].

Examples of analytical techniques for various biomonitors used for organic air pollutant monitoring are given in Table 12.

TABLE 12 **Common Analytical Techniques Used to Analyse Extracts from Biomonitors Used for the Monitoring of Organic Air Pollutants**

Biomonitor	Analytes	Instrument	References
Wild chard	PAHs	GC-FID	[218]
Pine needles	PAHs	GC-MS	[119,126]
Lichen	PAHs	HPLC-UV-V	[81]
Lichen	PAHs	HPLC-DAD-V-UV and FLD	[30]
Moss	PAHs and NPAHs	APGC-MS/Q-TOF	[107]
Lichen	PCDD/Fs	GC-HRMS	[43,85]
Lichen	PCDD/Fs	HRGC/HRMS	[63,70]
Lichen	PCBs	GC-HRMS	[85]
Pine needles	Pesticides	GC-ECD	[171]

APGC-MS/Q-TOF, atmospheric pressure gas chromatography coupled to a quadrupole hyphenated to a time-of-flight mass spectrometer; GC-HRMS: gas chromatography - high-resolution mass spectrometry; HRGC/HRMS, high resolution gas chromatography/high resolution mass spectrometry; GC-ECD, gas chromatography-electron capture detector.

9.2 Inorganic Analytes

ICP-OES and ICP-MS are the most common techniques used in the analysis of micro- and macro-elements from plant biomonitoring studies, as they are capable of high sample throughput and multi-element determinations, and they have large dynamic ranges [219]. Choice of nebuliser and spray chamber needs to be determined for each specific matrix, and argon humidifiers may be used in samples where salt concentrations are high. Standards and samples need to be closely matrix-matched as any deviations may result in signal drift [220].

ICP-MS is able to analyse concentrations of analytes in the ppb to ppt range, which is important when monitoring elements which may have environmental impacts at very low levels, such as Pb and Cd. This technique is also able to analyse isotopes and thus can be used in source appointment studies. ICP-MS is, however, not as robust as ICP-OES, and problems are experienced with samples having high total dissolved solids (TDS) [220]. The tolerance of ICP-MS for TDS is typically within the range of 0.1−0.2%, and samples are usually filtered prior to analysis. Additionally, high concentrations of acids in samples have a negative effect on the lifetime of the sampling cones [221], therefore when using HF for sample digestion, platinum cones should be used. Analysis of samples containing high concentrations of carbon and matrix ions may result in deposits forming on the cones leading to signal drift. Several techniques are employed to reduce this, such as sample dilution, the use of ion-exchange columns, and the introduction of oxygen to burn off excess carbon. Chloride ions which are present in HCl matrices are not only extremely corrosive but also pose a problem in the analysis of low concentrations of As, as $^{40}Ar^{35}Cl$ may form in the plasma which has the same nominal mass as ^{75}As. Unfortunately As is monoisotopic, and so the use of an alternative isotope is not an option. Correction equations, collision or reaction gases may therefore be applied to reduce or remove the interference.

10. CONCLUSIONS, CHALLENGES AND FUTURE OUTLOOK

One of the biggest challenges in biomonitoring of air pollution is that the measured concentrations are not necessarily fully reflective of ambient conditions. Real-time monitoring has advantage in this context; however it may incur significant costs.

The advantages of using biomonitors are a result of their ability to accumulate compounds and elements above their essential requirements. The application of biomonitors to monitor air pollution is favourable as a result of the low cost involved and ease of sampling since no bulky equipment is required. They also provide an integrated pollution profile over an extended

period of time and allow for air pollutant mapping of large geographical regions. These features suggest that biomonitoring will continue to be applied in air pollution studies for the foreseeable future. The limitations of the use of biomonitors include that the comparison of results from different types of biomonitors cannot be made as a result of morphological and ecophysiological differences, resulting in different pollutant profiles being detected. There is, however, value in using the results in a complimentary way in order to obtain a comprehensive understanding of regional air pollutant profiles.

In order to establish the monitoring of air pollutants using biomonitors, an understanding of the uptake and release mechanisms of each individual subspecies is required. This is a result of various studies highlighting differences in accumulation rates, such as the study on PAHs in lichens by Blasco et al. [78]. In this context, plant species such as mosses and lichens may require expert identification in biomonitoring studies [98]. Furthermore, dose–response relationships need to be well defined so that the accumulation, release and preservation of pollutants within the organism and the effects thereof on the organisms can be better understood.

Biomonitoring has been successfully used to monitor the spatial behaviour of pollutants and to assess the variation in pollutant levels with changes in land use, along with the potential human and environmental health risks related to this. In order to meaningfully contribute towards risk assessment and management, the impact of various emission sources needs to be understood. Studies such as Augusto et al. [74], which correlate the PAH values found in lichens with atmospheric data, are important in this regard although biological processes in plants employed as biomonitors may complicate such studies [114]. One of the analytical challenges with biomonitoring studies is matrix effects due to chlorophyll and other biological compounds present in the sample extract, which require effective cleanup prior to analysis in the case of organic target analytes.

An area in which biomonitors show promise is in source apportionment of air pollutants. Caution must be exercised when applying any pollutant-specific diagnostic ratios to the results of biomonitoring studies, however, since photodegradation, varying analyte stabilities and differing sensitivities of biomonitors to analytes may impact on the results. Phase transport and degradation also need to be considered, since conservation of the pollutant from the source to the sampling point needs to be demonstrated in order to use existing diagnostic ratios [222]. Therefore, the development of new source diagnostic ratios with conditions specific to biomonitors would be beneficial. Multi-component studies that include trace metals and a range of organic air pollutants would also further develop the understanding of source apportionment in this field. Finally, standardisation in the methods and approaches employed with respect to biomonitoring studies is highly desirable.

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Appendix C

Poster presented at Analitika 2014, Sep 7-11, Parys, South Africa.

Preliminary investigation into the use of lichens as biomonitors of air pollution in South Africa

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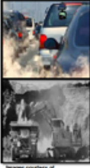
Introduction

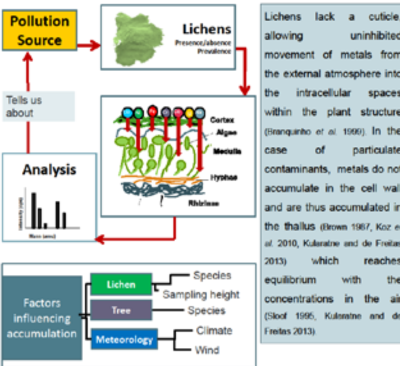


Air pollution is known to adversely affect human health. It is therefore imperative to monitor the presence of pollutants, including heavy metals, in air resulting from motor emissions (Giordano et al. 2010), consumption of fossil fuels, industrial processes and mining, for example.

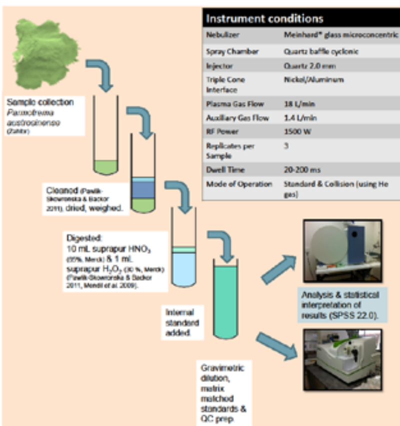
Determination of air pollution is usually done through the use of theoretical dispersion models and *in situ* measurements of air pollutants (Sloof 1995), the latter often requiring the use of expensive equipment for collection. Economical options have been explored for this type of monitoring, where biomonitors, such as lichens, are deemed as valuable alternatives, and have been shown to accurately reflect environmental concentrations (Branquinho et al. 1999, Pawlik-Skowrońska and Bačkor 2011, Demiray et al. 2012, Koz et al. 2010, Kularatne and de Freitas 2013, Jeran et al. 2007, Forbes et al. 2009).

In this study we aim to investigate the accumulation of metals in lichens at two sites of varying degrees of potential environmental impacts and to determine the suitability of lichens as biomonitors of air pollution in South Africa. Methodology considerations will also be assessed.

Principle



Methodology



Instrument conditions	
Nebulizer	Meinhard® glass microconcentric
Spray Chamber	Quartz baffle cyclonic
Injector	Quartz 2.0 mm
Triple Cone Interface	Nickel/Aluminum
Plasma Gas Flow	18 L/min
Auxiliary Gas Flow	1.4 L/min
RF Power	1500 W
Replicates per Sample	3
Dwell Time	20-200 ms
Mode of Operation	Standard & Collision (using He gas)

Results

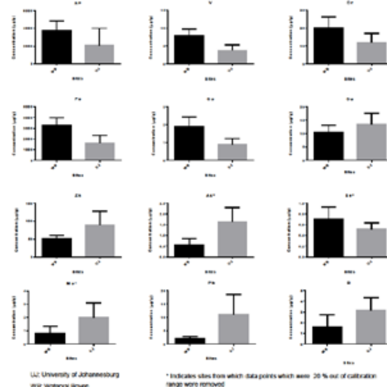


Fig 1. Concentrations of metals which showed significant differences between sites.

As shown in Figure 1 above, Cu, Zn, As, Mo, Pb and B were found to be significantly ($P < 0.05$) higher in the University of Johannesburg site in comparison to the Waterval Boven site, whilst V, Cr, Fe, Co and Se were found to be significantly ($P < 0.05$) higher in the Waterval Boven site.

QA & QC

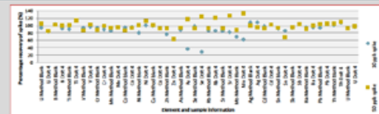


Fig. 2 Spike recoveries (%) of simple (spiked blank) & complex matrices

Spike recoveries were within an acceptable range (USEPA 1987) for most elements (Fig. 2), although the recoveries for the 10 ppb spike on As, Se and Mo for the more complex matrix (Dogfish Liver; DOLT 4) were poor and higher than expected recoveries were found for As, Se, Rb, Sr and Mo on the 50 ppb spike of the DOLT 4 CRM. The observed enhancement of Se is thought to be attributed to interference by Ca, Ar and Br.

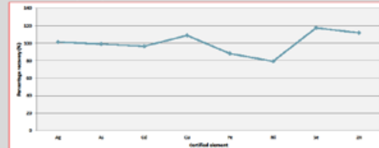


Fig 3. DOLT 4 recoveries (%)

All CRM recoveries were between 90-110 % with the exception of Ni which was poorly recovered at 79 % and Se which was recovered at 117 % (Fig 3.).

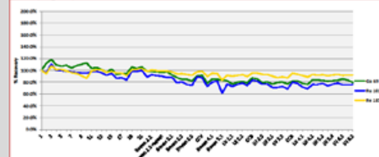


Fig. 4 Internal standard recoveries (%) during sample run

All internal standard recoveries were within an acceptable range of between 60-125% (Fig. 4) of the original blank response reading (USEPA 1992) although long term drift was observed during the sample run. Continuing calibration verification standards (CCVs) were included to account for the observed decline.

Discussion

Waterval Boven (also known as Emgwenya) is situated in Mpumalanga on the edge of the escarpment. Surrounding areas between Machododorp and Carolina contain a quarry and three mines (McCarthy and Humphries 2012, Cockburn 2013). In areas where mining takes place, stock piles are often susceptible to drying, and thus metals present in the fine, mined dust can be dispersed by wind (Branquinho et al. 1999, Sloof 1995) and taken up by lichens. Metals released as emissions in industrial and mining processes are also dispersed and taken up in this manner. Considering these mines focus on the abstraction of Ni, Cu, Co, PGMs, coal (McCarthy and Humphries 2012), ferrochrome and Cr (Cockburn 2013), this may explain the significantly higher concentrations of Al, V, Cr, Fe, Co and Se found in lichens from this site.

Significantly higher concentrations of Cu, Zn, Pb, As, Mo and B in lichens from the University of Johannesburg site could originate from the high population densities, high traffic density, smelting, coal mining, scrap production (Cu; USEPA 1985), mine dumps, coal fly ash (Zn; ATSDR 2011), iron and steel production (Zn; ATSDR 2011), gold mining activities, crematoriums, medical waste incineration (As; USEPA 1998), combustion of fossil fuels (Mo; TDSHS 2012) and gas works, all of which are within close proximity to the site.

Conclusion

The findings from this study appear to support the use of lichens as sensitive, cheaper alternatives for monitoring the pollution of air by heavy metals in South Africa, where levels of metals in lichens appeared to correlate with metals mined or emitted near the site.

Recommendations and future work include:

- the pooling of samples per tree in order to ensure homogeneity,
- the determination of bark and soil sample concentrations to support the hypothesis that lichens gain all of their nutrients from the atmosphere alone,
- assessment of the consistency of these findings over a larger sampling area within the sites, and
- the determination the effect of seasonal variations on bioaccumulation.

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Appendix D

Poster presented at the Canadian Society for Chemistry Conference, 28 May-1 June 2017, Toronto, Canada.

Sample preparation strategies for the determination of metal(loid) fractionation patterns in lichen air biomonitors

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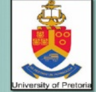


Introduction

Lichens have been used extensively in air pollution biomonitoring initiatives, where they are considered to be cheap and effective alternatives to air filter monitoring [1, 2]. Their use, however, does pose some complex challenges, as sample pretreatment, preparation and storage conditions have not been standardized (Fig 1). This is especially problematic in studies which aim to assess the bioavailability of metals since these conditions can affect the chemical species, as has been found in sediments [3] and may also have an impact on the role that plants play in the biotransformation of metals [4]. Furthermore, the existing studies of metals in lichens have focused predominantly on the evaluation of total metals, or the intracellular and extracellular metals [5,6,7,8]. As far as we are aware, there are currently no studies which have evaluated the bioavailability of these metals.

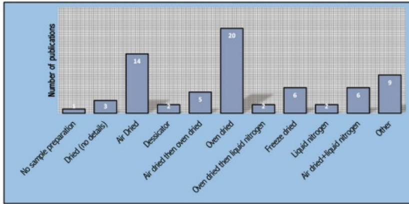
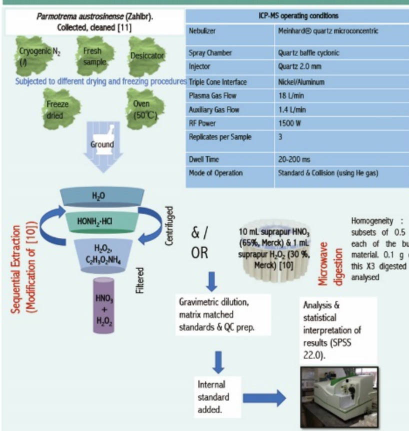


Figure 1 Number of publications for different sample fixation strategies published between 2000-2016 according to Science Direct, Springer Link, RCS, Taylor & Francis online, ACS using the search term "metals in lichen", limited to results from the first 4 pages.

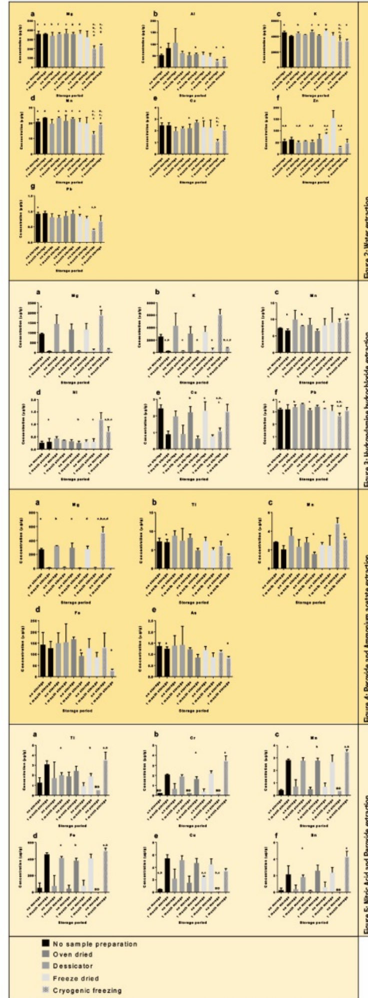
Lichens gain all of their nutrients from metals adhering to dust particles, dissolved metals in rainwater and the diffusion of gases [9]. Therefore, a sequential extraction developed for use in street dusts as a monitor for air pollution [10] may be useful to evaluate the bioavailability of metals in both the surface dusts on lichens and that which has been assimilated by the lichen.

Methodology



ICP-MS operating conditions	
Nebulizer	Meinhard® quartz microconcentric
Spray Chamber	Quartz baffle cyclonic
Injector	Quartz 2.0 mm
Triple Cone Interface	Nickel/Aluminum
Plasma Gas Flow	18 L/min
Auxiliary Gas Flow	1.4 L/min
RF Power	1500 W
Replicates per Sample	3
Dwell Time	20-200 ms
Mode of Operation	Standard & Collision (using He gas)

Results



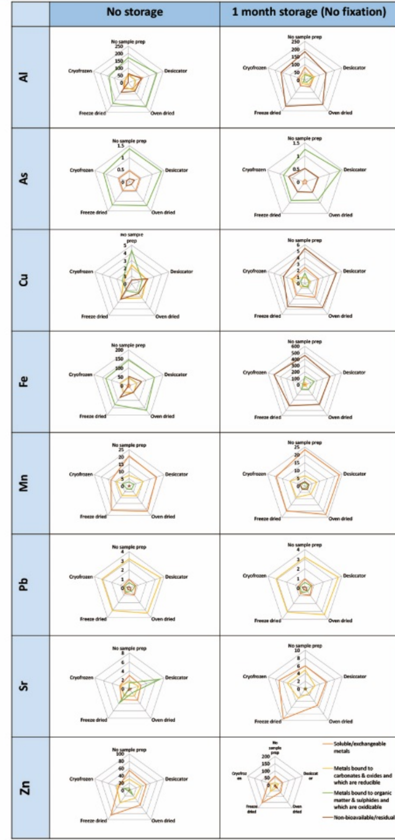


Figure 6: Average concentrations (blank subtraction values) of selected metal(loid)s in the bulk lichen sample between different extraction media and sample preparation strategies. Bulk material was stored for a period of one month in a fridge at 4 °C without fixation. Values reflected are in µg/g dry weight and only results which has a statistically significant difference ($P < 0.05$) are shown here.

Discussion

Homogeneity (results not shown)

The bulk sample was analysed using ICP-MS and was determined to be adequately homogenous (< 5 % RSD) for Li, Mg, Al, K, Sc, V, Cr, Mn, Fe, Co, Cu, Zn, Sn and Pb but not for Na, Ca, Ti, Ni, As, Sr, Se and U.

Quality Control and Quality Assurance

Internal standards (Ga, Rh, Re) were found to be within an acceptable range (70-130 %) as specified by the USEPA [12]. An initial calibration verification standard (ICV) was run prior to analysis to verify that the calibration was valid against an external standard which was prepared by a different scientist. A continuing calibration blank (CCB), and lower and higher concentration continuing calibration verification standards (CCVs) were run after every 10 samples and the method recalibrated every 20 samples. A certified reference material (CRM) of tea leaves (INCT-TL-1) was analysed, where analytes Na, Mg, K, Ca, Sc, V, Cr, Mn, Co, Ni, Cu, Sr and Pb were found to be within the 95 % confidence interval of the certified value. Al, Zn, As and Cd were found to be outside these limits where the 127 % recovery of Al was likely to be due to the measured concentration being more than 10 % higher than the last calibration standard. Zn recoveries of 198 % could be indicative of Zn contamination and As and Cd concentrations in the CRM were below the method detection limits (MDLs).

Combined Extraction Efficiencies (results not shown)

Combined extraction efficiencies were good for As, Cu, Mn, Pb, Sr and Zn (>82 %) but poor for other elements, where the best recoveries were found in samples that were dried in a desiccator and fresh samples. Although these recoveries are low, they are higher than that which has been reported in other speciation studies such as 2-9.3 % found in a sequential extraction of the foliose lichen *Parmelia sulcata* performed by Farinha *et al.* [13]. Losses may be explained through adhesion of metals to the walls of the vessels used during the extraction steps and losses of sample via decanting of the supernatant. In some cases the concentration for an analyte was below the MDL which lead to readings with a low bias. Furthermore, poor interaction of the sample with the extraction matrix due to the hydrophobic nature of plants [14] could also contribute to poor extraction recoveries when compared to the total extractable metal as was achieved during microwave digestion.

Effect of Sample Preparation Strategy

As can be seen in Figure 2, the cryo-frozen samples had statistically lower ($P < 0.05$) recoveries of Mg, Al, K, Mn, Cu, Zn and Pb than any other sample preparation technique.

Furthermore, Figures 3,4 and 5 show statistically significant differences for cryo-frozen samples in most cases which indicates that this method is not suitable for the sequential extraction of metals from plant materials and in determining the metal species present. This contrasts to opinions of Krämer *et al.* [15] and Laurette *et al.* [16] who recommend cryo-fixation for speciation analyses. A possible explanation for this observation may be the pronounced poor interaction between the sample and the extraction matrix in comparison to the other sample preparation techniques, however this would need to be confirmed through further experimentation. Significantly higher concentrations of Zn in non-stored, freeze-dried samples in the water extraction step may be due to the variance of 4.8 % in the homogeneity of this element in the bulk material, or the action of vacuum on the sample, resulting on the Zn being drawn into the intracellular spaces for easy extraction [17].

Although lower concentrations of many elements were observed in the water extraction step for cryo-frozen samples, these were largely recovered in the subsequent extraction steps, where this was statistically significant for Mg (3a, 4a), K (3b), Mn (3c, 4c, 5c), Ni (3d), and Cu (3e).

Stability over 1 month without fixation and sample preparation techniques

As can be seen in Figure 6, many elements were found to biotransform over a period of a month without fixation. Sample preparation techniques caused quite a large degree of variance in Cu concentrations in the different extraction media, where Cu was noted to transform mostly into the non-bioavailable form over a period of a month, which may indicate a detoxification mechanism of the lichen through sequesteration of Cu from the thallus into insoluble forms on the lichen surface. Storage over a period of a month without fixation may be suitable for some elements such as Zn and Sr. Although Zn exhibited changes in the average concentrations of the non-bioavailable and oxidizable fractions, this was not statistically significant. Cryogenic freezing of samples showed statistically significant differences over the two months and thus is not recommended for long-term storage.

Conclusion

Sequential extractions can provide useful information about the bioavailability of metals, however sample preparation strategy can have a significant effect on both the partitioning of metals and long-term stability of chemical species within the lichen sample. Furthermore, it is necessary to be aware that sequential extractions provide incomplete information when it comes to chemical species, as co-extraction of these species is common.

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PLEASE NOTE THAT THE DATA SHOWN HERE HAS BEEN SUBMITTED FOR PUBLICATION

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