Lichen biomonitors: factors affecting response behaviour

Rita Mendes Godinho

DEPARTMENT OF RADIATION, RADIONUCLIDES & REACTORS

Invitation

To attend the defence of the
Ph.D. thesis:

Lichen Biomonitors: Factors Affecting Response Behaviour

of

Rita Mendes Godinho

on

Wednesday 17 February 2010
at 10.00 hours

in the Senaatszaal of the Auditorium
of the Delft University of Technology,
Mekelweg 5, Delft

You are welcome to the gathering
that will take place after
the thesis defence

Stellingen, behorende bij het proefschrift
"Lichen biomonitors: factors affecting response behaviour"
door Rita Mendes Godinho, 17 februari 2010

1. De bepaling van de concentratie van elementen in een korstmos aan het einde van een blootstelling gedurende een bepaalde periode leidt tot een tijdsbeeld dat zowel kan overeenstemmen maar ook een onderschatting of overschatting kan geven van hoe de korstmos in die betreffende periode de beschikbaarheid van elementen uit de omgeving weergeeft.

   R.M. Godinho, H.Th. Wolterbeek, T. Verburg, M.C. Freitas. Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. Environmental Pollution 151 (2008), 318-325

2. De wijze van opname door een korstmos zou gezien kunnen worden als verband houdend met fysiologie van de korstmos maar ook, en wellicht is dat zelfs beter, als een gevolg van de condities van het milieu in de omgeving ervan.

   R.M. Godinho, H.Th. Wolterbeek, T. Verburg, M.C. Freitas. Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. Environmental Pollution 151 (2008), 318-325

3. De keuze van een onderdeel van de korstmos voor monitoring zou kunnen afhangen van het element waar de belangstelling naar uitgaat, en er kan daardoor geen algemene richtlijn gegeven worden voor de aanpak van het onderzoek.

   R.M. Godinho, T.G. Verburg, M.C. Freitas, H.Th. Wolterbeek. Accumulation of trace elements in the peripheral and central parts of two species of epiphytic lichens transplanted to a polluted site in Portugal. Environmental Pollution 157 (2009), 102–109.

4. Transplanten hebben als voordeel dat het materiaal waarmee onderzoek wordt gedaan gestandaardiseerd is. Dat rechtvaardigt de voorkeur ervoor boven het gebruik van materiaal dat van nature aanwezig is.

   Dit proefschrift, Hoofdstuk 6

5. Een gemakkelijker manier van monitoring wordt verkregen door levende bio-materialen te vervangen door dode, waardoor verstoringen ten gevolge van het metabolisme worden omzeild en een direct vergelijk tussen de resultaten van onderzoeken mogelijk wordt.

   Adamo, P., Crisafulli, P., Giordano, S., Minganti, V., Modenesi, P., Monaci, F. et al. Lichen and moss bags as monitoring devices in urban areas. Part II: Trace element

content in living and dead biomonitors and comparison with synthetic materials. Environmental Pollution *146 (2007), 392-399.*

Giordano, S., Adamo, P., Monaci, F., Pittao, E., Tretiach, M., Bargagli, R. Bags with oven-dried moss for the active monitoring of airborne trace elements in urban areas. Environmental Pollution 157 (2009), 2798-2805.

6. Gezondheid versus biomonitoring: Biomonitoring biedt de mogelijkheid om een breed scala van verontreinigende stoffen te meten, waardoor de gelegenheid wordt geboden om te onderzoeken of specifieke verontreinigende stoffen de oorzaak zouden kunnen zijn voor gezondheidsgerelateerde effecten die bij luchtverontreinigings studies worden waargenomen. De gepubliceerde gegevens wijzen erop dat studies naar de correlaties tussen de luchtverontreiniging door metalen, zoals bepaald door biomonitoring, en (epidemiologische) informatie over gezondheid van waarde kunnen zijn om de aandacht te richten op specifieke zaken, en om verder onderzoek te richten naar mogelijke dosis-respons mechanismen in de epidemiologie van metalen in de lucht.

   Sarmento, S.F.M., Wolterbeek, H.T. , Verburg, T.G. Freitas, M.C. Correlating element atmospheric deposition and cancer mortality in Portugal: Data handling and preliminary results. Environmental pollution, 151 (2008), 341-351. Wolterbeek, H.T. , Verburg, T.G. Atmospheric metal deposition in a moss data correlation study with mortality and disease in the Netherlands. Science of the total environment 319 (2004), 53-64.

7. Reizen verbetert je wetenschap.

   Watson, J. D.; in Avoid Boring People: Lessons from a life in science. New York, Knopf (2007)

8. Creativiteit (waartoe behoort het vermogen om niet alleen problemen te erkennen en oplossingen ervoor te vinden, maar ook om problemen te ontdekken en originele oplossingen te vinden) is een essentiële eigenschap voor een succesvolle toekomstige vakman, en behoort daarom te worden onderwezen op school. "Voorstellingsvermogen is belangrijker dan kennis".

   The Expanded Quotable Einstein, Collected and edited by Alice Calaprice, published by Princeton University Press.

9. Duurzame ontwikkeling steunt op het vaststellen van de ecologische prijs van goederen en diensten. Een evaluatie van die prijs zou daarom prioriteit moeten hebben binnen de ecologische wetenschap en economie.

10. Bio-brandstof, een oplossing of de start van een nieuw probleem ?

Deze stellingen worden opponeerbaar en verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotor, Prof. Dr. H.Th.Wolterbeek.

Propositions accompanying the thesis
"Lichen biomonitors: factors affecting response behaviour"
Rita Mendes Godinho

1. Measuring the lichen elemental concentration at the end of an exposure experiment of a specific duration sets a time window that can be either fitting, or too small or too large for the lichen to reflect the selected period of environmental availability.

   R.M. Godinho, H.Th. Wolterbeek, T. Verburg, M.C. Freitas. Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. Environmental Pollution 151 (2008), 318-325

2. Lichen accumulation performance should be regarded as related to lichen physiology, but as a result or maybe better expressed, as a consequence, also to environmental surrounding conditions.

   R.M. Godinho, H.Th. Wolterbeek, T. Verburg, M.C. Freitas. Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. Environmental Pollution 151 (2008), 318-325

3. Tissue selection in lichen monitoring may depend on the element of interest, and cannot be made into a generalized approach in survey set-ups.

   R.M. Godinho, T.G. Verburg, M.C. Freitas, H.Th. Wolterbeek. Accumulation of trace elements in the peripheral and central parts of two species of epiphytic lichens transplanted to a polluted site in Portugal. Environmental Pollution 157 (2009), 102–109.

4. The advantage of transplants in permitting the standardization of the experiment material justifies its preferential use over the use of native material.

   This thesis, chapter 6

5. Replacing living with devitalized bio-materials, avoiding interference due to metabolism thereby allowing more direct comparison between the results of the surveys is a more convenient way of monitoring.

   Adamo, P., Crisafulli, P., Giordano, S., Minganti, V., Modenesi, P., Monaci, F. et al. Lichen and moss bags as monitoring devices in urban areas. Part II: Trace element content in living and dead biomonitors and comparison with synthetic materials. Environmental Pollution 146 (2007), 392-399.
   Giordano, S., Adamo, P., Monaci, F., Pittao, E., Tretiach, M., Bargagli, R. Bags with oven-dried moss for the active monitoring of airborne trace elements in urban areas. Environmental Pollution 157 (2009), 2798-2805.

6. Health vs biomonitoring: The possibility offered by biomonitoring methods of measurement of a wide range of pollutants offers the opportunity to investigate whether specific pollutants might be responsible for the health effects observed in air pollution studies. The presented data suggest that correlation studies between biomonitoring data on metal air pollution and (epidemiological) health data may prove valuable in turning attention to specific issues and in directing further study into possible dose-response mechanisms in air-associated metal epidemiology.

   Sarmento, S.F.M., Wolterbeek, H.T. , Verburg, T.G. Freitas, M.C. Correlating element atmospheric deposition and cancer mortality in Portugal: Data handling and preliminary results. Environmental pollution, 151 (2008), 341-351. Wolterbeek, H.T. Verburg, T.G. Atmospheric metal deposition in a moss data correlation study with mortality and disease in the Netherlands. Science of the total environment 319 (2004), 53-64.

7. Travel makes your science better.

   Watson, J. D.; in Avoid Boring People: Lessons from a life in science. New York, Knopf (2007)

8. Creativity (encompasses the ability to not just recognize and find solutions to problems, but also to discover the problems and find original solutions) is an essential characteristic to the future successful professional and therefore it must be learned at school. "Imagination is more important than knowledge."

   The Expanded Quotable Einstein, Collected and edited by Alice Calaprice, published by Princeton University Press.

9. Sustainable developments rely on setting the ecological price of goods and services. Evaluation of that price should be a priority for ecological science and economy.

10. Biofuel, solution or the rising of a new problem?

These propositions are considered defendable and as such have been approved by the supervisor Prof.dr. H.Th. Wolterbeek.

Lichen biomonitors: factors affecting response behaviour

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Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus, Prof.ir. K.C.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen
op woensdag 17 februari 2010 om 10.00 uur

door

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Dit proefschrift is goedgekeurd door de promotor:
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Samenstelling promotiecommissie:

Rector Magnificus, voorzitter
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This thesis was developed with the financial support of the Portuguese Foundation for Science and Technology.

ISBN 978-1-60750-482-5

Keywords: Biomonitoring, Lichen, Trace Elements, Dynamics, Air Pollution

Published and distributed by IOS Press under the imprint Delft University Press

Publisher
IOS Press
Nieuwe Hemweg 6b
1013 BG Amsterdam
The Netherlands
tel: +31-20-688 3355
fax: +31-20-687 0019
email: info@iospress.nl
www.iospress.nl
www.dupress.nl

PRINTED IN THE NETHERLANDS
The research described in this thesis was performed at the Reactor department of the Nuclear and Technological Institute, EN 10, Apartado 21, 2686-953 Sacavém, Portugal and Section of Radiation and Isotopes for Health of the Department of Radiation, Radionuclides and Reactors, Faculty of Applied Sciences, Delft University of Technology, Mekelweg 15, 2629 JB Delft, The Netherlands.

Contents

Chapter 1

GENERAL INTRODUCTION

1.1. Biomonitoring

Longest standing attention for widespread air pollution stems from our concern over its ecological effects. In addition to the ongoing concern for ecosystem performance as such, attention has been and becomes increasingly more directly focused on human health. An increasing body of epidemiological data systematically demonstrates the adverse effect of air particulate matter on human health (see e.g. Dockery and Pope, 1994; Beeson et al., 1998; Laden et al., 1999, 2000; Schwartz et al., 1996).

Monitoring anthropogenic air pollution is a complex problem because of the high number of potentially dangerous substances, the difficulty of estimating their synergistic or antagonistic effects, the large spatial and temporal variation of pollution phenomena, the high costs of recording instruments, and hence, the low sampling density in a purely instrumental approach (Nimis et al., 2000; Wolterbeek, 2002).

The necessary information on air pollutants can be obtained by dispersion modelling (source-orientation, a priori known emission sources) and by field measurements of the immission (receptor/effect orientation).

Immission measurements, however, should be regarded as necessary and indispensable: they validate dispersion models and they indicate the presence of unknown/unregistered sources. In order to ensure the temporal and spatial representativeness of in-field measurements, sampling is required on a long-term basis, and at a large number of sites (Slanina et al., 1990).

It is here that biomonitoring comes in.

Biomonitoring is commonly defined as the use of organisms and biomaterials to obtain quantitative information on certain characteristics of the biosphere. The use of bioindicators in field studies has the great advantage of the permanent and common occurrence of the selected organism in the field, and ease of sampling, permitting long-term monitoring of large-scaled multiple-sites programs without widespread establishment and maintenance of sophisticated and costly equipment (Beeby, 2001).

The use of living organisms in the study of environmental quality is now widely accepted in many countries, and corroborative information obtained from biomonitoring surveys to support the limited data derived from physico-chemical measurements is strongly recommended. Recent studies addressed the correlations between biomonitor concentrations and epidemiological data on health and mortality (Wappelhorst et al., 2000; Wolterbeek and Verburg, 2004). Overall, these data suggested that correlation studies between biomonitoring data on metal air pollution and (epidemiological) health data may prove valuable in tuning attention to specific metal health issues and in directing further study into possible dose–response mechanisms in air-associated metal epidemiology.

1.2. Lichens

It may be clear that the framework discussed above implies that biomonitoring data, and, hence, biomonitoring approaches should be of the highest possible quality. Questions needing ongoing attention comprise: Which bio-organisms should be selected, which specific needs should they meet? Which survey protocols and analytical techniques should be used or developed? How does the bio-organism reflect the polluting compound of interest: what are the characteristics of the 'dose-response' relationships, and what is the extent of possible interferences with the monitor's physiology? What do we know about the elements or compounds of interest, what do we know about possible sources and source profiles, and how do the biomonitor data relate to existing data on environmental and human health? (Wolterbeek and Freitas, 2002)

Of all biological species used in biomonitoring air pollution, lichens and mosses have the most common occurrence. They are highly dependent on the atmosphere for nutrients and are lacking a waxy cuticule and stomata allowing many contaminants to be absorbed over the whole thallus surface being bio-concentrators. The morphology of lichens and mosses does not vary with seasons; thus accumulation can occur throughout the year. Lichens and mosses usually have considerable longevity, and are present both in remote areas and in areas near pollution sources.

Lichens are one of the most valuable biomonitors of atmospheric pollution: they can be used as 1) sensitive indicators to estimate biological effects, by measuring changes at the community or pollution levels (the air pollution is indexed by geographical variances in biodiversity and biomonitor's species

richness). The variety of responses of different species towards pollution permits detailed patterns to be obtained even at low levels of pollution. 2) Cumulative monitors of persistent pollutants by assaying trace element content.

1.3. Dose-response relationships

Analysing the elemental content of naturally occurring lichens or those transplanted into a polluted area is a most useful way to monitor the sphere of influence of a pollution source or the effectiveness of any pollution control measures (Garty, 2001). Mapping regional distribution patterns of elemental concentrations in biomonitor materials has been extensively used (e.g. Sloof, 1993; Steinnes et al., 1992; Freitas et al., 1997; Bennett and Wetmore, 2003; Harmens et al., 2008). Estimating rates or time-integrated deposition values from these data is much more difficult. Averaged or integrated elemental contents of filter-trapped air particulate materials (APM) or atmospheric (total) deposition data were compared with biomonitor's averaged or integrated metal concentrations (Sloof, 1993; Berg et al. 1995; Jeran et al., 2000; Bari et al., 2001) the biomonitor being traditionally regarded as a passive accumulator reflecting a long-term integration of atmospheric pollution (Berg et al., 1995; Berg and Steinnes, 1997; Fernandez et al., 2000; Bennett and Wetmore, 2003; Tabors et al., 2004). Although, reports exist of rapid equilibration of lichen contents to new environmental levels (Sloof and Wolterbeek, 1992; Garty, 1993; Loppi and Pirintsos, 2003), Bennet et al. (1996) reports that changes in element

concentrations do not appear to be related with time. Moreover, Ayrault et al. (2007) suggested that while in heavily polluted sites constant accumulation rates can be observed, in sites proximate to diffuse pollution sources discontinuous trends are recorded.

The calibration of the biomonitor's elemental content to atmospheric element dispersion or deposition asks for a clear understanding of bioconcentration and biomagnification, if possible predicting its extent beyond the few measurements done in verification. This knowledge is essential to interpret the monitoring data, and may permit the modelling of observed phenomena ultimately allowing for predictions to be made.

Using biomonitoring as a means to get insight in trace element concentrations in aerosols and deposition implies that the monitor should quantitatively reflect its ambient conditions (Wolterbeek et al., 2002). Analytical data from biological samples may not provide information on 'the state of the environment' straightforwardly. Rather, they represent results of a relationship among (or/and food) concentration levels and biochemical uptake and transportation processes.

Over the last several decades, the uptake, retention and release of trace elements by biomonitor organisms has got major attention (Garty, 1993; Sloof 1995; Reis et al. 1999, Conti and Cecchetti, 2001; Bargagli and Michaelova, 2002). The Chernobyl accident also provided extensive information on accumulation efficiencies and release rates in biomonitors (Sloof, 1993; Sloof and Wolterbeek, 1992). However, still much work is to be done in combining mechanistic findings from the lab with results from the field. These processes must be unravelled and quantified for judging the

meaning of data derived from environmental analytics on biological samples (Markert, 2007).

1.4. Effects on lichens

The impact of trace element air pollution may be discussed in terms of effects on ecosystems, or on human health, but it should also be discussed in terms of possible effects on the biomonitor's behaviour (Gonzalez and Pignata, 1999). The latter means that, although the information on impact also serves its own additional purposes (Markert et al., 2000), if we regard the elemental levels in the biomonitor as a response to ambient elemental levels (air deposition = the dose), and if we restrict ourselves to the context of the dose–response relationship, impact on biomonitor physiology should be seen as relevant because it may cause changes in the nature of this dose–response relationship (Wolterbeek and Verburg, 2008).

In this effects-context many questions may be addressed such as:

- What are possible effects of ambient conditions on the monitor's performance?,
- Zoning of metal accumulation in lichen thalli is of relevance in sampling and/or analytical approaches, is this zoning affected by ambient conditions? (Bargagli et al., 1987; Bargagli, 1995),
- What is the consequence of seasonal fluctuations in element concentrations on survey time-scales? (Markert and Weckert, 1989),

- What may be the effects of rainfall and desiccation of thalli, both having effects on the monitor's soluble element content (Brown, 1995)?,
- Which consequences may have relationships between altitude and metal concentrations on survey's dimensional characteristics (Zechmeister, 1995)?,
- Which consequences may have species-specific responses and ambience-directed changes in behaviour on possibilities to intercalibrate monitor species (Schmid-Grob et al., 1992)?,
- Since individual chemical determinations show the size of mineral pools rather than the rate of through-put (Brown and Brown, 1991), what may be the consequences of possible differences or variations in the rate of acquisition, redistribution or loss of soluble elements, among species, geography, time or further ambient conditions?

1.5. Objectives

As suggested above, accurate evaluation of how and to what extent lichens reflect current or time-integrated abundances of heavy metals in the atmosphere or in deposition is essential for their use in biomonitoring: data should be compared within and between survey areas. Therefore the present thesis focuses on the element distribution within lichens, it addresses biological and physico-chemical lichen parameters to judge the possible impact from ambient conditions, and it gives attention to the dynamics of elemental uptake and release in lichens (see also Reis et al., 1999).

The above three themes are presented in 6 chapters (Chapters 2-7).

Chapters 2-3 address element distribution in lichens, both by element determination of separated lichen parts and by micro-PIXE scanning of whole lichen sections. Underlying reasoning was that, apart from interspecies and intra-species differences in responses, there may be response-differences also within individual lichens: results may be important in judging validity of the monitor's outcomes and may have consequences for sampling and sub-sample mass taken into element determinations. The present work compares the element concentrations and the elemental microdistributions of thin sections of peripheral and central parts of foliose lichen *Flavoparmelia caperata* after field exposure to industrial pollution.

Chapters 4-5 focus on comparability issues: variations in effects from ambient conditions may render lichens as difficult-to-compare. Comparability is at stake both for in-situ lichens as for transplants, since hardly anything is known about the time elapsed in developing effects from changes in conditions. The thesis addresses two different lichen characteristics: several keys on vitality were assessed, such as membrane permeability and chlorophyll levels (physiology) , as were some physico-chemical aspects, such as the hydrogen affinity and capacity of lichen ion exchange sites (non-physiological exchange behaviour).

Chapters 6-7 address lichen dynamics in field experiments: transplants were used to judge rates of uptake and release of elements into and from lichens. The reasoning was that fast release phenomena indicate that lichens do not reflect long fore-going deposition periods, the underlying general thought was that lichens will eventually reach equilibrium with any steady ambient

condition (Reis et al. 1999). The "time-length of the lichen's reflection period is often called remembrance-time or "memory".

The very nature of this memory predicts element-specific outcomes: a lichen may be a short-term monitor for the one element and at the same time be a long-term monitor for the other element. The chapters focus on the specifics of this phenomenon, its consequences for the interpretation and comparability of monitor's outcomes, thereby using mostly transplanted thalli of *Flavoparmelia caperata.*

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Chapter 2

ACCUMULATION OF TRACE ELEMENTS IN THE PERIPHERAL AND CENTRAL PARTS OF TWO SPECIES OF EPIPHYTIC LICHENS TRANSPLANTED TO A POLLUTED SITE IN PORTUGAL[1]

2.1. Introduction

The ability of lichens to accumulate levels of elements in excess of physiological requirements in close correlation with atmospheric elemental levels has led to their wide-scale application as practical biomonitors of inorganic atmospheric contamination.

Considering lichens as biomonitors means that the plant characteristics of lichens should be seen relative to the atmospheric metal availability. The inferred accumulation of the elements implies that both morphology and

[1] R.M. GODINHO, T.G. VERBURG, M.C. FREITAS, H.TH. WOLTERBEEK. Accumulation of trace elements in the peripheral and central parts of two species of epiphytic lichens transplanted to a polluted site in Portugal. Environmental Pollution 157 (2009), 102–109.

physiology of the lichen should be taken into account (Wolterbeek *et al.*, 2002).

The amount of a given element accumulated by lichens depends on morphological and structural features (Di Lella *et al.*, 2003), as well as eco-physiological properties (Nimis *et al.*, 2001). Goyal and Seaward (1982) report morphological modifications in lichen exposed to metal polluted environments. This suggests that biomonitoring with lichens is strongly species dependent. Bergamaschi *et al.* (2007) argue that the choice of a biomonitor species should be based on both the accumulation characteristics and the purpose of the study.

The comparability of results obtained with different biomonitor species is actually under debate (Bargagli and Mikhailova, 2002; Bennett and Wetmore, 1999; Bergamaschi *et al.*, 2007; Cercasov *et al.*, 2002; Yenisoy Karakas and Tuncel, 2004).

Growth should also be regarded as an important lichen parameter. Firstly growth dilutes internal element concentrations due to increases in the mass and secondly, growth dictates that the lichen consists of a continuum of old to new plant mass.

Zoning of metal accumulation in lichen thalli is an issue associated with the use of lichens as biomonitors (Bargagli *et al.*, 2002). There are reports of significantly higher concentrations of most metals in the inner, older zone of the thalli than in the peripheral parts of foliose native lichen of polluted sites (Bargagli *et al.*, 1987; Bargagli and Mikhailova, 2002). Senhou *et al.* (2002) describes an increase in thallus concentrations with lichen size in *E. prunastri*. Patterned accumulation of Pb, Cr, V and Hg has been reported for some species of foliose lichens (Nimis *et al.*, 1993; Nimis *et al.*, 2001).

However this zonation pattern is not always evident and several elements seem to be rather mobile within the thallus. Element translocation, especially with Cu, Mn and Zn, has been reported (Goyal and Seaward, 1982). Loppi *et al.* (1997) found a high variability in the trace element content of the peripheral and central parts of F.caperata from an unpolluted location. There are reports of rapid equilibration of lichen contents to new metal levels (Boonpragob and Nash, 1990; Garty, 1993; Loppi *et al.* 2003). Loppi *et al.* (2004) showed that despite their slow growth rate, lichens respond rapidly to decreasing concentrations of air pollutants, allowing annual changes to be detected.

There are also evidence that metals, including both mineral nutrients and heavy metals, are lost due to leaching depending on meteorological conditions and seasonal growth (Conti *et al.*, 2004; Giordano *et al.*, 2005; Godinho *et al.*, 2008).

While some biomonitoring protocols recommend sampling only the younger, marginal zone of the thalli of autochthonous foliose lichens (Bargagli, 1998; Nimis *et al.*, 2001), Bennett and Wetmore (2003) state that measurements performed only on lobe tips rather than on the whole thallus can lead to an underestimation of concentrations.

The accumulation of trace elements by lichens can be seen both as a passive phenomena (Bari *et al.*, 2001), reflecting a long term integration, or as a dynamic process involving uptake and release until equilibrium. According to Wolterbeek *et al.* (2002), after a change in ambient deposition conditions, the period towards a new equilibrium is associated with the remembrance time (memory length) of the lichen, which in this context gives an expression of the length of the foregoing environmental availability period

reflected by the lichen elemental content. This period is element specific and depends on ambient and lichen morphological/physiological conditions. Apart from interspecies and intra-species differences in responses, there may be differences in response within individual lichens; differences between tissues may result in differences in response.

The present paper compares the element concentrations of young and older lichen thallus parts, of one foliose and one fruticose lichen during a transplant experiment to a polluted site. The study focuses on the dynamics of the metal accumulation, thus addresses the lichen memory length (Godinho *et al.*, 2008; Reis *et al.*, 1999), thereby aiming to increase the possibilities to improve biomonitoring results, interpretation and comparability. *Flavoparmelia caperata* and *Evernia prunastri* are the two species presently used; they are largely used as biomonitors and with different morphologic characteristics that may suggest different accumulation proprieties.

2.2. Methods

2.2.1. *Sampling and exposure*

Samples of two lichen species *Evernia prunastri* (L.) Ach. and *Flavoparmelia caperata* (L.) Ach. were collected from pine trees located in a clean rural zone in the centre of Portugal (39º30' N, 8º00' W), near Tomar. The average diameter of *F. caperata* was 3 to 4 cm and average length of *E. prunastri* was 4 to 5 cm. Samples were cleaned to remove dust,

leaf debris, fungus contamination or degraded material, and rinsed three times, for 5 s each, in double distilled water (Garty *et al.*, 2001, Smodis *et al.*, 2004). Lichens were then cut into individual thallus, keeping the substrate, and exposed inside a polyamide net (61 μm porosity) bound with a petri slide (Machado *et al.*, 2004) and hanging, protected from direct rain, as described by Freitas *et al.* (2001). Samples were transplanted (1) into the same zone of initial collection, and (2) to a polluted area, 180 km southwest of Tomar, near Sines' industrial complex composed of a refinery, a thermo-electrical power station and a petrochemical industry. Previous work in the Sines area revealed high contents of various elements related to human activities such as Zn, Se, Pb and Hg (Freitas *et al.*, 1997).

The lichens were exposed for four months, from March to July, and samples were collected periodically at both sites (Godinho *et al.*, 2004). In Tomar, samples of native populations of *F. caperata* were also collected as controls. Each time three replicates of material were collected.

Meteorological data collected are described in Godinho *et al.* (2004).

2.2.2. *Analytical procedures*

In the laboratory the samples were rinsed three times with double distilled water for 5 s (Garty *et al.*, 2001) and were dried before being analyzed. The outermost 3-4 mm of the thallus was detached and analyzed separately from the central part. In *F. caperata* this corresponds to the lobes distinguishable by their colour and their lack of rhizinae, which corresponds to an age of about 1 year (Loppi and Pirintsos, 2003). The superior tips of *E. prunastri* were cut before the first branching (Stone and McCune, 1990).

Elemental contents were determined by k0-standardised, instrumental neutron activation analysis (k0-INAA) following procedures described in Machado *et al.* (2004) and Freitas *et al.* (2006).

2.2.3. *Statistical analysis*

Data were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Of the 20 elements analysed in the peripheral and central parts of the thallus of *F. caperata* and *E. prunastri*, only for Mg in the peripheral *F. caperata* and K in peripheral *E. prunastri* data were not normally distributed. On basis of these results, differences between groups were further tested for significance using the Student's t-test.

Experimental ratios and slopes were compared against expected ratios and slopes using the Z-score for any statistically significant difference. The Z-score value denotes the difference as the number of standard deviations from the expected value.

Standard weighted linear regression analysis was used to estimate the strength of the association between species or thallus parts and association in time series.

All the significant results were given at a 5% level of significance ($p = 0.05$), unless mentioned otherwise.

2.3. Results and Discussion

2.3.1. *Initial element concentration (background situation)*

Table 1 summarizes the analytical data for the peripheral and internal parts of *F. caperata* and *E. prunastri* from Tomar, the reference site.

Compared to biomonitoring reports carried out in Europe the site chosen can be considered a fairly unpolluted place (Adamo *et al.*, 2003; Nimis *et al.*, 2001; Sloof, 1995) although Table 1 shows higher values than remote locations like Himalayan or northern Canada (Bergamaschi *et al.*, 2002; Chiarenzelli *et al.*, 2001). The values for the *F. caperata* reference sample are similar to the ones reported for background areas (Loppi *et al.*, 2004; Rizzio *et al.*, 2001). Elemental concentrations in *E. prunastri* are lower than reported by Pirintsos *et al.* (2004), and in the same order of those reported by Cercasov *et al.* (2002), with the exception of Zn and Cr that are higher in our study.

The variability observed in the present study, with maxima standard deviations of around 30%, reflects the inter-individual variability and is similar to that reported by other authors in background areas. Chiarenzelli *et al.* (2001) report ranges of concentrations within species of an order of magnitude which are attributed to differences in sample age, morphology and microclimatic factors unique to the sampling site. Loppi *et al.* (1997) reports a variation range of 24-63 % in external part and of 20-95 % in internal part of *F. caperata*, while Rizzio *et al.* (2001) presents a median variability of 50%. For *E. prunastri* both Cercasov *et al.* (2002) and Conti *et al.* (2004) found a variability of around 20% while Ayrault *et al.* (2007)

report 30%. Loppi *et al.* (1997) hypothesized that variability in older parts could be due to patterned retention of some elements (for example particle entrapment and mineral immobilization) although in our study younger and older parts present similar variability with the exception of Fe in *F. caperata*.

Table 1. Initial element concentrations contents before exposure: average ($mg.kg^{-1}$) and standard deviation (%) of 3 replicates of *Flavoparmelia caperata* and *Evernia prunastri* with P values of the Student's t-test. P-tissue for differences between tissue and P-sp. for differences between species (Peripheral and Central). Significant differences between groups are shown in bold. n.d. = not detected.

	Al	As	Br	Ca	Cl	Co	Cr	Fe	K	La	Mg	Mn	Na	Rb	Sb	Sc	Sm	Ti	V	Zn
Flavoparmelia caperata (peripheral tissue)																				
Average	150	0.22	1.3	2380	203	0.18	13.1	93	5088	0.08	745	13	285	3.9	0.022	0.02	n.d.	n.d.	0.6	142
St. dev.	15	32	24		24	30	25	15	14	25	17	17	23	27	14	33			32	17
Flavoparmelia caperata (central tissue)																				
Average	610	0.19	5.6	4109	439	0.21	10.5	210	2962	0.29	525	17	252	5	0.131	0.07	0.06	41	2.22	157
St. dev.	13	30	26	28	13	17	7	44	27	30	6	28	33	23	1	30	30	32	12	29
P-tissue	**7E-04**	0.824	**0.008**		**0.0052**	0.504	0.475	0.096	**0.026**	0.057	**0.041**	0.256	0.625	0.269		0.086	0.103		**9E-04**	0.635
Evernia prunastri (peripheral tissue)																				
Average	177	0.22	5.8	2445	1783	0.22	7.9	122	2504	0.14	613	17.8	199	3.4	0.06	0.04	0.03	21	0.8	188
St. dev.	6	30	29	30	6	30	16	30	19	30	7	31	8	30	17	30			9	26
Evernia prunastri (central tissue)																				
Average	254	0.2	9.2	4208	1823	0.11	6.9	105	1588	0.15	445	14.8	124	1.7	0.08	0.04	0.03	36	1.07	201
St. dev.	13	30	15	30	14	18	20	24	12	32	7	9	10	23		25			30	31
P-tissue	**0.018**	0.833	**0.05**	0.326	0.8107	0.178	0.431	0.624	**0.039**	0.891	**0.005**	0.418	**0.003**	0.157		0.872	0.733		0.319	0.826
P-sp.(Per.)	0.192	0.997	**0.011**	**<0.0001**		0.602	0.191	0.388	**0.007**	0.15	0.156	0.266	0.094	0.703		0.211	0.384		0.159	0.216
P-sp. (Cent.)	**0.002**	0.912	**0.033**	0.955	**0.0007**	**0.015**	**0.016**	0.131	**0.045**	0.176	0.039	0.428	0.06	**0.01**	0.208	0.159		0.531	**0.016**	0.471

In order to test the significance of possible differences in the initial element concentrations in the peripheral and central part of the two lichen species a Student t-test was performed. The central part of *F. caperata* revealed higher concentrations of Al, Br, Cl and V, while in the peripheral part K and Mg concentrations were higher. The central part of *E. prunastri* revealed higher concentrations of Al and Br while in the peripheral part K, Mg and Cl concentrations were higher (see P-tissue, Table 1).

With the exception of Cl, the pattern of content distribution agrees with descriptions given by Loppi *et al.* (1997); elements of little metabolic significance are more abundant in older parts whereas essential elements are more abundant in younger parts. Differences in element content in the two lichen species were also observed. In *E. prunastri* higher concentrations were found for Br and Cl in both the peripheral and central part. Higher concentrations of K were found in both parts of *F. caperata* and higher concentrations of Al, Co, Cr, Rb and V were found in the central part (see P-sp, Table 1). The comparability of different biomonitor species is under debate. Although species- and morphology-specific element accumulation is reported (Bergamaschi *et al.*, 2007; Cercasov *et al.*, 2002), some studies from clean remote sites report similar multi-element patterns between lichen genera and species (Bergamaschi *et al.*, 2002, Chiarenzelli *et al.*, 2001). According to Cercasov *et al.* (2002) initial low element content is one criterion to compare the suitability of two species for biomonitoring.

2.3.2. *Transplant effects*

The effect of the transplantation procedure was tested at Tomar, the "clean" reference area, by comparing the behaviour of *F. caperata* (the most abundant species), native populations and "in situ" transplanted lichen thallus. Elemental concentrations are presented in Table 2. The transplant apparatus was such as to minimise and/or standardize the ambient interferences (tree trunk run-off, heavy rain, insulation) and to maximize the surface exposure in order to maximize the lichen atmospheric capture (collection efficiency).

Ayrault *et al.* (2007) defend the use of a non-metallic roof, allowing the air to circulate and to protect the lichen from direct rain. The exposure method involves flattening the lichens to promote surface exposition but to prevent particle retention by branching. Lichen thallus was maintained attached to the substrate to maintain the integrity of the thallus and to avoid scratches. In order to assess the significance of a possible transplant effect the element concentration ratio (transplant/native) was calculated after 2 and 3 months of exposure. If transplantation has no impact a ratio equal to 1 is expected (Figure 1). A Z-test was used to compare the calculated ratio with the expected ratio. Comparisons revealed no major significant effects, except for minimal differences for Sm and Zn after 2 months exposure time (results of the Z-test are not shown). Increáses in surface exposure seem to have no accumulation benefits, maybe due to the influence of the net and/or reduced branching particle retention. Bari *et al.* (2001) reports a small change in the efficiency of exposure between boards and bags, finding stress to affect the accumulation in the first case. In our experiment no impact of the transplant method in lichen vitality was observed (Godinho *et al.*, 2004).

Table 2. Element concentrations, average ($mg.kg^{-1}$) and standard deviation (%) of 3 replicates of *Flavoparmelia caperata* from a relatively unpolluted site near Tomar. Comparison of native lichens with in situ transplanted lichens after 2 and 3 months of exposure. n.d. = not detected.

		Al	As	Br	Ca	Cl	Co	Cr	Fe	K	La	Mg	Mn	Na	Rb	Sb	Sc	Sm	Ti	V	Zn
Initial	Average	420	0.3	2.8	6369	247	0.1	8.5	170	4980	0.2	577	11	109	3	n.d.	0.1	0.1	34	1.4	58
	St. dev.	26	25	2	31	3	16	12	26	16	25	14	1	30	10		19	24	17	23	9
Native																					
2 months	Average	370	0.3	2.8	3957	184	0.1	7.3	166	5218	0.2	623	15	86	3	n.d.	0.1	0.05	15	1.4	63
	St. dev.	32	30	13	26	18	3	22	19	14	25	16	20	5	9		20	4	30	18	9
3 months	Average	590	0.3	n.d.	6128	282	0.1	10	289	5070	0.3	606	15	129	3	0.1	0.1	0.1	44	2.2	46
	St. dev.	30	19		30	8	30	29	30	9	30	11	15	12	14		31	30	22	12	9
Transplanted																					
2 months	Average	430	0.3	3	3409	161	0.1	6.8	193	5334	0.2	672	14	105	3	0.1	0.1	0.1	25	1.8	134
	St. dev.	24	7	13	20	2	7	7	19	10	15	4	6	9	30		18	12	21	3	17
3 months	Average	n.d.	0.3	3.8	4283	n.d.	0.1	8	215	3906	0.3	n.d.	n.d.	113	3	0.1	0.1	0.1	n.d.	n.d.	53
	St. dev.			0.1	18		3	11	9	19	1			0	0	22	6	0.4			20

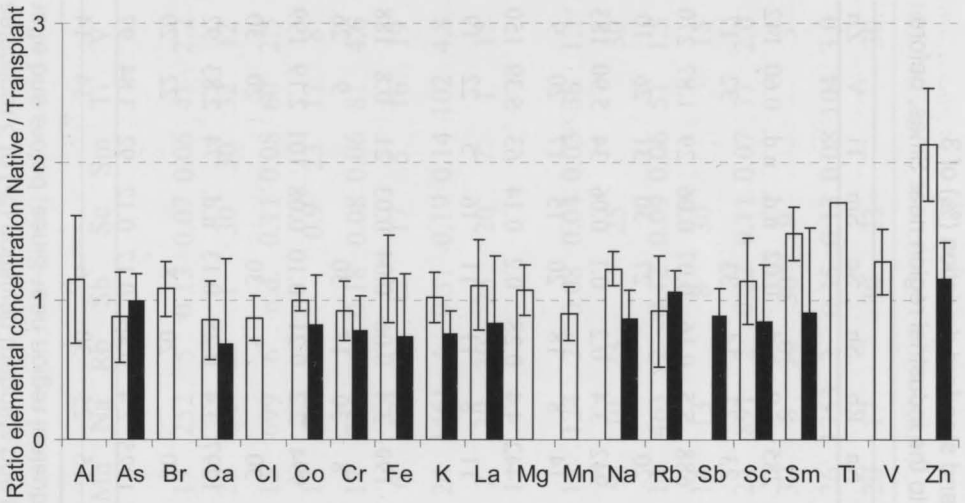

Figure 1. Ratios of element concentrations in transplanted and native *Flavoparmelia caperata* originating from a relatively unpolluted site near Tomar. White, two months exposure; Black, 3 months exposure.

2.3.3. *Element accumulation*

Table 3 and 4 present the element concentrations in both lichen parts of both species transplanted to Sines along the experiment time. Generally there was a rise of concentrations after transplantation to the polluted site.

Table 3. Element concentrations in peripheral tissue, average ($mg.kg^{-1}$) and standard deviation (%) of 3 replicates of *Flavoparmelia caperata* and *Evernia prunastri* transplanted to the industrial region near Sines, before and after 2, 3 and 4 months of exposure. n.d. = not detected.

	Al	As	Br	Ca	Cl	Co	Cr	Fe	K	La	Mg	Mn	Na	Rb	Sb	Sc	Sm	Ti	V	Zn
Flavoparmelia caperata (peripheral tissue)																				
Initial	150	0.22	1.3	2380	3	0.18	13.1	93	5088	0.08	745	13	285	3.9	n.d.	0.02	n.d.	n.d.	0.60	142
	15	32	24		24	30	25	15	14	25	17	17	23	27	14	33			32	17
2 mon.	290	0.25	6.3	4604	1006	0.32	17	242	4458	0.37	802	15	688	5.5	0.16	0.07	0.06	29	1.87	270
	24	8	6	3	18	19	30	30	18	30	15	10	20			27	30	31	26	10
3 mon.	310	0.26	8.8	2882	1255	0.46	13	191	4675	0.49	1167	20	942	3.4	0.2	0.1	0.06	34	5.90	183
	20	10	8	15	10	17	20	25	9	15	10	10	14	8	18	20	15	17	20	9
4 mon.	740	0.50	13	6064	2250	0.76	11	531	4803	0.87	1382	30	1442	4.4	0.28	0.2	0.14	63	5.39	150
	8	24	10	18	8	8	10	12	5	10	8	8	11	8	11	11	16	5	22	19
Evernia prunastri (peripheral tissue)																				
Initial	177	0.22	5.8	2445	1783	0.22	7.9	122	2504	0.14	613	17.8	199	3.4	0.06	0.04	0.03	21	0.8	188
	6	30	29	30	6	30	16	30	19	30	7	31	8	30	17	30			9	26
2 mon.	442	0.28	10.8	3641	2715	0.58	11.5	319	1656	0.54	1066	27.9	994	2.2	0.21	0.10	0.08	101	2.19	199
	30	11	22	19	24	16	17	30	8	17	20	11	30		5	30			20	30
3 mon.	642	0.70	n.d.	n.d.	2370	0.57	11.3	414	2660	1.2	1610	35.0	1297	3.4	0.25	0.13	n.d.	74	2.83	92
	8	16			7	14	3	4	12		29	19	30		20	8			22	20
4 mon.	844	0.32	17.3	4716	4721	0.66	8.8	591	1931	0.78	1519	37.8	1925	2.4	0.46	0.17	0.12	92	3.84	97
	9	25	2	4	13	5	19	9	12	10	11	12	15	25	30	6			14	14

Table 4. Element concentrations in central tissue, average ($mg.kg^{-1}$) and standard deviation (%) of 3 replicates of *Flavoparmelia caperata* and *Evernia prunastri* transplanted to the industrial region near Sines, before and after 2, 3 and 4 months of exposure. n.d. = not detected.

	Al	As	Br	Ca	Cl	Co	Cr	Fe	K	La	Mg	Mn	Na	Rb	Sb	Sc	Sm	Ti	V	Zn
Flavoparmelia caperata (central tissue)																				
Initial	610	0.19	5.6	4109	439	0.21	11	210	2962	0.29	525	17	252	5	0.13	0.07	0.06	41	2.2	157
	13	30	26	28	13	17	7	44	27	30	6	28	33	23	1	30	30	32	12	29
2 mon.	710	0.30	7.1	4450	1361	0.32	12	303	4043	0.51	672	15	699	6	n.d.	0.11	0.08	60	2.5	227
	9	7	26	18	4	17	30	4	20	22	27	13	19	14		0.9	23	17	8	30
3 mon.	790	0.22	7.7	3298	1457	0.3	10	253	3939	0.45	795	17	734	4	0.18	0.08	0.06	81	4.6	92
	7	14	23	11	4	15	30	24	17	22	9	6	17	4	12	17	5	16	19	30
4 mon.	1040	0.33	15	8886	2572	0.68	6.4	651	3877	0.86	992	24	1461	4	0.31	0.19	0.14	102	4.8	140
	14	33	12	19	37	23	6	21	19	15	8	2	30	30	25	20	7	1	15	24
Evernia prunastri (central tissue)																				
Initial	254	0.20	9.2	4208	1823	0.11	6.9	105	1588	0.15	445	15	124	2	0.08	0.04	0.03	36	1.1	201
	13	30	15	30	14	18	20	24	12	32	7	9	10	23		25			30	31
2 mon.	302	0.21	9.6	2062	2150	0.34	11	268	1478	0.44	625	14	463	2	0.12	0.09	0.06	51	1.5	101
	30		21	30	30	21	27	30	16	5	8	14	13		8	30			15	30
3 mon.	518	0.29	11	3389	2248	0.45	11	354	1325	0.50	1155	24	891	2	0.22	0.11	0.07	77	2.0	87
	8	24	8	30	15	30	18	15	17	30	30	30	8	30	30	9			3	30
4 mon.	705	0.32	14	4627	5213	0.45	9	423	1408	0.46	1132	26	1753	2	0.35	0.13	0.08	104	3.0	73
	30	22	20	30	30	27	23	32	18	28	21	20	17		30	23			30	32

2.3.3.1. *Accumulation ratio*

Table 5, part 1, shows the ratio of concentrations in exposed and unexposed samples. This is known as the enrichment factor (Cercasov *et al.*, 2002) or the control EC ratios (Frati *et al.*, 2005). This reveals the accumulation of some elements. The use of ratios allows us to interpret changes in element content without assuming a linear or non-linear model describing element accumulation and/or release during the time of exposure. Significant accumulation (ratio>1) during the exposure time were registered for the following elements: Al, Br, Cl and V (*F. caperata*, both parts); Co and Na (*E. prunastri*, both parts); Fe, La, Mn, Na, Sc and Sm (*F. caperata*, peripheral part); Al, Br and V (*E. prunastri*, peripheral part); Co (*F. caperata*, central part); Sc and Cr (*E. prunastri*, central part). Significant decreases (ratio<1) in element content were found for Cr (*F. caperata*, central tissue) and Zn (*E. prunastri*, both tissues). According to the EC ratio interpretative scale used by Frati *et al.* (2005), most of these accumulations correspond to "severe accumulation level".

Table 5. Comparison of accumulation behaviour of peripheral and central parts of *F. caperata* and *E. prunastri*, transplanted near Sines using: 1) Accumulation ratio; ratio element concentration at the start of the experiment and after 4 months exposure. In bold: ratio peripheral part significantly different from ratio central part. 2) Comparison of slopes calculated by weighted linear regression of time versus element concentration. + sign indicates accumulation, - sign indicates release and 0 indicates neither accumulation nor release. 3) Comparison of slopes calculated by weighted linear regression of peripheral tissue versus central tissue. <1 indicates *d*Central < *d*Pheripheral, 1 indicates dCentral = dPheripheral.

	Flavoparmelia caperata					*Evernia prunastri*				
	1-Accum. ratio		2-Time *vs* element conc.		3-Periph. *vs*	1-Accum. Ratio		2-Time *vs* element conc.		3-Periph. *vs*
	Central	Peripheral	Central	Peripheral	Central	Central	Peripheral	Central	Peripheral	Central
Al	**1.7**[a]	**4.8**[a]	+[c]	+[c]	1[b]	2.8	4.8[a]	+[b]	+[b]	<1[b]
As	1.7	2.3	0	+	<1	1.6	1.5	0	0	1
Br	**2.6**[a]	**9.8**[a]	+[c]	+[b]	<1	1.5	3.0[a]	+[c]	+[b]	1
Ca	2.2	2.5	0	+	1	1.1	1.9	0	+[b]	1
Cl	5.9[a]	11.1[a]	+[b]	+[b]	1[b]	2.9	2.6	+	+	1
Co	3.2[a]	4.3	+	+[b]	<1[c]	4.1[a]	3.0[a]	+[b]	+[c]	1[b]
Cr	0.6[a]	0.8	-[b]	0	1[b]	1.3[a]	1.1	0	0	1[b]
Fe	3.1	5.7[a]	+	+[c]	1[b]	4	5	+[b]	+[b]	1[b]
K	1.3	0.94	0	0	1	0.89	0.77	0	0	<1
La	**2.9**	**11.2**[a]	+[c]	+[b]	<1[b]	3.0	5.4	+	+[c]	<1
Mg	1.9[a]	1.9[a]	+[b]	+[c]	<1[b]	2.5[a]	2.5[a]	+[c]	+[b]	<1[b]
Mn	1.4	2.3[a]	+[b]	+[c]	<1[b]	1.8	2	0	+[b]	<1
Na	5.8	5.1[a]	+[b]	+[b]	1[b]	14.1[a]	9.7[a]	+[b]	+[b]	<1[b]
Rb	0.89	1.1	0	0	1[c]	0.95	0.71	0	0	1
Sc	**2.7**	**7.0**[a]	0	+[b]	1[b]	3.3[a]	4.3	+[b]	+[b]	1[b]
Sm	2.3	6.4[a]	+[c]	+[b]	1[b]	3.0	3.8	+[b]	+[b]	1
Ti	2.5		+[b]	+[b]	1	2.9	4.3	+[c]	+[b]	<1
V	**2.2**[a]	**9.0**[a]	+	+[c]	<1[b]	2.8	4.8[a]	+	+[b]	<1
Zn	0.89	1.1	0	0	<1	0.36[a]	0.52[a]	0	0	<1

[a]; ratio significantly different from 1 ($P_{Z\text{-score}}$<0.05),.[b]; significant linear regression (P<0.05).,[c]; significant linear regression (P<0.1).

2.3.3.2. *Time series*

When using accumulation ratios any information about element behaviour during the exposure time will be lost. Therefore a simple weighted linear model was used to identify possible differences in accumulation and/or release in both lichen parts.

A significant association with time was found for Al, Br, Cl, La, Mg, Mn, Na, Sm and Ti in both peripheral and central part of *F. caperata*, and Al, Br, Co, Fe, Mg, Na, Sc, Sm and Ti in both peripheral and central part of *E. prunastri*, showing element accumulation to be linear during the time of exposure in both lichen parts (see Figure 2).

Accumulation in only the peripheral part was found for Co, Fe, Sc and V in *F. caperata* and Ca, La, Mn, Sb and V in *E. prunastri*. Only the Cr content in the central part of *F. caperata* decreased significantly during the period of exposure (see Figure 2).

The different behaviour of the two lichen parts suggests different accumulation mechanisms. Time dependent linear accumulation behaviour could be indicative of a progressive passive uptake, while the absence of linear behaviour may suggest regulation mechanisms. Behind element specific accumulation mechanisms reported by several authors, the different lichen parts are dealing differently with each element. This would be in line with reported element translocation within lichen and internal regulation (Chiarenzelli *et al.*, 2001, Goyal and Seaward, 1982).

2.3.3.3. *Accumulation rate: dynamics*

A possible comparative evaluation of the accumulating/release rate for the different lichen parts may be based on a simple weighted linear regression

(peripheral versus central) (see Table 5, part 3). The slope is expected to be equal to 1 if the element accumulation sensibility of the two parts is equal. Differences in accumulation rates are found for Co, La, Mg, Mn and V in *F. caperata* and Al, Mg and Na in *E. prunastri*. The accumulation rate for all elements in both species is higher in the peripheral part of the lichen. Accumulation by ion exchange and especially particulate trapping are two well documented mechanisms by which elevated levels of elements are achieved in lichens. The initially higher metal concentrations found in older lichen thalli parts may be due to the longevity of the parts themselves: the central parts, being older, have been exposed for a longer time, and are likely to contain higher concentrations due to progressive uptake of particles (Nimis *et al.*, 2001), or to the possibility that older parts have higher cation exchange capacities, the latter presumably due to progressive cell senescence, in turn associated to the exposure of additional exchange sites (Wolterbeek *et al.*, 2002).

Because cell wall-bound elements are readily exchangeable (Brown 1987, Purvis *et al.* 2005), in the last case we would expect a rapid response to the environmental input.

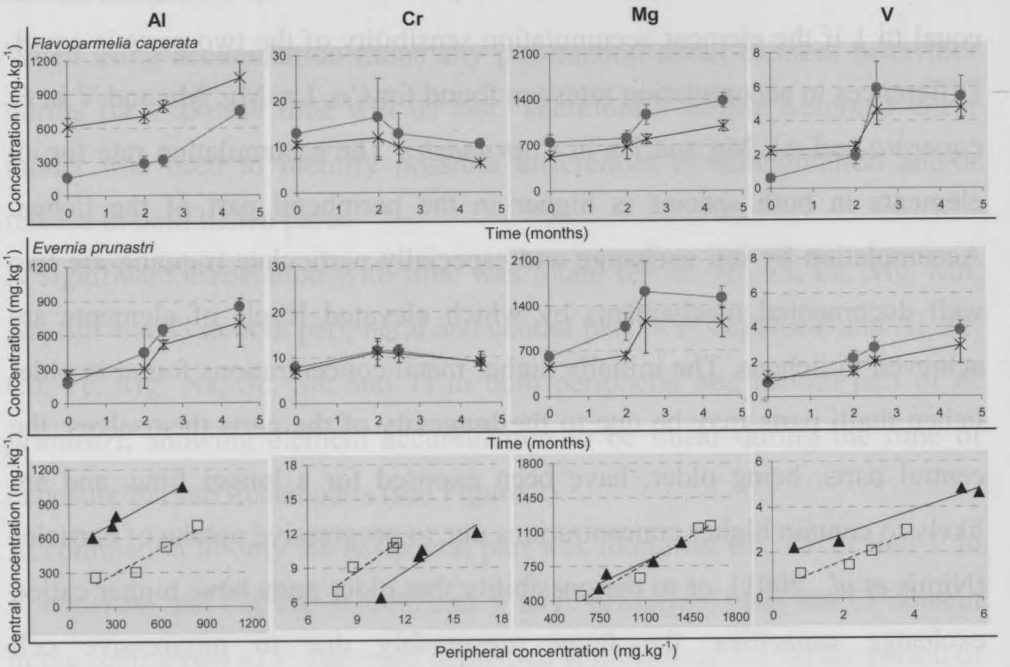

Figure 2. Example of element behaviour during the exposure time in peripheral and central parts of *F. caperata* and *E. prunastri*, transplanted near Sines, and the linear correlation (peripheral *versus* central part) of both lichen species. —●— Peripheral part of lichen thallus; —✕— Central part of lichen thallus; ▲ *F. caperata*; □ *E. prunastri*; —— linear (*F. caperata*); - - - · linear (*E. prunastri*).

In both species older parts are thicker (data not shown), with more hyphal mass. A higher ratio "mass-to- thallus surface area" may explain the smaller response in terms of concentrations verified in the older parts in respect to passive binding elements (element presence is presented as "amount per unit of mass"). The different accumulation behaviour between algal and fungal cells could account for the different performance of peripheral and central

tissues, since proportionally the algal is more abundant in the young tissue. It should be remembered however, that algae hardly contribute to the mass of the lichen. Another possibility therefore, may be that the loss of cytosol in older parts could result in lower cellular dry weights, indirectly rising element concentrations when expressed on a dry weight basis. The higher metabolic activity of peripheral younger parts could justify higher rates of active accumulation as suggested by Loppi *et al.* (1997). At the end of the exposure again peripheral parts present higher concentrations of metabolically essential elements although also others like La and Cr. Still Al concentration was higher in central parts of *F. caperata* while the peripheral parts had more Cr, Mg and Mn. In *E. prunastri* younger parts had significantly more Co, La and Mn (Student's t-test $p<0.05$, results not shown).

The final pattern of element allocation can be explained by the difference in rate accumulation of both lichen parts. For instance the difference in the change in vanadium content is due to higher rates of accumulation in *F. caperata* lobes. Loppi *et al.* (1997) hypothesized that elements occurring in higher amounts in central parts are likely to be trapped as particulates by loose hyphal weft of the medulla, which could be the case of Al in the centre of *F. caperata*.

To identify possible differences in accumulation rates in the two lichen species (*Flavoparmelia caperata* versus *Evernia prunastri*) a weighted linear regression of element concentrations was performed for both lichen parts, the results of which are shown in Table 6.

Table 6. Result weighted linear regression (*Flavoparmelia caperata* versus *Evernia prunastri*), peripheral and central tissue, transplanted near Sines. Significant correlations (P model) are shown in bold. Unequal accumulations rates (P slope, slope not equal to 1) are shown in bold.

	Peripheral tissue				*Central tissue*			
	slope	error in slope	P model	P slope=1	Slope	error in slope	P model	P slope=1
Al	1.26E+00	1.82E-01	0.0920	0.0666	1.25E+00	5.79E-01	**0.0423**	0.4584
As	2.13E-01	3.32E-01	0.4309	0.0546	1.78E-01	6.78E-01	0.8094	0.0938
Br	9.82E-01	1.88E-01	**0.0095**	0.8855	5.71E-01	3.76E-01	0.0584	0.1067
Ca	6.13E-01	3.01E-01	0.0613	0.1561	2.78E-01	4.24E-01	0.4561	**0.0424**
Cl	8.81E-01	1.94E-01	0.1112	0.3048	6.13E-01	3.65E-01	0.2318	0.1245
Co	4.67E-01	1.64E-01	0.1794	**0.0073**	1.04E+00	3.84E-01	0.1890	0.8436
Cr	4.64E-01	6.26E-01	0.4859	0.1851	-6.17E-02	5.29E-01	0.9376	**0.0277**
Fe	9.66E-01	2.05E-01	0.1741	0.7618	5.65E-01	4.53E-01	0.4639	0.1507
K	6.11E-01	2.26E+00	0.7271	0.7536	-1.78E-01	2.87E-01	0.1975	**0.0038**
La	8.50E-01	1.59E-01	0.0896	0.1562	5.09E-01	3.80E-01	0.3188	0.0814
Mg	1.32E+00	4.39E-01	0.0841	0.2415	1.53E+00	5.22E-01	**0.0222**	0.1345
Mn	8.69E-01	3.78E-01	0.1534	0.5381	1.24E+00	6.41E-01	0.1182	0.5032
Na	1.55E+00	3.10E-01	**0.0045**	**0.0384**	1.25E+00	3.07E-01	0.0703	0.1956
Rb	-5.95E-01	7.33E-01	0.1768	**0.0224**	3.38E-01	1.23E+00	0.5118	0.3596
Sb	1.48E+00	1.18E+00	0.2327	0.5509	1.88E+00	1.44E+00	0.2345	0.4021
Sc	7.04E-01	1.56E-01	0.1421	**0.0322**	3.82E-01	4.39E-01	0.5501	0.0670
Sm	7.91E-01	3.35E-01	0.0733	0.3934	2.02E-01	4.25E-01	0.8478	**0.0330**
Ti	4.25E-01	4.34E-01	0.6257	0.1489	1.04E+00	5.53E-01	**0.0091**	0.8915
V	5.72E-01	1.28E-01	0.0615	**0.0068**	3.42E-01	1.52E-01	0.0827	**0.0033**
Zn	-1.98E-01	6.16E-01	0.8378	**0.0301**	1.32E-01	4.88E-01	0.8157	**0.0378**

The slope is expected to be equal to 1 if element accumulation sensibility of the two species is equal. In the peripheral part different accumulation rates are found for Co, Na, Sc, V and Zn (at α=0.05). However, only the concentrations for Na in the two lichen species show a linear association at α=0.05 while the slope is >1 therefore the accumulation rate in *E. prunastri* is significantly higher (see Figure 3).

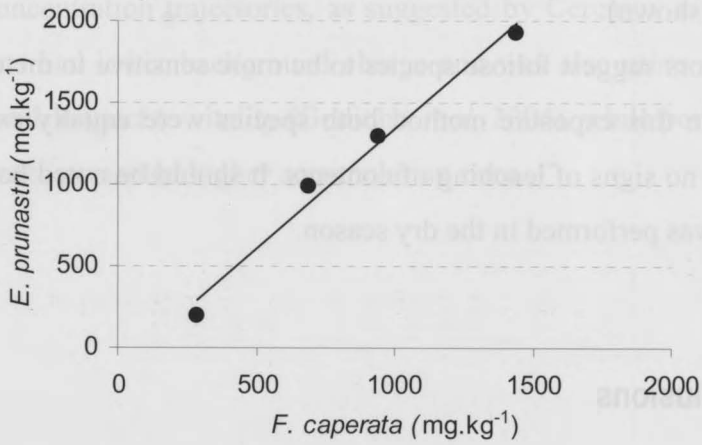

Figure 3. Linear association of Na in the lichen species *Flavoparmelia caperata* and *Evernia prunastri* (peripheral part), transplanted to Sines, where *r* is the correlation coefficient.

If we accept a significance level of α=0.1, a different accumulation sensibility is also found for Al (accumulation rate in *E. prunastri* is significantly higher) and V (accumulation rate in *E. prunastri* is significantly lower). In the central part a lower accumulation rate is found for V in *E. prunastri* at the significance level of α=0.1. Final elemental

contents are still different in the two species suggesting that they probably have different affinities for some elements. As lichens were flattened, these differences can not be attributed to branching but maybe to thallus constitution. However, a similar type of element distribution is found between the older and younger parts of the two species. Peripheral parts of both species differ in Br, Cl and Ti content, being higher in *E. prunastri* and also in K content, higher in *F. caperata*. Central parts of *E. prunastri* have more Cl while central *F. caperata* contains more K (Student's t-test $p<0.05$, results not shown).

Some authors suggest foliose species to be more sensitive to meteorological leaching. In this exposure method both species were equally exposed and there were no signs of leaching of contents. It should be noted however that the study was performed in the dry season.

2.4. Conclusions

Both at the beginning of the experiment, under a "clean" situation, and at the end of a pollution exposed period, central parts of the thallus of both species have higher concentrations of elements of limited metabolic significance, while elements of metabolic interest were more abundant in peripheral parts. Both lichen parts respond to environmental changes. Here, differential accumulation may suggest that differential constitution leads to differential uptake and release, and/or the overall behaviour is partly due to internal translocation and regulation mechanisms within the whole lichen. Considering thallus parts, internal translocation should be taken into account

as one more factor affecting lichen "memory length". Young parts of the thallus presented higher rates of change, but different lichen parts accumulate different elements to different extents. This observation makes that tissue-selection in monitoring may depend on the element of interest, and cannot be made into a generalized approach in survey set-ups: the choice depends on the element.

Both species presented similar trends in accumulation but showed different element affinities: this makes it difficult to compare species over large element concentration trajectories, as suggested by Cercasov *et al.* (2002). *E. prunastri* had lower background charge and was also more sensitive to pollution with respect to vitality (Godinho *et al.* 2004), while peripheral part of *F. caperata* presented higher accumulation rates.

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Chapter 3

MICRO-SCALE ELEMENTAL DISTRIBUTION IN THE THALLUS OF *FLAVOPARMELIA CAPERATA* TRANSPLANTED TO POLLUTED SITE[2]

3.1. Introduction

In the context of air pollution biomonitoring by lichens, it is crucial to understand the relations between the lichen and the pollutants in terms of lichen eco-physiology.

The patterned accumulation and zoning of metal concentrations in lichen thalli is an actual issue associated with the use of lichens as biomonitors. Element-specific partitioning between old and younger parts of foliose lichen thallus is reported and suggested as influencing the biomonitoring results (Loppi *et al.*, 1997; Bargagli and Mikhailova, 2002; Godinho *et al.* 2009; Nimis *et al.*, 2001).

[2] R.M. GODINHO, H.TH. WOLTERBEEK, M.T PINHEIRO, ALVES L.C, T.G. VERBURG, M.C. FREITAS, "Micro-scale elemental distribution in the thallus of *Flavoparmelia caperata* transplanted to polluted site", Journal of Radioanalytical and Nuclear Chemistry (2009) 281: 205-210.

It was also hypothesized that differences in the lichen thallus constitution (algae, fungi) may cause the different patterns of element distribution in peripheral and central lichen parts (Godinho *et al.*, 2009).

In the general dynamics of element uptake and release, specific components which affect total behaviour, or which are relevant in sampling, or in the handling of the lichen samples should be extensively studied. In this context the comparability of lichen sub-parts should be investigated in terms of particle entrapment, and uptake and release processes. Here, differences between pseudo-tissues (e.g. heterogeneities in internal distribution of myco- and photobionts) may result in differences in response.

The internal spatial distribution of the elements is a reflection of the pathways of transport and influences the extraction potential and toxicity of the elements. Therefore insight in distribution patterns may help to understand the lichen rates of accumulation and release being related with lichen remembrance time (Reis *et al.*, 1999; Godinho *et al.*, 2008).

Various methods have been used to study spatial distributions, such as sequential elution techniques (Brown, 1995), X-ray microanalysis (Hauck *et al.*, 2002) and microscopic histochemistry methods (Garty and Theiss, 1989).

Proton microprobe techniques based on focused proton beams generated in particle accelerators permit the direct microanalysis of individual biological samples. It delivers images of the sample morphology and elemental distributions providing not only low detection limits, but also spatial information necessary to understand physiological and biochemical relationships (Mesjasz-Przybylowicz and Przybylowicz, 2002). Moreover, it allows the estimation of element accumulation without the risk of taking

into account the metals present in dust on the lichen surfaces. However, so far, only few Micro-PIXE studies have been carried out on lichens (Clark *et al.*, 1999; Budka *et al.*, 2002; Ayrault *et al.*, 2007; Ohnuki *et al.*, 2003).

Most of these microdistribution studies highlight particle entrapment in interhyphal spaces and mineralised structures as the main cause of high metals contents in thalli with detoxifying mechanisms emphasizing the fungus toxicity protection role (Branquinho *et al.*, 1999; Garty *et al.*, 1979). However, these interpretations do not explain the relatively short time-scale changes in lichen mineral composition in a fast response to changes in ambient elemental availability (Godinho *et al.*, 2009; Sloof and Wolterbeek, 1992).

In addition to particle entrapment lichens can acquire substances from the environment by extra- or intra-cellular uptake of ions in solution. The uptake of metals from solution by lichens has been studied in laboratory experiments but information on the participation of both symbiotic partners in the uptake and final localization of compounds is scarce (Mrak *et al.*, 2007).

The present work focuses on the elemental microdistributions of thin sections of peripheral and central parts of foliose lichen *Flavoparmelia caperata* after field exposure to industrial pollution, in order to better understand the elements distribution patterns in relation to the lichen constitution, thereby increasing our knowledge on uptake and release mechanisms.

The foliose lichen *F. caperata* was chosen for this work because it is one of the most abundant species in Portugal and is often used as a biomonitor including by our research group.

3.2. Experimental

3.2.1. *Sampling and exposure*

F. caperata thalli, average diameter 3-4 cm, were collected from pine trees located in a clean rural zone in the centre of Portugal (39º30' N, 8º00' W), near Tomar, and transplanted to a polluted location, 180 km southwest of Tomar, near Sines' coal powered station. Transplants were exposed inside a polyamide net (61 μm porosity) hanging, protected from direct rain, during 4 months. Experiment details are described elsewhere (Godinho *et al.*, 2009; Godinho *et al.*, 2004).

3.2.2. *Sample analysis*

At the end of the exposure the samples were rinsed three times with double distilled water for 5 s, air dried for 24 hours at room temperature, freeze dried by immersion in liquid nitrogen and kept frozen at -50ºC until analysis. The lichen thalli were sectioned using a cryo microtome at -65ºC with a glass knife. Sectioning was accomplished by immobilizing the sample in a drop of water and immediately freezing by immersion in liquid nitrogen (modified from[12]). Sections were generally 1-2 mm long (long samples allow better results), 0.5 mm wide and 15μm thick. Thinner samples 10-12 μm break easily during manipulation and often burn during analysis.

Sections were mounted self-supported onto sticky carbon tabs, glued to an Al-holder.

Sections were classified from young (peripheral), the outermost 3-4mm of the thallus, the lobes distinguishable by their colour and their lack of rhizinae, equivalent to an age of about 1 year, to older (central), more central part of the thallus (Godinho *et al.*, 2009).

The samples were examined at the proton microprobe set up of Instituto Tecnológico e Nuclear of Sacavém[21]. Shortly, a proton beam of 2 MeV energy with a current of 100 pA and of 3 μm resolution was used to scan the sample areas of interest. Particle induced X-ray emission (PIXE), Rutherford backscattering spectrometry (RBS), and scanning transmission ion microscopy (STIM) were used simultaneously to obtain morphological and quantitative elemental distribution data. PIXE technique provides minor and trace elemental information. RBS enables the measurement of matrix composition, depth variations and sample stoichiometry. STIM provides measures of density variations, and high-resolution images (<0.5μm) of the sample morphology. The combination of PIXE and RBS data allows quantitative measurements of elemental concentrations. Also the density information obtained through the STIM spectra can also be used to normalise PIXE data for quantitative elemental determinations (Alves *et al.*, 2000; Verissimo *et al.*, 2007).

Maps of the scanned sample regions were generated assigning the various detector signals to a digital X, Y positional coordinate. The relative amount measured is represented by a colour gradient (Grime and Dawson, 1995). The size of the areas scanned matched the size of the thalli that was of the order of 150 x 150 μm. Detailed images of specific features and point analyses along transects rendering concentration profiles were also produced.

Previous to analysis the integrity of the sections were checked under the light microscope.

3.3. Results

F. caperata is a foliose lichen dorsiventrally flattened against the substrate and attached to the substrate by small root-like structures call rhizines. The thallus is stratified.

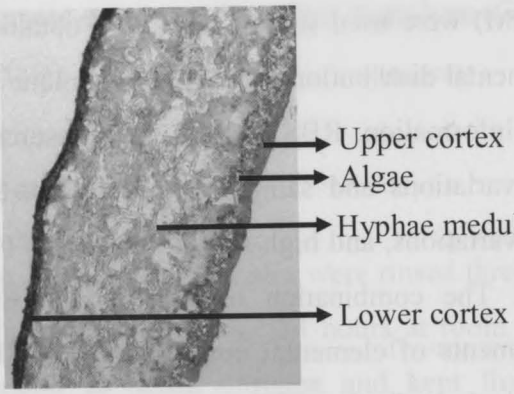


Figure1. Light microscope image (10X magnification) *Flavoparmelia caperata* cross section. Structural layers are labelled. The rectangles indicates the zone and direction of the point analysis performed: 1, Zone scanned in young lichen thallus; 2, Zone scanned in old lichen thallus.

The micrograph in Figure 1, illustrates the typical cross-section of the thallus inspected by Nuclear Microprobe analysis. The microprobe scans were performed along the thallus layered structure. The upper and lower surfaces consist of a dense conglutinated layer of fungal filaments, upper and lower cortex. The central area corresponds to medulla region, which are

less dense, consisting of relatively thick layer of spongy fungi hyphae. The algal layer is located in the upper portion of medulla, immediately beneath the upper cortex.

Table 1 shows the elemental distribution in transversal cuts of young and old parts of *F. Caperata* thallus. The 156x156 μm scan covered all thallus area, as illustrated in Figure 1. The 53x53 μm rows depict a detail of the above scanned area corresponding to the algae layer.

Main features were evidenced by the P, S, Cl, K, Ca, and Fe distributions. In general elemental distributions correlate with the mass distribution, being the superior layer (upper cortex and algae layer), followed by the lower cortex that concentrates most of these elements.

Non-metal elements like S, P, and Cl are more abundant in the upper layer. In young lichen parts P and S distribution clearly defines the algae layer while at the old lichen parts the distribution of these elements is more diffuse.

Ca exhibits a somewhat granular distribution especially in the older thallus, it is abundant in the superior zone but also in some zones of medullar hypha. In the younger part of the thallus Ca appears more regularly distributed in the zone around the algae layer.

Iron is most abundant in algae, and shows a second peak of concentration in the lower cortex.

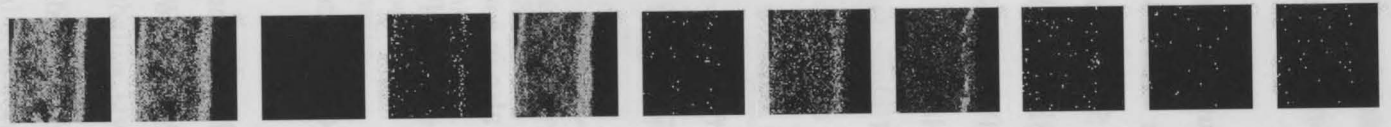
The superior cortex presents the smallest element concentrations while the inferior cortex is rich in chlorine, phosphorous, potassium and iron. The medullar layer generally shows low element quantities except for some spots of high chlorine concentration, especially in younger thallus, and calcium concentration.

Table 1. Elemental distribution in transversal cuts of peripheral and central parts of the thallus of *Flavoparmelia caperata*. The relative amount is represented by the gradient from dark (minimum) to white (maximum).The thallus orientation corresponds to the figure 1 being the algae layer on the right side of the picture.

Ca	Cl	Cu	Fe	K	Mn	P	S	Ti	V	Zn

Central lichen part 159x159 μm scan

Peripheral lichen part 159x159 μm scan



Central lichen part 53x53 μm scan in algae zone

Peripheral lichen part 53x53μm scan in algae zone

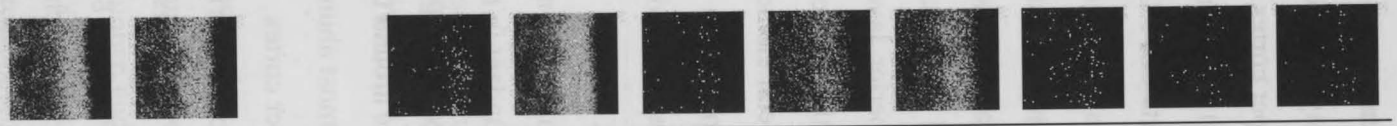


Figure 2 presents the elemental concentration profiles obtained by sequential point analysis performed along transversal transects of the young and old part of the lichen (see Figure 1).

Differences between the older and younger parts of the thallus can be seen in the distributions of Ni, Si and Ti that are more abundant in the old parts of the thallus. Ni is only present in the old medulla, it appears dispersed, not concentrated in particles or crystallized structures. Si and Ti are present in the algae layer of both parts, but especially in some spots of the old medulla. Zn and Mn had a relatively uniform level across the thallus. Mn presented one point of high concentration in the young part of the thallus. Cu and Al were found in the algae layer of both lichen parts and showed an irregular distribution in the medulla. High Cu concentration spots were found in the medulla of old lichen parts.

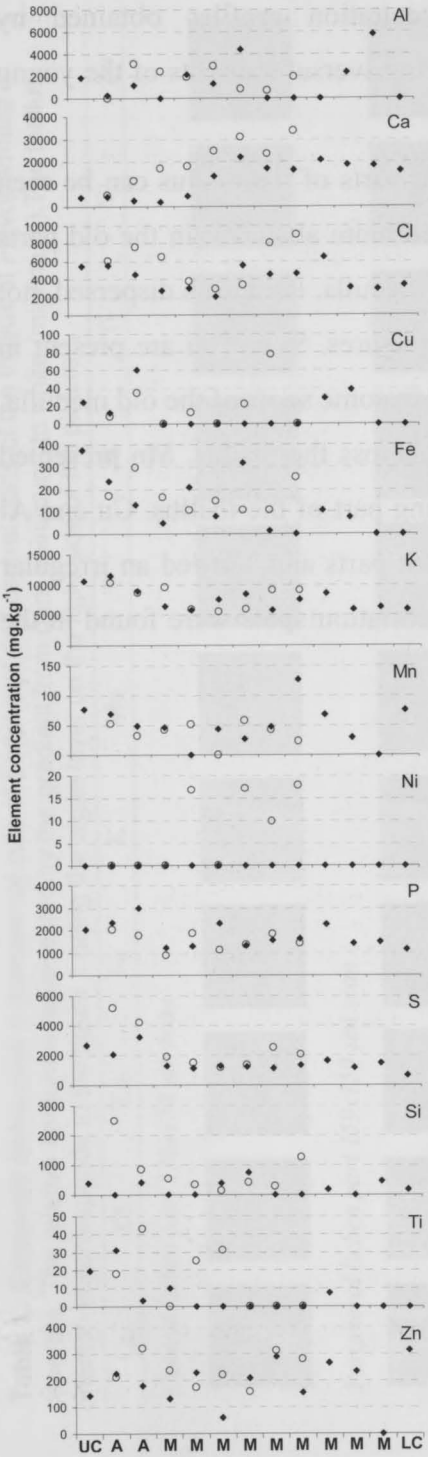


Figure 2. Concentration values obtained in the point analysis transect performed on the central and peripheral *Flavoparmelia caperata* thallus parts (see figure12). Empty circles refer to central lichen part; Filled squares refer to peripheral lichen part. Categories in the X axis refer to spatial localization in the lichen: UC, upper cortex; A, algae layer; M, medullar hypha layer; LC, lower cortex.

Table 2 shows the correlation coefficients between the element concentration values shown in figure 13.

Table 2. Results of correlations between the element concentrations shown at figure 2. n.d. = not determined.

	Al	Si	P	S	Cl	K	Ca	Ti	Mn	Fe	Ni	Cu	Zn
Correlation between concentration distributions in central and peripheral lichen parts													
	-0.98	-0.06	0.30	0.75	0.36	0.33	0.84	n.d.	-0.69	-0.14	n.d.	n.d.	0.08
Central lichen part													
Al	1.00	0.20	-0.28	0.32	-0.04	0.01	-0.54	0.87	-0.49	0.63	0.67	-0.38	0.19
Si		1.00	0.44	0.81	0.36	0.68	-0.51	-0.53	-0.03	0.40	0.52	-0.48	0.04
P			1.00	0.62	0.20	0.20	-0.45	-0.38	0.19	0.01	-0.58	-0.05	0.12
S				1.00	0.46	0.71	-0.78	-0.10	-0.08	0.49	-0.76	-0.13	0.38
Cl					1.00	0.82	-0.06	0.35	-0.87	0.60	-0.37	0.69	0.84
K						1.00	-0.45	-0.20	-0.42	0.44	-0.48	0.25	0.57
Ca							1.00	0.27	-0.25	-0.18	0.39	0.35	-0.08
Ti								1.00	-0.97	0.73	n.d.	0.99	0.83
Mn									1.00	-0.79	-0.04	-0.53	-0.80
Fe										1.00	0.43	-0.07	0.62
Ni											1.00	-0.98	-0.63
Cu												1.00	0.74
Zn													1.00
Peripheral lichen part													
Al	1.00	0.56	-0.65	-0.42	-0.14	-0.50	0.72	-0.45	-0.64	0.34	n.d.	0.29	0.22
Si		1.00	-0.17	0.09	0.08	0.21	0.12	0.22	-0.80	0.57	n.d.	0.45	-0.36
P			1.00	0.81	0.37	0.68	-0.31	-0.13	0.05	0.04	n.d.	0.40	-0.31
S				1.00	0.42	0.71	-0.55	-0.11	0.06	0.37	n.d.	0.43	-0.31
Cl					1.00	0.68	0.12	0.25	0.26	0.48	n.d.	-0.47	0.33
K						1.00	-0.34	0.74	0.22	0.65	n.d.	-0.53	-0.27
Ca							1.00	-0.24	-0.07	-0.17	n.d.	-0.06	0.37
Ti								1.00	0.61	0.63	n.d.	-0.85	0.07
Mn									1.00	0.33	n.d.	-0.60	-0.03
Fe										1.00	n.d.	-0.51	-0.09
Ni											n.d.	n.d.	n.d.
Cu												1.00	-0.19
Zn													1.00

Ca was the only element for which significant positive correlation was found between the distribution concentration in the young and old lichen parts. Al presents a significant but negative correlation meaning that where

older part present higher Al concentrations the younger part present low. Comparing the patterns of element associations in each lichen part it can be observed K associates with S and Cl in both lichen parts. With this exception elements combine differently in the different lichen parts.

3.4. Discussion

The data shows a significant degree of element partitioning in thalli of the foliose lichen *F. caperata.*

Physiological elements like K, P and S presented similar distribution in both young and old lichen parts. In contrast to the progressive decreasing of concentrations from the external layers described for *Xanthoparmelia chlorochroa* by (Clark *et al.*, 2001), the elemental distributions observed suggest selective transport according to metabolic activity. The more pronounced pattern on younger lichens parts reinforces this idea.

In this work the, algae layer of young and older lichen parts concentrated the majority of the elements, both those of physiological importance and metals such as Fe and Cu. Although, microdistributions in exposed lichens reported are not consensual, the high levels of elemental concentrations in the algae layer may suggest lichen sensitivity to high environmental concentrations, namely sulphur.

On the other hand crustal and non metabolic elements like Ni, Si and Ti presented different patterns of distribution in young and in older lichen parts. In younger lichen the second peak of concentrations is mainly localized in the lower cortex. A concentration rise in lower cortex of lichens

exposed, to pollutants was reported (Clark *et al.*, 1999; Budka *et al.*, 2002) suggesting a protective role of this structure. In the older parts of the lichen the localised high concentrations of Ca in the medulla may indicate the presence of calcium oxalate crystals as described by Clark *et al.* (2001) and Wadsten and Moberg (1985).

In both young and older lichen parts particle entrapment in the hypha medullar area could not be observed.

The distribution data of the elements studied suggests that beside passive accumulation there is biological regulation of internal concentrations. Results indicate that elements are not only entrapped but can be mobilized in the thallus suggesting an easy interchange with environment. This agrees with high and fast accumulation previously observed, maybe explained by high capacity of the lower cortex, and points to low remembrances times.

Considering thallus parts, element-specific internal translocation should be taken into account as one more factor affecting lichen "memory length". This observation suggests that tissue-selection in monitoring should be dependent on the element of interest, and cannot be made into a generalized approach in survey set-ups: the choice depends on the element.

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Chapter 4

ASSESSMENT OF LICHEN VITALITY DURING A TRANSPLANTATION EXPERIMENT TO A POLLUTED SITE[3]

4.1. Introduction

In the context of air pollution and damage, several studies have emphasized the potential of in-situ or transplanted lichens as effective biomonitors of air quality (Garty *et al.*, 1996; González *et al.*, 1996; Gries, 1996). Lichen physiological parameters have been shown to reflect the stress caused by pollution giving biologically relevant information on pollution impact (Winner, 1994). Moreover they also correlate with the content of airborne elements accumulated in lichen transplants (Garty *et al.*, 2000). One of the visible signs of pollution damage in lichens is the bleaching or discoloration of the thallus, which is caused by the breakdown of the chlorophyll molecules. Transplanted materials generally show chlorophyll degradation positively correlated with pollution intensity (Garty *et al.*, 1993). Another

[3] M. GODINHO, M. C. FREITAS, H. TH. WOLTERBEEK. Assessment of lichen vitality during a transplantation experiment to a polluted site. Journal of Atmospheric Chemistry 49 (2004), 355–361.

main effect of pollutants is the disturbance of the integrity and organization of the membrane. Changes in membrane permeability to ions have been pointed out as one of the most sensitive physiological responses to environmental stress (Garty *et al.*, 2001), having the advantage of relating to the whole lichen rather than just the photobiont. The loss of electrolytes, measured as the conductivity of the leachate has been shown to correlate with the presence of gaseous pollutants such as SO_2, O_3 and NO_2, catalysts of lipid membrane peroxidation, and also with some high heavy metal cellular concentrations like Cu (Conti and Cecchetti, 2001).

The present work evaluates the stress effects occurring in a transplantation experiment from a clean to a polluted site of two lichen species with different thalli morphology, the foliose *Flavoparmelia caperata* and the fruticose *Evernia prunastri*.

4.2. Methods

4.2.1. *Collection of Lichen Material, Site description, and Transplantation*

Samples of two lichen species *Evernia prunastri* (L.) Ach. and *Flavoparmelia caperata* (L.) Ach. were collected from pine trees located in a clean rural zone in the centre of Portugal (39º30' N, 8º00' W), near Tomar. In the laboratory, samples were cleaned to remove dust, leaf debris, fungus contamination or degraded material, and rinsed three times, for 5 s each, in double distilled water (Garty *et al.*, 2001). Lichens were then cut into individual thallus, and air-dried for 24 h in the laboratory before being

transplanted. Samples were transplanted (1) into the same zone of initial collection, and (2) to a polluted area, 180 km southwest of Tomar, near the Sines industrial complex which includes a refinery, a thermo-electrical power station and a petrolchemical industry. Previous work in this area revealed high contents of various pollution-related elements such as Zn, Se, Pb and Hg (Freitas *et al.*, 1997).

Samples were exposed inside a polyamide net (61 μm porosity) bound with a petri slide (Machado *et al.*, 2003) hanging, protected from direct rain, as described by Freitas *et al.* (2001).

The lichens were exposed during four months, samples being collected periodically in both sites. In Tomar, samples of natural populations were also collected as control. Each time three replicates of material of each species were collected and rinsed three times with double distilled water for 5 s and air-dried for 24 h before being analyzed (Garty *et al.*, 2001).

4.2.2. *Measurement of Photobiont Chlorophyll Content and Integrity*

Samples of 100 mg, 24 hours air-dried lichen material, were immersed in 15 ml of dimethyl sulfoxide (DMSO; Merk, analytical grade) and kept overnight in the dark for pigment extraction. Chlorophyll *a*, *b*, total chlorophyll (*a* + *b*), and the ratio of chlorophyll *a* to phaeophytin *a* (A_{435nm}/A_{415nm}) were determined according to Barnes *et al.* (1992) by means of a Schimadzu V-160 spectrophotometer.

4.2.3. *Assessment of Cell Membrane Integrity in Lichen Thalli*

Samples of 100 mg, 24-hour air-dried material, were immersed in 10 ml double-distilled water for 60 minutes. The electric conductivity of the water was measured by an electric conductivity meter (Consort K 220; Schott Gerate) (modified from Garty *et al.* (2001)).

4.2.4. *Data analyses*

Analysis of variance (ANOVA) with a significant level of $\alpha = 0.05$, and analysis of correlation (CORREL function) were performed in Excel, Office 2000 version.

4.3. Results

4.3.1. *Meteorological data*

Figure 1 presents the meteorological conditions for the two sites, during the experiment period. The meteorological conditions were similar between the two stations except for the total amount of rain. It may be noted that season affects the three parameter values: March and April show the highest rainfall, the lowest temperature and the highest humidity.

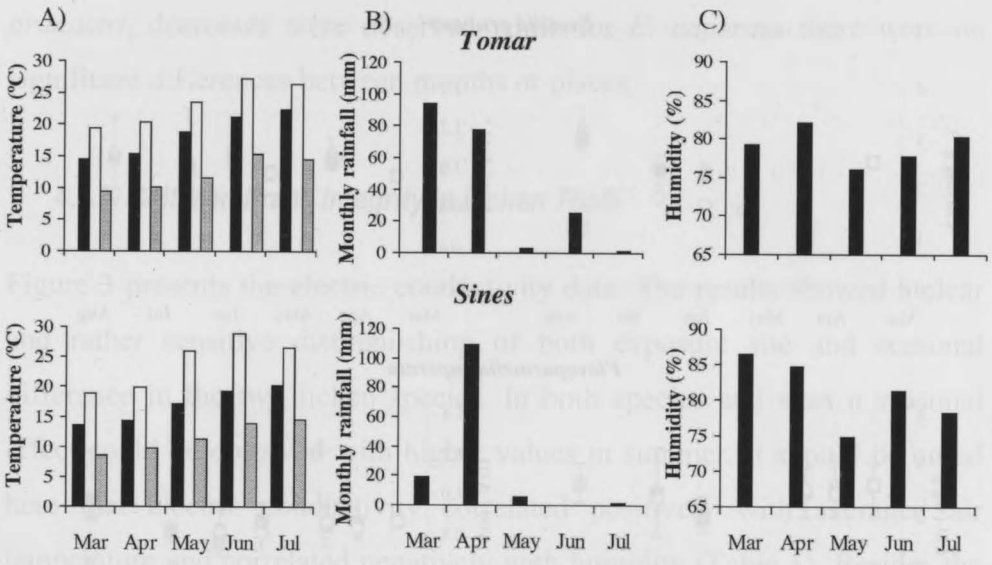

Figure 1. Monthly meteorological parameters measured by the Meteorology Institute of Portugal in the stations Sines and Tomar. (A) Mean monthly temperature (black), mean maximum monthly temperature (white), and mean minimum monthly temperature (gray); (B) Monthly rainfall; (C) Mean monthly relative humidity.

4.3.2. *Chlorophyll Content and Integrity*

The mean values of pigment content and integrity found for the transplant and in-situ samples are illustrated in Figure 2. For both chlorophyll concentration and pigment integrity, there were no significant differences between native and transplanted lichens of both species in Tomar. Chlorophyll *a* / phaeophytin *a* ratio was higher in *E. prunastri* than in *F. caperata* in native specimens and in transplants in both stations.

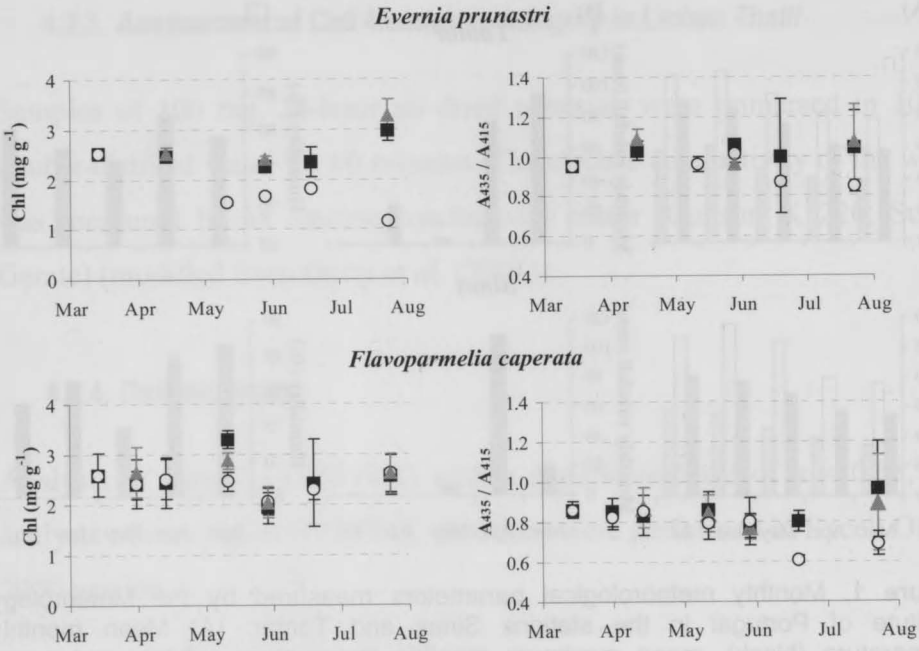

Figure 2. Mean values and standard deviation (n = 3) of chlorophyll content, chlorophyll (*a+b*) and chlorophyll integrity, expressed as A_{435}nm/A_{415}nm ratio, observed at the different sampling times, for *E. prunastri* and *F. caperata* transplanted to Sines (circle) and Tomar (square), and Tomar local populations (triangle).

In spring, the first two months of exposure, the lichens pigments seem not to be influenced by transplantation site, as the integrity values were fairly similar and constant in time and between sites. The samples collected at the end of May and June presented the lowest values for both lichen species in the two places.

In summer, Tomar transplants somewhat recovered, while Sines transplants (especially *E. prunastri*) showed largest chlorophyll degradation. In Sines, total pigment concentration differed between the two lichen species: for *E.*

prunastri, decreases were observed while for *F. caperata* there were no significant differences between months or places.

4.3.3. *Cell Membrane Integrity in Lichen Thalli*

Figure 3 presents the electric conductivity data. The results showed a clear and rather sensitive distinguishing of both exposure site and seasonal difference in the two lichen species. In both species and sites a seasonal effect could be observed with higher values in summer. It should be noted here that electric conductivity correlated positively with averaged air temperature and correlated negatively with humidity (Table 1). Besides the effect from the summer period, there was a significant difference between Sines and Tomar transplants, with higher values for the polluted Sines site.

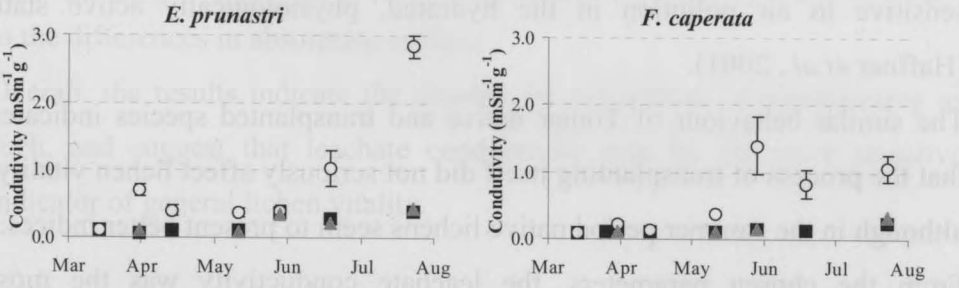

Figure 3. Electric conductivity of water expressing electrolyte leakage in thalli of *E. prunastri* and *F. caperata*, from Tomar (triangle), and transplanted to Sines (circle) and Tomar (square). Each point represents the mean of three replicates, vertical bars denote standard deviations.

Table 1. Correlation coefficients between meteorological variables and electric conductivity values measured in *E. prunastri* and *F. caperata* transplanted to Sines and Tomar.

		Temperature	Humidity
Sines	*E. prunastri*	0.77	
	F. caperata	0.90	-0.95
Tomar	*E. prunastri*	0.81	-0.92
	F caperata	0.82	-0.73

4.4. Discussion

Lichen vitality, as measured with the studied parameters, showed seasonal fluctuations related principally with temperature and humidity. Differences could be found between Sines and Tomar, indicating a possible site (pollution) effect. Lichens seemed more sensitive during summer hot and drier months, although previous studies indicate that lichens are more sensitive to air pollution in the hydrated, physiologically active state (Haffner *et al.*, 2001).

The similar behaviour of Tomar native and transplanted species indicates that the process of transplanting itself did not seriously affect lichen vitality, although in the summer period native lichens seem to present better indices.

From the chosen parameters, the leachate conductivity was the most sensitive, in the sense that it showed higher variability between site and season. Total chlorophyll concentration only showed a decrease in *E. prunastri*, and $A_{435}nm/A_{415}nm$ values only varied in the summer. This result agrees with previous reports of the order of sensitivity of various lichen physiological responses to air pollution (Garty *et al.*, 2000, 2001).

The $A_{435}nm/A_{415}nm$ values of the present study, even those from the control site, were rather low when compared with literature, indicating some degree of phaeophytinization. Ratios are cited to change from 1.41 for non-acidified solution of pure chlorophyll and 0.56 for an acidified solution (Ronen and Galun, 1984). Garty *et al.* (1997) also presents values between 1.40 and 1.45 for lichens transplanted to clean sites against 0.86 to 1.02 to lichens from polluted sites.

E. prunastri was the most sensitive species. When transplanted to Sines it showed an immediate and continuous rise in conductivity values while *F. caperata* managed to recover from initially increasing values from spring onwards to decreasing again but still being more affected at the driest and hottest period. Also, *E. prunastri* was the only that showed a reduction in the total chlorophyll concentration, although that was not in agreement with the $A_{435}nm/A_{415}nm$ values indicating molecule degradation. Haffner *et al.* (2001) also found pigments of foliose species to be more resistant, relating it to the differences in absorption surface.

Overall, the results indicate the absence of any effects of transplanting as such, and suggest that leachate conductivity may be the more sensitive indicator of general lichen vitality.

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Chapter 5

ASSESSMENT OF ACID-BASE BUFFERING PROPERTIES OF *FLAVOPARMELIA CAPERATA*. INFLUENCE OF AGE AND POLLUTION EXPOSURE

5.1. Introduction

Despite the strong interest in lichens for environmental monitoring and biogeochemical cycling studies (Brown, 1991; Richardson, 1995; Garty, 2001), and despite the widespread use of lichen element concentrations in comparative geographical surveys on (trace) element air pollution, the chemical properties and mechanisms governing trace element uptake and sequestration by these organisms remain unclear.

It is generally accepted that element accumulation in lichens reflects the element deposition from the atmosphere. Trace elements are deposited on the lichen surface either as dry particulates or as material dissolved and/or suspended in precipitation. Moreover, several processes besides direct nutrient supply from the atmosphere, such as windblown soil dust, canopy throughfall and uptake of elements from soil water, have been found to affect elemental concentrations in biomonitor pseudotissues.

There are three main mechanisms proposed for trace element uptake of lichens (Richardson, 1995):

(1) Extracellular uptake by an ion exchange process. Lichens operate as ion exchange resins absorbing extracellularly metal ions from rainwater and releasing H^+ ions or metal ions of low binding affinity as the uptake proceeds (Richardson and Nieboer, 1981; Richardson, 1992). This passive (chemical) process, occurring in lichens, is rapid and unaffected by metabolic inhibitors (Nieboer *et al.*, 1976; Burton *et al.*, 1981). Is thought to be one of the mechanisms to escape metal toxicity and this fraction of elements accumulated can be desorbed and recovered.

(2) Intracellular accumulation via passage of an element across the plasma membrane. Unlike the process of extracellular uptake, the uptake at intracellular sites is using an appropriate carrier system and is a slow process (e.g. Nimis *et al.* (2001) observed Cd uptake is light stimulated, suggesting a close relationship existed between uptake and metabolism).

(3) Trapping of metal-rich particulates. Depend on morphology and ecophysiology. The surface characteristics of lichen thalli may determine the efficiency of particle entrapment (Garty *et al.*, 1979; Puckett and Finegan, 1980; Lawrey and Hale, 1981).

Lichens may adapt to their environmental conditions, among others by changes in their properties mentioned under 1-3 above. Therefore, in surveys, lichens may be or may not be comparable in these properties due to strong variations in e.g. pollution levels, and macro/micro climatological conditions. One of the possible protocols to monitor lichen's comparability in terms of uptake mechanism 1 and/or 2 (see above) is the assessment of their acid-base buffering characteristics. The argument here is that lichens

should only be taken into a comparison in terms of accumulated element levels if their comparability in terms of acid-base properties is acceptable. It should be noted that for a full comparison, simultaneous attention should also be devoted to both uptake mechanisms 1, 2 and 3.

As a set up, the present paper focuses on the lichen's binding sites. Considering the acid-base properties, proton binding stands central, and is a very common and simple chemical reaction. The protonation of a molecule controls its charge and thus greatly influences the physical and chemical properties of the molecule. Understanding protonation equilibria is therefore crucial for understanding the chemistry and the reactivity of molecules. Quantification of the chemical thermodynamic properties of lichen surfaces may help to improve our understanding of the relevance of surface complexation mechanisms for element accumulation. Lichens may facilitate this process by optimizing cell-surface properties to maximize the efficiency of ion acquisition or cell-mineral attachment.

Therefore the aim of the present study was to investigate the extracellular ionizable functional groups or binding sites which can play a (binding) ligand's role in young (peripheral) and older (central) lichen thallus parts of the epiphytic lichens *Flavoparmelia Caperata* collected under different pollution load environments. Potentiometric acid-base titration was used for obtaining the capacity of lichens to retain extracellularly bound H^+ and pK_a distributions describing the acid-base properties of lichen.

5.2. Material and Methods

5.2.1. *Exposure, sampling and biomass preparation*

The titration experiments were performed on lichen samples from an experiment previously described in Godinho *et al.* (2008). Tomar samples refer to native *Flavoparmelia caperata* (L.) Ach. collected from pine trees located in a clean rural zone in the centre of Portugal (39º 30' N, 8º 00' W), near Tomar. Lisboa samples refer to lichens transplanted and exposed during 8 months in an industrial urban area, Sacavém, near Lisbon.

After collection the lichens were cleaned and separated in peripheral (younger) and central (older) parts as described in Godinho *et al.* (2009). Peripheral correspond to the outermost 3–4 mm of the thallus corresponding to the lobes distinguishable by their color and their lack of rhizinae. Central corresponds to the inner central part of the lichen thallus. The different parts were then freeze dried and milled in an inert mill (PTFE balls and capsules) under liquid nitrogen and kept dried until use.

5.2.2. *Volume/mass (V/m) ratio and the number protons bound to the ionizable group*

Van Elteren *et al.* (2004) reports it is essential to get insight information on what volume/mass ratios to use in order to obtain the correct extractability of ions. A too small V/m ratio may lead to an underestimate of exchangeability and extractability.

To gain insight in optimal V/m ratio, a separate titration study was performed using the complete thallus of the epiphytic lichen *Parmelia sulcata* sampled from a rural area in the north of Portugal (Freitas *et al.*, 1997) using 3 different volume (V) to lichen mass (m) ratios, V/m respectively 50, 100 and 200 (using respectively 0.5, 0.25 and 0.125 gram lichen in 25 ml MilliQ water).

The number of anionic binding sites in the lichen cell wall that can be presented by ligands is unknown. Therefore a two-site and three-site model was tested and compared (see also "data processing"). These two models are "nested", this means that the two-site model is a simpler case of the three-site model. In that case an F-test can be used to find the most appropriate model.

5.2.3. *Titrations*

All fully automated potentiometric titrations were performed in an electronic titration apparatus (Mettler Toledo model DL-53) equipped with a pH electrode, calibrated with standard buffers at pH 4, 7 and 9, at 25°C temperature. 40ml of MilliQ water or 0.1 M $NaNO_3$ lichen suspension were dispensed into polypropylene vessels, secured to the burette assembly, and mixed allowed to equilibrate for 15min. During experiments a positive pressure of N_2 was maintained in the vessel. Prior to titration the pH of the lichen solution was measured. Fresh titration-grade 0.1 M NaOH and 0.1 M HCl (Titristar) standard solutions were used for titration experiments. Titrant standard solutions were calibrated against potassium hydrogen phthalate (for NaOH) and TRIS (for HCl). Titrations were performed by

adding 10 mL of HNO_3 (0.025 M), acid titration, or 10 mL of NaOH (0.025 M), base titration. Titrant was instrumentally added in volumes of 0.033 mL, after achieving a pH drift at each step of equal to or less than 0.1 mV/10 s. The volume of titrant added and pH were recorded after each addition. Titrant volumes were accounted for in calculating dilution factors. Each titration was performed in duplicate.

5.2.4. *Data processing*

Titration data were modelled using a least squares fit in Micromath Scientist (Micromath Scientific Software, Salt Lake City).

Assuming the lichen to be a 3 protic (organic) acid (see also "Results and Discussion") a model was defined by choosing the added amount of H^+/OH^- as the independent variable and pH as dependent variable. Since the total lichen ligand concentration (A_{tot}) is not known it will be treated as fitted parameter along with the ligand dissociation constant K's (K_{HA}, K_{H_2A}, K_{H_3A}) and the total amount of proton initially present ($H_{tot\text{-}initial}$) as the unknown parameters.

For a triprotic acid the equilibrium conditions are:

$$K_{HA} = \frac{H^+.A^{3-}}{HA^{2-}}$$

$$K_{H_2A} = \frac{H^+.HA^{2-}}{H_2A^-}$$

$$K_{H_3A} = \frac{H^+.H_2A^-}{H_3A}$$

Rewriting these equations gives:

$$HA^{2-} = \frac{H^+.A^{3-}}{K_{HA}}$$

$$H_2A^- = \frac{H^+.HA^{2-}}{K_{H_2A}}$$

$$H_3A = \frac{H^+.H_2A^-}{K_{H_3A}}$$

In the model *HAA* is defined as the concentration of HA^{2-} divided by A^{3-}:

$$HAA = \frac{H^+}{K_{HA}}$$

And similar for H_2AA and H_3AA:

$$H_2AA = \frac{H_2A^-}{A^{3-}} = \frac{H^+.HA^{2-}}{K_{H_2A}} \frac{1}{A^{3-}} = \frac{H^+.HAA}{K_{H_2A}}$$

$$H_3AA = \frac{H_3A}{A^{3-}} = \frac{H^+.H_2A^-}{K_{H_3A}} \frac{1}{A^{3-}} = \frac{H^+.H_2AA}{K_{H_3A}}$$

Sequently *HAA*, H_2AA and H_3AA can be solved.

The mass balance for A can be written and rewritten as:

$$A_{tot} = H_3A + H_2A^- + HA^{2-} + A^{3-}$$

$$= A^{3-}\left(\frac{H_3A}{A^{3-}} + \frac{H_2A^-}{A^{3-}} + \frac{HA^{2-}}{A^{3-}} + 1\right)$$

$$= A^{3-}\left(H_3AA + H_2AA + HAA + 1\right)$$

There for A^{3-} and remaining HA^{2-}, H_2A^- and H_3A can be solved.

$$A^{3-} = \frac{A_{tot}}{H_3AA + H_2AA + HAA + 1}$$

The final equation in the model is the proton balance:

$H^+ + HA^{2-} + 2H_2A^- + 3H_3A - OH^- = H_{tot\text{-}initial} - OH^-_{add}$ ($OH^- = K_{water}/H^+$)

Thus for a given value of added OH^- (OH_{add}), Scientist will iteratively seek the unknown values of the parameters to give a pH that causes the proton mass balance to be satisfied after all the other species concentrations are calculated.

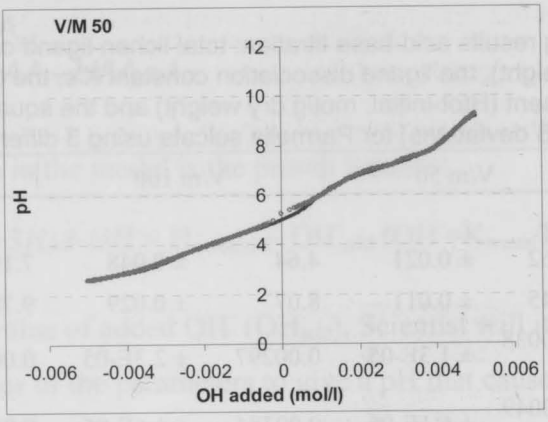
5.3. Results and Discussion

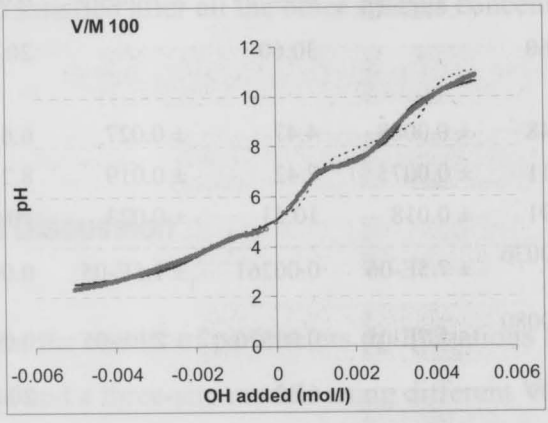
Table 1 summarizes the results of parameters optimizations for the titrations based on a two-site and a three-site models using different V/m ratio's.
For V/m 50 and 100 a three-site model provided an improved fit to the titration data compared with the two-site model (P value F-test<0.0001). This observation is consistent with results from a previous study on lichen (Haas, 1999).

Table 1. Modelling results acid-base titration: total lichen ligand concentration (Atot, mol/g dry weight), the ligand dissociation constant K's, the total amount of proton initially present (Htot-initial, mol/g dry weight) and the squared and summed fitting residuals (SS deviations) for Parmelia sulcata using 3 different V/m ratio's.

	V/m 50		V/m 100		V/m 200	
2 protons						
pK-H_2L	4.62	± 0.021	4.64	± 0.048	7.19	± 0.048
pK-HL	7.45	± 0.011	8.07	± 0.029	9.78	± 0.077
A_{tot}	0.00383	± 1.3E-05	0.00297	± 2.3E-05	0.00154	± 3.2E-05
$H_{Tot\text{-}initual}$	0.00497	± 6.1E-06	0.00384	± 1.6E-05	0.00307	± 5.9E-05
SS deviations	7.60		30.60		20.43	
3 protons						
pK-H_3L	4.48	± 0.0088	4.47	± 0.027	6.63	± 0.082
pK-H_2L	7.11	± 0.0075	7.42	± 0.019	8.26	± 0.110
pK-HL	9.91	± 0.018	10.51	± 0.023	10.12	± 0.19
A_{tot}	0.00361	± 7.5E-06	0.00261	± 1.5E-05	0.00105	± 3.8E-05
$H_{Tot\text{-}initual}$	0.00805	± 1.7E-05	0.00576	± 2.6E-05	0.00303	± 1.1E-4
SS deviations	1.09		6.80		20.82	

Figure 1 presents the titration curves of the different V/m ratio's. It can be observed that the lichen suspension displays relatively weak inflection points especially at the lower V/m ratio's. Taking in account these results the three-site model was accepted using a V/m ratio of 200 (by adding 30/40 mL of MilliQ water or $NaNO_3$ to 130-250 mg of dry weight lichen) to process the Tomar and Lisboa lichen samples.





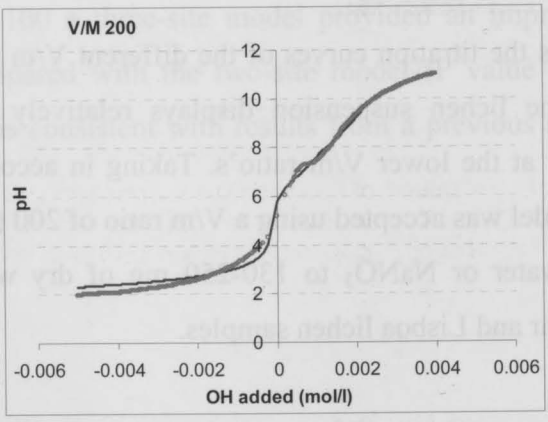


Figure 1. Titration curves of lichen suspension from whole thallus of *Parmelia sulcata* using different V/m ratios. In the graph the fitted two- and three-site is presented by respectively a dashed and a solid line.

Figure 2 presents the measured pH values of initial lichen suspensions. The pH at immersion ranged from 4.26 to 5.09, values within the range reported for other lichen species such as *Cladina* sp. *Umbilicaria* sp. and *Usnea* sp. (Haas, 1999).

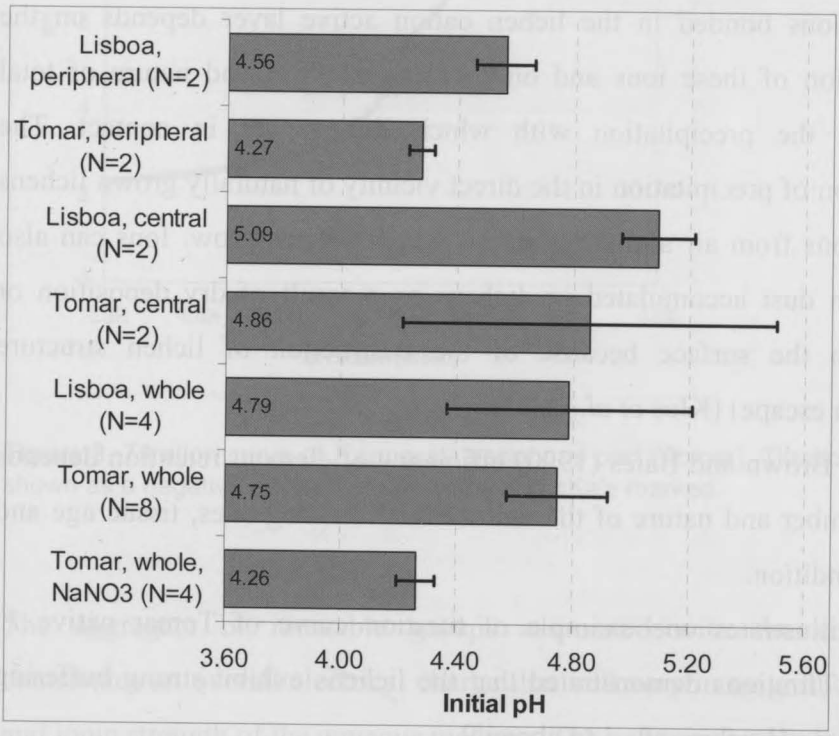


Figure 2. Measured pH values of initial lichen suspension (respectively in MilliQ water and 0.1M $NaNO_3$ for Tomar only) from complete (whole) thallus and central and peripheral thallus parts of *F. caperata* from two sampling locations (Tomar and Lisboa).

A significant differences (student's t-test, P=0.043) in initial pH values can be observed for the central and peripheral part from Lisboa and for the

peripheral part from Lisboa and Tomar (P=0.020), the central parts showing somewhat higher initial pH values.

Klos *et al.* (2006) indicate that the concentration of hydrogen ions in lichens is influenced by the substrate in which the lichen grow and as well as by the composition of atmospheric precipitation. The concentration of mobile hydrogen ions bonded in the lichen cation active layer depends on the concentration of these ions and on the concentration and nature of total cations in the precipitation with which lichens are in contact. The composition of precipitation in the direct vicinity of naturally grown lichens includes ions from air and substrate on which lichens grow. Ions can also come from dust accumulated on lichens as a result of dry deposition or deposit on the surface because of the destruction of lichen structure (potassium escape) (Klos *et al.*, 2005).

Following Brown and Bates (1990) efficiency of element retention depends on the number and nature of the extracellular binding sites, tissue age and growth condition.

Figure 3 illustrates one example of titration curve of Tomar native *F. caperata*. Titrations demonstrated that the lichens exhibit strong buffering capacities at pH values of ~3 to above 9.

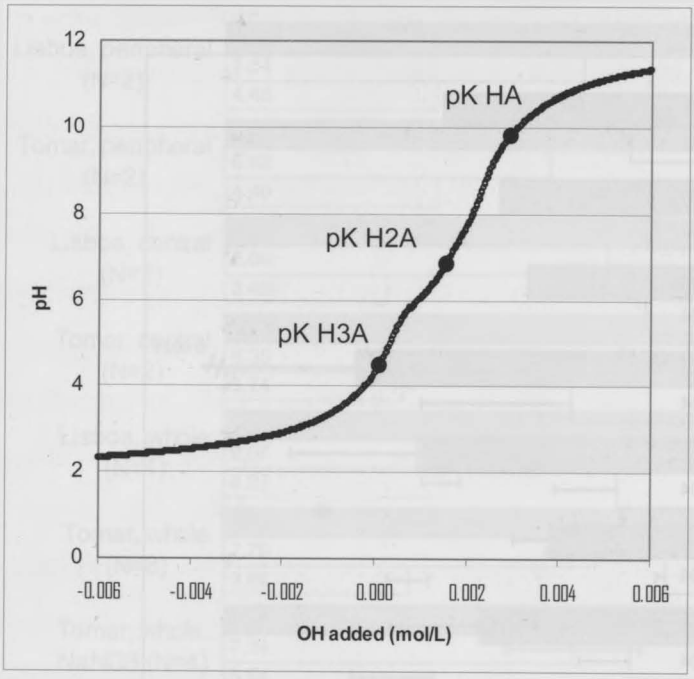


Figure 3. Titration curve of *F.caperata* peripheral part (Tomar). Titration with H^+ is shown as a negative addition of OH^- with fitted pKa's marked.

The aggregate of ionizable groups associated with the cell surface contributes an overall electrical charge that will vary in magnitude with pH and ionic strength of the aqueous medium. The cell membrane displays a net negative charge at pH greater than ~3 and is neutrally charged at lower pH values.

The effect of ionic strength on proton binding can be explained by competition of Na for the electrostatic binding to the biomass: at higher ionic strength Na balances most of the negative charges in the biomass.

The Figures 4 and 5 presents the results of optimal fits to the titration of *F. caperata* constrained according to 3 protic acid model.

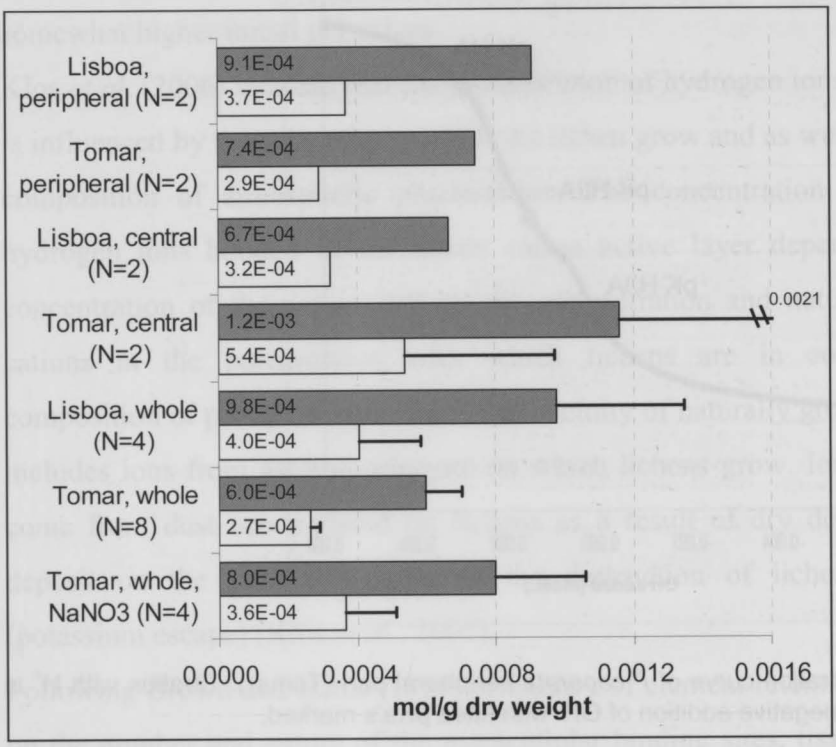


Figure 4. Modelled total lichen ligand capacity, A_{tot} ($=A^{3-} + HA^{2-} + H_2A^{-} + H_3A$) (White) and initial total H concentration, $H_{tot\text{-}initial}$ ($=H^{+}+HA^{2-}+2H_2A^{-}+3H_3A$) (Grey), of lichen suspensions (respectively in MilliQ water and 0.1M $NaNO_3$ for Tomar only) from complete (whole) thallus and central and peripheral thallus parts of *F. caperata* from two sampling locations (Tomar and Lisboa).

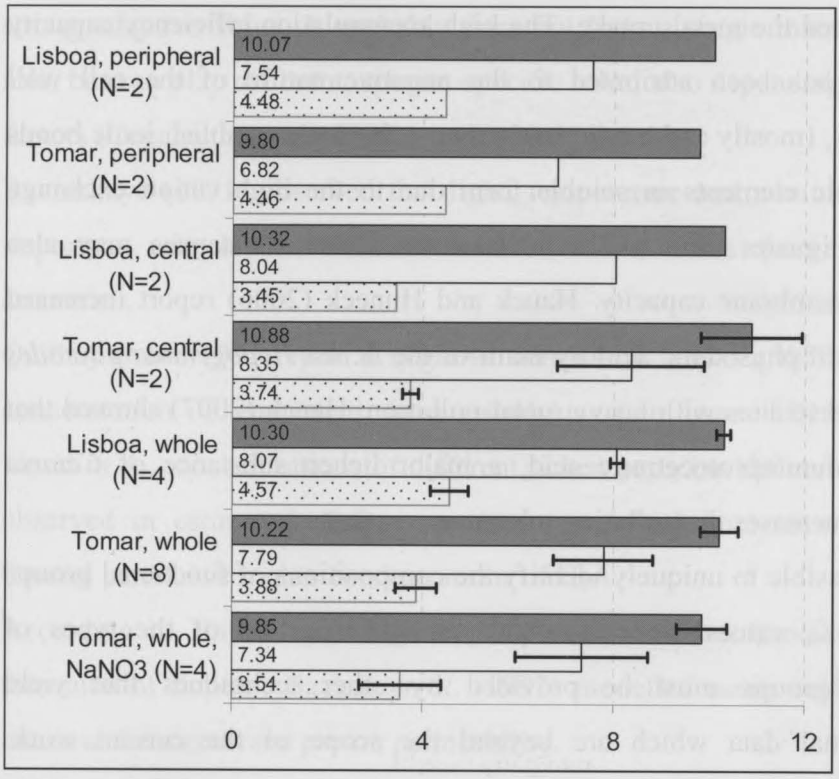


Figure 5. Modelled pKa's values of lichen suspensions (respectively in MilliQ water and 0.1M $NaNO_3$ for Tomar only) from complete (whole) thallus and central and peripheral thallus parts of *F. caperata* from two sampling locations (Tomar and Lisboa). Grey, pKa-HA; White, pKa-H_2A; Dotted, pKa-H_3A.

No significant difference can be observed in different lichen parts and sample locations in both capacities and pK values. pK_a-H_3A varies from 3.54-4.57; pK_a-H_2A varies from 7.34-8.35; pK_a-HA varies from 9.80-10.88. Although previous work also reports three site models to best describe lichen pH titration data (Haas, 1999), some differences in acid-dissociation constant values were found. Haas (1999) found for *Usnea mammulata* pK_a~6.5, ~7.6, and 9.9 being the one-site model with the smallest pK_a that

best described the metal uptake. The high accumulation efficiency capacity of lichens has been attributed to the negative nature of the cell wall constituents, (mostly carboxylic acid groups) that may establish ionic bonds with cationic elements in soluble form due to the high cation exchange capacity (Figueira *et al.*, 2002). However lichen substances may also influence membrane capacity. Hauck and Huneck (2006) report increased production of physodalic acid by thalli of the lichen *Hypogymnia physodes* transplanted to sites with heavy metal pollution. Hauck (2007) showed that depsidone fumarprotocetraric acid, a major lichen substance of *Cetaria islandica*, increases the pollution tolerance.

It is not possible to uniquely identify the compositions of functional groups by their pK_a values alone. Unequivocal identification of the types of functional groups must be provided by other techniques that yield compositional data which are beyond the scope of the current work. Constrained pK_a values, may, however, be used to suggest or exclude possible functionalities.

F. caperata contains usnic, protocetraric, and caperatic acids, and atranorin (Brodo *et al.*, 2001). Usnic acid is giving the *F. Caperata* cortex a very distinctive pale yellow green color when dry.

Sharma *et al.* (1966) reports pK's of usnic acid: 4.4, 8.8 and 10.7, values within the range of the described by the present work. Although Cansaran Duman *et al.* (2008) report usnic acid concentration in *Flavoparmelia caperata* to range between 0.47-2.38 percent dry weight while A_{tot} found in our study (figure 21) varies from ≈ 9.3-18.6 %.

Usnic acid is one of the most common lichen substances, spread in several even phylogenetically distant genera, suggesting an ancient origin and a

strong adaptive value. It probably does not have a single main biological role, but can play different, species-specific roles, also depending on habitat factors (Cocchietto *et al.*, 2002).

Ravinskaya (1991) observed that light, temperature and humidity play an important role in the concentration of lichen acids (usnic acid and atranorin).

Quilhot *et al.*, 1991 report a negative correlation between thallus age and concentration of usnic acid. In our study no significant differences were found in A_{tot} concentrations of different lichen parts but differences can be observed in estimated surface speciation at initial immersion (table 2). Central parts have higher concentrations of H_2A^-

According to Hauck and Jurgens (2008) usnic acid controls the acidity tolerance of lichens. Backor *et al.* (1997) modelled usnic acid ability to increase the membrane proton permeability.

Table 2. Initial speciation as % of Atot calculated with initial measured pH and modelled pKa's.

	N	A^{3-}	HA^{2-}	H_2A^-	H_3A
Lisboa, peripheral thallus	2	<0.01	0.06 ± 0.02	54.8 ± 5.8	45.1 ± 5.8
Tomar, peripheral thallus	2	<0.01	0.11 ± 0.02	39.2 ± 2.5	60.7 ± 2.5
Lisboa, central thallus	2	<0.01	0.1 1 ± 0.03	97.6 ± 0.6	2.29 ± 0.63
Tomar, central thallus	4	<0.01	0.09 ± 0.09	89.8 ± 6.7	10.1 ± 6.7
Lisboa, whole thallus	4	<0.01	0.05 ± 0.07	57.3 ± 26.3	42.7 ± 26.4
Tomar, whole thallus	8	<0.01	0.11 ± 0.18	70.6 ± 37.4	29.3 ± 37.5
Tomar, whole thallus, 0.1M $NaNO_3$	4	<0.01	0.34 ± 0.38	76.6 ± 20.8	23.1 ± 21.1

5.4. Conclusion

The results indicate that for *Flavoparmelia caperata*, neither ambient conditions nor specific lichen thallus parts affect capacity and pK-values of ionizable groups. This suggests that *F. caperata* may be used in surveys comprising variable ambient conditions.

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Chapter 6

BIOACCUMULATION BEHAVIOUR OF TRANSPLANTS OF THE LICHEN *FLAVOPARMELIA CAPERATA* IN RELATION TO TOTAL DEPOSITION AT A POLLUTED LOCATION IN PORTUGAL[4]

6.1. Introduction

Atmospheric particulates have attracted great environmental concern over the last few decades because of evidence that they are associated with respiratory and cardiovascular diseases in humans (Dockery and Pope, 1994).

During the last 30 years many studies have underlined the relevance of using lichens as biomonitors of air quality. Owing to their dependence on atmospheric inputs for their nutrient supply and their capacity for biomagnification of environmental concentrations, lichens can provide information on the bioavailability of persistent atmospheric pollutants and their biological effects. In addition, biomonitoring offers other advantages compared to instrumental methods such as: low cost, independence of

[4] R.M. GODINHO, H.TH. WOLTERBEEK, T. VERBURG, M.C. FREITAS.Bioaccumulation behaviour of transplants of the lichen *Flavoparmelia caperata* in relation to total deposition at a polluted location in Portugal. Environmental Pollution 151 (2008), 318-325.

power supply as well as easier sampling, sample handling and easier determination of trace elements.

Lichens may be used as bioindicators and/or biomonitors in different ways (Conti and Cecchetti, 2001; Szczepaniak and Biziuk, 2003; Wolterbeek, 2002). Although considerably less active than passive biomonitoring studies have been carried out in recent years, the use of transplants bear additional important advantages: possibility to sample even in areas devoid of lichens, standardization of experimental material in terms of physiological condition and lastly capacity for bioconcentration (Fernandez *et al.*, 2000). With the transplant technique there are no differences in the content or in lichen vitality at the beginning of the research and the exposure time is well defined, which allows the comparison between wet and dry deposition.

Some authors found linear relationships between transplants and native organisms' responses, although differences due to acclimatization of native populations have also been described (Sloof, 1995; Fernandez *et al.*, 2000).

It is generally accepted that element accumulation in lichens reflects the element deposition from the atmosphere. Traditionally, the biomonitor has been regarded as a passive accumulator reflecting a long-term integration of atmospheric pollution (Sloof, 1995; Galsomies *et al.*, 2003; Bennett and Wetmore, 2003). Trace elements are deposited on the lichen surface either as dry particulates or as material dissolved and/or suspended in precipitation. Trace elements may be retained by particulate entrapment, physiochemical processes such as ion exchange, as well as by passive and active intracellular uptake (Tyler, 1989). In view of the complexity of these processes, it is not accurate to regard lichens as mere passive filters (Bari *et al.*, 2001); instead accumulation may be viewed as a dynamic process,

involving uptake and release processes until equilibrium with the surrounding environment is reached (Reis *et al.*, 1999). Previous attempts of correlating element concentrations in lichen thalli with those in the atmosphere also suggest that lichens might reflect mainly the bulk (wet and dry) deposition (Sloof, 1995).

Many environmental factors contribute to fluctuations and sometimes intermittent changes in metal levels in the atmospheric environment (Samecka-Cymerman *et al.*, 2005). In natural conditions, as opposed to laboratory conditions, stable availability values do not exist. Instead, biomonitors may be subjected to either situations of typically low availability, meaning that some sparse high availability episodes may occur, or to situations of typically high availability conditions, with sparse low availability episodes. The question is how the time-related variability in element concentrations in lichens reflects this changing ambient scenario?

Besides the variations due to element deposition, the metal content in lichens varies with time during periods of accumulation and release, either due to environmental causes (meteorology, pH) or to physiological conditions (metabolism, detoxification mechanisms).Moreover, several processes besides direct nutrient supply from the atmosphere, such as windblown soil dust, canopy through fall and uptake of elements from soil water, have been found to affect metal concentrations in biomonitor pseudotissues.

The memory loss concept of Reis *et al.* (1999) defines a remembrance time l, which is essentially an exponent parameter expressing the rate in time at which a moment in atmospheric element availability is losing relevance in being reflected by the lichen element content or concentration. The term l

may be regarded as reflecting the time during which the biomonitor ''preserves'' the memory of a given environmental availability condition, based on uptake and release processes. It may be clear that the lichen's release rate correlates to l, and that the lichen's rate of element uptake correlates with the lichen transplant's progressive reflection of new ambient conditions.

Accurate evaluation of how and to what extent lichens reflect current or some time-integrated concentrations of heavy metals in the atmosphere is essential for their use in biomonitoring. The preceding discussion implies that lichens may reflect element deposition periods which are longer than the transplantation period (i.e. they preserve background area information), or periods that are shorter than the total transplantation period (i.e. possibly reflecting shorter sub-periods of exposure). The present study uses the cross transplant technique in order to determine ''lichen remembrances''. This aim is achieved by investigating the correlations between element concentrations in exposed lichen transplants with those in atmospheric element deposition for different exposure periods.

The foliose lichen *Flavoparmelia caperata* was chosen for this work because it is one of the most abundant species in Portugal and is often used as a biomonitor including by our research group.

6.2. Materials and Methods

6.2.1. *Sampling, transplantation and site characterization*

Samples of *Flavoparmelia caperata* (L.) Ach. were collected from pine trees located in a clean rural zone in the centre of Portugal (39º 300'N, 8º 00'W), near Tomar, and transplanted: (1) into the same zone of initial collection; and (2) to an industrial-urban area, Sacavém, near Lisbon. Lichens were cleaned, sorted and exposed attached to the substratum in nylon net bags (2 mm porosity) with protection from direct rain, as described by Freitas *et al.* (2001).

The experiment lasted one year (November 2003 to November 2004) and consisted of two types of exposure: in the cumulative exposure mode, the lichens were exposed at the beginning of the experiment and were sampled every two months until 12 months of exposure. In the short exposure mode, in each sampling date, a new set of lichens were exposed for two months (Figure 1). At each sampling date two replicates of material were collected.

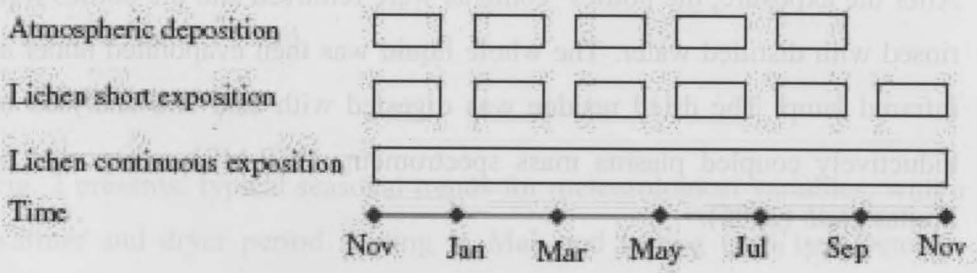


Figure 1. Experiment setup.

Atmospheric deposition was measured as bulk deposition. Collectors consisting of a funnel connected to a polyethylene bottle (previously washed with acid) were exposed to the atmosphere for two-month periods. Precipitation thus collected consisted of the wet deposition flux and the portion of dry deposition flux associated with gravitational sedimentation. The funnel was protected with nylon net in order to avoid fouling of insects. Meteorological data was supplied by the Portuguese Meteorological Institute.

6.2.2. *Analytical procedures*

In the laboratory, lichen samples were firstly cleaned from dust, leaf debris, fungus contamination and degraded material. Then they were rinsed three times for 5 s, in double distilled water (Godinho *et al.*, 2004; Garty *et al.*, 2001), freeze dried and milled in an inert mill (PTFE balls and capsules) under liquid nitrogen. Elemental content were determined by means of k0-standardized, instrumental neutron activation analysis (k0-INAA), following procedures described in Machado *et al.* (2004) and Freitas *et al.* (2006).
After the exposure, the bottles' contents were removed and the bottles were rinsed with distilled water. The whole liquid was then evaporated under an infrared lamp. The dried residue was digested with acid and analysed by inductively coupled plasma mass spectrometry (ICP-MS) as reported by Freitas *et al.* (2006).

6.2.3. *Assessment of cell membrane integrity in lichen thalli*

Samples of 100 mg, 24-h wet chamber material, were immersed in 10 ml double distilled water for 60 min. The electric conductivity of the water was measured by an electric conductivity meter (Consort K 220; Schott Gerate). The acidity of the leakage solution was checked with a digital pH meter before and after the lichens immersion (modified from Marques *et al.*, 2005).

6.2.4. *Data analysis*

Data handling was performed in Excel. Lichen vitality, assessed by their electric conductivity, was compared in lichens exposed in the polluted and clean site by means of Analysis of Variance (ANOVA function) with a significance level set at α=0.05. Correlations were performed with the CORREL function.

6.3. Results and discussion

6.3.1. *Meteorology*

Fig. 2 presents' typical seasonal trends for meteorological variables, with a warmer and dryer period starting in May and lasting until late October; humidity was relatively high throughout the whole experiment period.

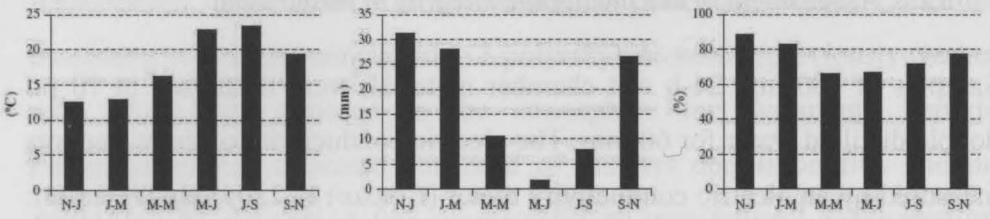


Figure 2. Meteorological parameters measured by the Meteorology Institute of Portugal at Lisboa: From left: mean bimonthly temperature, mean bimonthly rainfall, and bimonthly relative humidity.

6.3.2. *Lichen vitality*

The pH of the leakage solutions varied between 5.8 and 6.5, high enough to discard conductivity pH influence on conductivity values. Some authors also pointed out that particulate matter deposited on the lichen thallus surface could bias conductivity values.

We emphasize the sample washing before the conductivity measurements as well as the samples immersion in water with no mixing as procedures to avoid this source of perturbation.

The values of electric conductivity presented in Fig. 3 are lower than those previously reported for *Flavoparmelia caperata* collected in polluted areas of Portugal (Godinho *et al.*, 2004; Marques *et al.*, 2005). Nevertheless there is a clear difference between exposure sites, with higher values for the polluted site. Garty *et al.* (1998) reported differences of a factor of 2 in electrical conductivity of lichen batches from industrial polluted sites compared with those from rural sites. This was not verified in this experiment for short periods of exposition that did not present significant

differences between clean and polluted sites, suggesting that it took some time for the lichen to be influenced by the exposition.

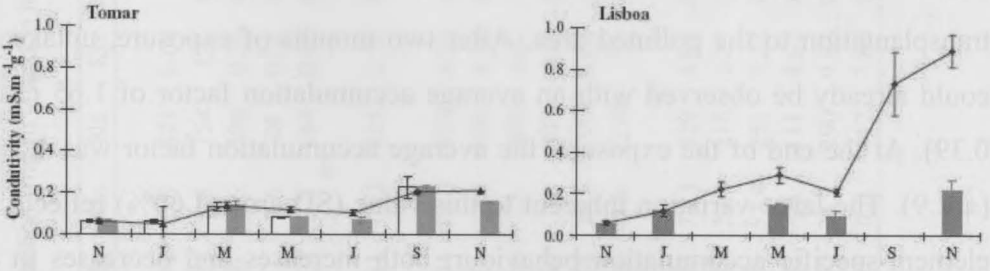


Figure 3. Electric conductivity of water expressing electrolyte leakage in thalli *F. caperata*, from Tomar, and transplanted to Tomar and Lisboa. Tomar natives, white column; Short exposition, grey column; Cumulative exposition, line. Each result represents the mean of three replicates, vertical bars denote standard deviations.

Both exposure sites show a seasonal trend where the onset of the dry hot season is associated with higher conductivity values. A seasonal effect on lichen membrane permeability, mostly associated with meteorological causes (temperature and humidity), has also been reported in previous studies (Godinho *et al.*, 2004; Marques *et al.*, 2005), and suggests that the better performance of lichens exposed for short periods at the polluted site might be due to the high humidity levels found in Lisbon.

In Tomar, the fact that native and *in situ* transplanted species showed similar behaviours indicates that the process of transplanting itself did not seriously influence lichen vitality.

6.3.3. *Elemental concentrations*

The elemental concentrations values found in lichen and bulk deposition samples are shown in Table 1. Results obtained with both analytical techniques (INAA and ICP-MS) has been presented elsewhere (Freitas *et al.*, 2006).

The data shows an increase in lichen elemental concentrations after transplantation to the polluted area. After two months of exposure, uptake could already be observed with an average accumulation factor of 1.65 (± 0.39). At the end of the exposure, the average accumulation factor was 4.3 (± 2.9). The large variation inherent to this value (SD around 60%) reflects element-specific accumulation behaviour: both increases and decreases in concentrations occurred during the 12 months of exposure, as previously described (Bari *et al.*, 2001); whereas for some elements no changes in concentrations were observed.

According to Wolterbeek *et al.* (2002), after a change in ambient deposition conditions, the period towards a new equilibrium is associated with the lichen's remembrance time, which in this context gives an expression of the length of the foregoing environmental availability period reflected by the lichen elemental content. This period is element specific, and depends on ambient and lichen morphological/physiological conditions. Measuring the lichen elemental concentration at the end of an exposure experiment of a specific duration sets a time-window that can be either fitting, or too small or too large for the lichen to reflect the selected period of environmental availability.

Table 1. Trace elemental concentrations in bulk atmospheric deposition ($mg.m^{-2}$) and in lichen *Flavoparmelia caperata*. Uncertainties are between 5 and 10%. ($mg.kg^{-1}$).

	As	Ca	Co	Cr	Cs	Fe	Hf	K	La	Mg	Mn	Na	Rb	Sb	Sc	Sm	Zn
Total deposition																	
Nov-Jan	0.04	602	0.02	0.2	0.004	24	0.002	58	0.02	111	0.6	821	0.09	0.1	0.01	0.002	5.2
Jan-Mar	0.19	1715	0.17	1.5	0.067	440	0.05	270	0.47	233	6.8	754	1.3	0.65	0.17	0.08	35.5
Mar-May	0.05	510	0.03	0.3	0.013	82	0.01	69	0.08	60	1.6	296	0.28	0.11	0.03	0.01	4.9
May-Jul	0.01	321	0.01	0.3	0.002	13	0.001	17	0.01	11	0.6	24	0.05	0.01	0.01	0.002	2.3
Jul-Sep	0.12	1110	0.1	0.9	0.048	283	0.03	162	0.26	103	3.7	164	0.85	0.17	0.14	0.04	8.8
Background lichens																	
	0.34	13100	0.12	7.86	0.07	260	0.03	6300	0.27	581	14.2	835	2.76	0.11	0.155	0.044	53
Cumulative exposure																	
Jan	0.49	14400	0.23	9.93	0.14	354	0.06	5360	0.45	804	23.3	929	4.93	0.29	0.286	0.082	69
Mar	0.45	6400	0.24	34.7	0.09	294	0.09	4960	0.37	712	20.5	669	4.74	0.45	0.221	0.056	95
May	0.49	18900	0.56	45.2	0.12	1130	0.13	6260	0.62	766	25	400	n.d.	0.9	0.26	0.18	189
Jul	0.47	15200	0.3	10.9	0.13	733	0.16	6140	0.67	660	20	803	5.4	1.01	0.284	0.085	n.d.
Sep	0.42	21600	2.42	17.3	0.16	987	0.14	6400	0.84	758	21.5	1380	8.64	1.02	0.247	0.149	192
Nov	0.51	8860	0.67	19.7	0.25	1270	0.22	3150	0.82	n.d.	n.d.	1210	7	1	0.3	0.22	73
Short exposure																	
Nov-Jan	0.49	14400	0.23	9.93	0.14	354	0.06	5360	0.45	804	23.3	929	4.93	0.29	0.29	0.082	69
Jan-Mar	0.75	n.d.	0.23	15.2	0.25	518	0.09	n.d.	0.05	663	22.3	210	n.d.	0.28	0.145	0.263	86
Mar-May	0.6	21700	0.9	17.2	0.17	635	0.14	6530	0.67	581	57.4	251	7.22	0.36	0.19	0.19	60
May-Jul	0.36	7320	0.21	9.58	0.09	375	0.09	3950	0.51	632	25	111	4.52	0.17	0.12	0.11	79
Jul-Sep	0.62	1380	0.23	10	0.11	n.d.	n.d.	4190	0.73	772	24.1	290	9.88	0.24	0.172	0.205	110
Sep-Nov	0.58	14300	0.53	18.2	0.13	624	0.16	5330	0.7	n.d.	n.d.	417	7.86	0.27	0.2	0.2	100

n.d. = not detected

Table 2. Results of the correlations between trace element concentrations values in the lichens and in the bulk atmospheric samples. Artwork in the top of each column refer to figure 1 and illustrate the periods of atmospheric deposition (on the top) and lichen exposure (below) that are being related. S= slope; I= intercept; R= correlation coefficient; P= probability; Y(i)= calculated lichen concentration; C1, C2= constants; x(i)= atmospheric deposition period.

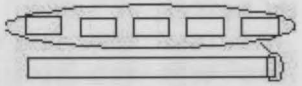
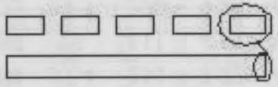
Short exposition					Cumulative exposition											
					 y(i)=C1*sum x(0…i)+C2				 Y(i)=C1*x(i)+C2				 y(i)=C1*x(i-1)+C2			
	S	I	R	P	C1	C2	R	P	C1	C2	R	P	C1	C2	R	P
As	*1.79*	*0.42*	*0.91*	*0.03*	-0.23	0.51	-0.53	0.11	-0.35	0.48	-0.44	0.2	0.55	0.41	0.7	0.05
Ca	-14.3	20283	-0.55	0.45	1.11	10256	0.28	0.5	*-4.5*	*16805*	*-0.52*	*0.19*	4.04	9863	0.35	0.5
Co	-1.15	0.43	-0.25	0.68	*3.07*	*-0.07*	*0.54*	*0.1*	2.03	0.4	0.21	0.55	-1.8	0.72	-0.19	0.65
Cr	1.77	11.3	0.27	0.66	3.58	14	0.25	0.54	11.23	12.8	0.44	0.27	*19.3*	*15*	*0.67*	*0.15*
Cs	*1.31*	*0.12*	*0.61*	*0.27*	0.26	0.12	0.37	0.36	0.06	0.14	0.04	0.92	-0.52	0.15	-0.32	0.53
Fe	0.25	437	0.38	0.62	0.6	406	0.55	0.13	-0.17	724	-0.08	0.84	*1.19*	*596*	*0.72*	*0.07*
Hf	-0.09	0.1	-0.06	0.94	*0.72*	*0.08*	*0.75*	*0.02*	-0.16	0.12	-0.09	0.81	0.03	0.12	0.03	0.94
K	-2.22	5179	-0.11	0.89	-2.31	6089	-0.33	0.42	*-4.72*	*5889*	*-0.4*	*0.33*	5.94	4486	0.46	0.35
La	-0.79	0.62	-0.59	0.3	*0.29*	*0.44*	*0.64*	*0.12*	-0.17	0.58	-0.21	0.65	0.08	0.58	0.09	0.88
Mg	0.27	663	0.23	0.71	-0.15	795	-0.43	0.21	0.08	732	0.12	0.75	*0.39*	*690*	*0.67*	*0.07*
Mn	-1.5	34.4	-0.26	0.67	-0.02	25	-0.02	0.96	-0.84	27.3	-0.5	0.14	*1.31*	*22*	*0.78*	*0.02*
Na	0.6	110	0.66	0.22	-0.09	887	-0.13	0.78	0.04	715	0.05	0.92	*-0.74*	*1060*	*-0.8*	*0.06*
Rb	*6.5*	*4.59*	*0.97*	*0.03*	0.71	5.2	0.56	0.25	-0.13	6.12	-0.06	0.91	-1.76	6.59	-0.14	0.86
Sb	0.05	0.26	0.19	0.77	*0.55*	*0.27*	*0.69*	*0.03*	-0.44	0.76	-0.38	0.28	-0.1	0.77	-0.12	0.78
Sc	-0.3	0.2	-0.36	0.55	0.1	0.22	0.17	0.63	0.14	0.23	0.16	0.67	*0.3*	*0.24*	*0.42*	*0.3*
Sm	*2.07*	*0.11*	*0.91*	*0.03*	0.8	0.07	0.68	0.09	-0.08	0.13	-0.04	0.94	0.6	0.13	0.29	0.64
Zn	0.38	77	0.27	0.66	*1.19*	*72*	*0.51*	*0.13*	0.04	118	0.01	0.98	0.85	118	0.28	0.5

Table 2 presents different ways of correlating elements in lichens and in atmospheric deposition, accounting for different remembrance times: long (column 2), intermediate (column 4) or short (columns 1 and 3). The best correlation found for each element is presented in italics. Elements for which no correlation could be found in any of the trials may be interpreted as elements for which the lichen's remembrance time is outside the time-window of the experiment.

In column 2 the lichen final concentration is correlated with the bulk element deposition, summed over the total previous periods. This is the closest approach to the traditional view on moss-deposition relationships (Berg and Steinnes, 1997; Berg *et al.*, 1995) that assumes an infinite remembrance time coupled to a metal specific efficiency in the moss uptake/retention process. This may be the case for Sb and Hf whose lichen concentrations were mostly rising irrespective of variations in the element deposition; the periods of small atmospheric availability were not reflected. For Sb its concentration in the lichen seemed to progress up to a certain plateau (Fig. 4). Also for elements such as Sm and Co rather reasonable correlations with the sum of longer-termed deposition were found, although the lichen did not fully reflect the summed atmospheric deposition. For these two metals, strong fluctuations were observed in both lichen concentrations and atmospheric deposition, but for both highest values were obtained at the end of the experiment period.

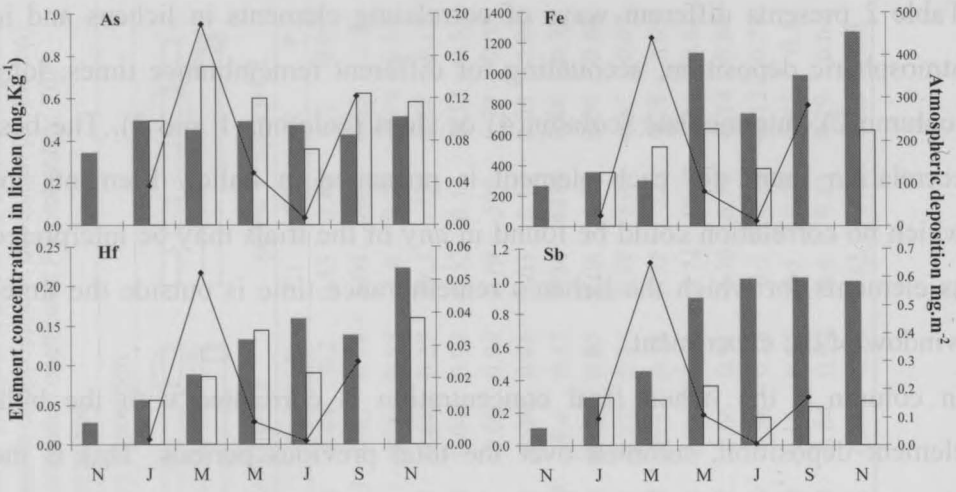


Figure 4. Trace element concentrations in bulk atmospheric deposition samples and in lichen *Flavoparmelia caperata*. Grey, lichens in cumulative exposition; white, lichens in short exposition; line, bulk atmospheric deposition.

The first column of Table 2 correlates each two-month period of lichen exposure with the corresponding period of atmospheric deposition. This approach highlights the elements for which the lichen has a relatively short remembrance time. As can be seen from Table 2, and although two months of exposure were enough to show increases in lichen element concentrations, these increases were not reflecting the observed atmospheric deposition during the exposure period, meaning that the two months did not fit the lichen remembrance time. However, significant correlations were obtained for As, Rb and Sm. This result suggests that the lichen's remembrance time for As, Rb and Sm is about two months. This in turn may imply that even if the lichen is exposed for say one year, the final lichen concentrations only reflects the last two months of atmospheric deposition. It should be noted here, however, that the column 3 in Table 2, does not

show significant correlations for As, Rb and Sm. This result may be interpreted as meaning that lichens in cumulative exposure (Table 2, column 3) are behaving differently compared to lichens in short exposure periods (Table 2, column 1). For instance, Fig. 4 shows that the lichen exposed to As deposition for a short time exhibits higher variations in As concentration than the lichen in the cumulative exposition, which shows smoother concentration curves. This behaviour can be considered as similar to the acclimatization behaviour described for native species (Fernandez *et al.*, 2000). In the cumulative exposure trial, the arsenic content in the lichen was significantly correlated to the last-but-one period of deposition (Table 2, column 4), meaning that the lichen is taking more than two months to "forget" the previous environmental situation.

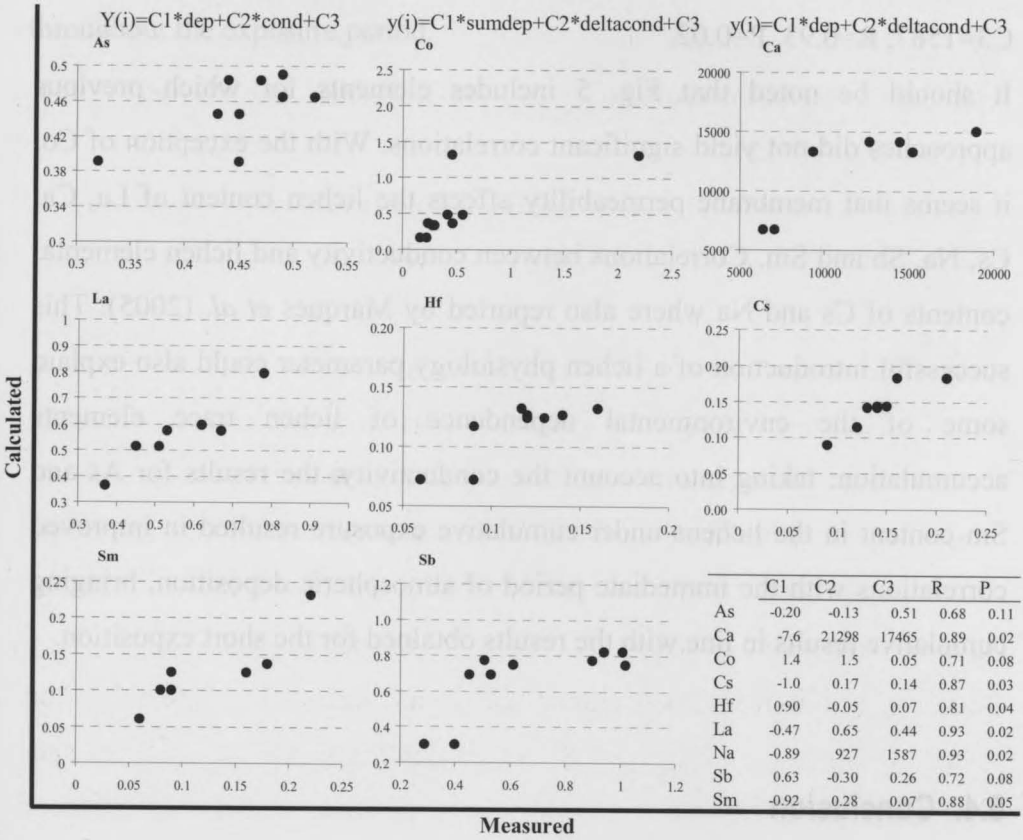
It is interesting to address the apparently slower response of lichens that are used in cumulative exposure trials. As time progresses, the lichen's initial situation at every two month's exposure period is becoming more and more different between the lichens of short and cumulative exposition trials. The first ones come from a low availability situation (Tomar background) whereas the last ones are departing from a foregoing situation of higher initial lichen element content and possibly related different lichen physiological conditions. This reasoning is underlined by data shown in Fig. 3, which indicate the decrease in vitality of lichens towards the end of the cumulative exposure period (Fig. 3). Bari *et al.* (2001) and Freitas and Pacheco (2004) also noticed a general decrease in metal accumulation in transplants during the last month of a 12 months exposure experience.

The changes in lichen vitality and the results shown in Table 2 suggest that lichen accumulation performance should be regarded as related to lichen

physiology, but as a result, or maybe better expressed, as a consequence, also on environmental surrounding conditions. These conclusions are in line with earlier literature (Adamo *et al.*, 2003; Freitas and Pacheco, 2004; Giordano *et al.*, 2005).

Following the reasoning related to the double layer model of Reis *et al.* (1999), which drives the differences between atmospheric and lichen element concentrations, there are rate constants that depend on lichen characteristics (physiology or morphology), and any significant change in rate constants influences both lichen equilibrium levels and remembrance times. Changes in membrane permeability to ions have been pointed out as one of the most sensitive physiological responses to environmental stress (Garty *et al.*, 2001; Godinho *et al.*, 2004), and have the advantage of relating to the whole lichen rather than just to the photobiont.

Based on the above considerations and in an attempt to characterise the lichen physiological status quo, we introduced the lichen conductivity data into the correlations between element deposition and lichen element concentrations. As conductivity is changing in time, we considered both the conductivity at the last period and the average conductivity over the whole period of exposure.



	C1	C2	C3	R	P
As	-0.20	-0.13	0.51	0.68	0.11
Ca	-7.6	21298	17465	0.89	0.02
Co	1.4	1.5	0.05	0.71	0.08
Cs	-1.0	0.17	0.14	0.87	0.03
Hf	0.90	-0.05	0.07	0.81	0.04
La	-0.47	0.65	0.44	0.93	0.02
Na	-0.89	927	1587	0.93	0.02
Sb	0.63	-0.30	0.26	0.72	0.08
Sm	-0.92	0.28	0.07	0.88	0.05

Figure 5. Plots of model calculated results against observed data of the correlations between trace element concentrations values in the lichens and in the bulk atmospheric samples accounting for lichen membrane permeability. Y(i)= calculated lichen concentration; dep= bulk atmospheric deposition; cond= lichen leakage solution conductivity; sum= sum; delta=difference between the higher and smallest; C1, C2, C3= constants; R= correlation coefficient; P= probability.

Again, for each element specific answers were obtained: curves for the elements for which significant correlations ($p < 0.1$) were found are shown in Fig. 5. For Na significant, not illustrated, correlation was found for the

model Y(i)=C1*sumdep+C2*sumcond+C3 with C1=0.89, C2=927, C3=1587, R=0.93, P=0.02.

It should be noted that Fig. 5 includes elements for which previous approaches did not yield significant correlations. With the exception of Co, it seems that membrane permeability affects the lichen content of La, Ca, Cs, Na, Sb and Sm. Correlations between conductivity and lichen elemental contents of Cs and Na where also reported by Marques *et al.* (2005). This successful introduction of a lichen physiology parameter could also explain some of the environmental dependence of lichen trace elements accumulation: taking into account the conductivity, the results for As and Sm content in the lichens under cumulative exposure resulted in improved correlations with the immediate period of atmospheric deposition, bringing cumulative results in line with the results obtained for the short exposition.

6.4. Conclusion

The lichen (transplant) elemental content does not unequivocally represent the average environmental availability of the exposure period. Reflection characteristics depend on the element and the lichen physiological conditions. In summary, lichens do not act like a measuring instrument; instead they reflect ambient element availability that is somehow biased by biological effects. This means that the design of a biomonitor experiment should involve transplants of similar and well-defined initial condition: here may be thought of similar morphology, well-characterized initial contents, and comparable physiological status quo. In addition, lichen physiological

parameters should be monitored along with the lichen elemental content throughout the exposure period.

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Chapter 7

DYNAMICS OF ELEMENT ACCUMULATION AND RELEASE OF *FLAVOPARMELIA CAPERATA* DURING A LONG TERM FIELD TRANSPLANT EXPERIMENT

7.1. Introduction

Individual chemical analyses show the size of mineral pool, not the rate of through-put. The rate of acquisition, redistribution or loss of soluble elements should be studied further (Brown and Brown, 1991). Lichens are one of the most valuable biomonitors of atmospheric pollution. They can be used as sensitive indicators to estimate the biological effects of pollutants by measuring changes at the community or population levels, and as accumulative monitors of persistent pollutants, by assaying their trace element content (Conti and Cecchetti, 2001). Nowadays interest has been focused on how data obtained by atmospheric deposition measurements via bioindicators/biomonitors can be related to human health aspects (Markert *et al.*, 2008)

In this context the calibration of the biomonitor elemental contents against atmospheric elements dispersion variables like total deposition or APM (air

particulate matter) concentration is essential and still not resolved. One question still to be answered is the response-time of the lichen to the environmental change.

As slow-growing organism lichens have been used as long terms biomonitors of air pollution. Temporal studies in urban and industrial areas showed the reappearance of lichens in areas previously devoid of these organisms ('lichen desert') and the improvement of lichen biodiversity (Showman, 1997; Purvis *et al.*, 2003; Hultengren *et al.*, 2004, McClenahen *et al.*, 2007). This recolonization studies are of great interest but only possible when old data or periodic observations are available.

Because the concentrations of trace elements in lichen thalli can be correlated with environmental levels of these elements (Sloof *et al.*, 1995; Bari *et al.*, 2001; Godinho *et al.*, 2008.), variation in lichen trace element contents in time can provide useful evidence for trends in ambient pollution burdens and evolution of emissions (McClenahen *et al.*, 2007; Cuny *et al.* 2004; Loppi *et al.*, 2004; Zschau *et al.*, 2003 Walther *et al.*, 1990). Time frame of most studies has been years or occasionally months in the case of transplant studies and frequently these studies are conducted at one point in time.

Element accumulation is the result of two competitive phenomena: the deposition of particles on the lichen surface and metal release by several mechanisms (biological regulation, passive dislodgment, rain run-off, or cell leakage under oxidative stress).

Individual chemical analyses show the size of mineral pool, not the rate of through-put.

The question is what is the availability period which is reflected by each sample?

According to Wolterbeek *et al.* (2002), after a change in ambient deposition conditions, the period towards a new equilibrium is associated with the lichen's remembrance time (Reis *et al.*, 1999), which in this context gives an expression of the length of the foregoing environmental availability period reflected by the lichen elemental content.

Accurate evaluation of how and to what extent lichens reflect current or time-integrated concentrations of heavy metals in the atmosphere is essential for their use in biomonitoring: we should know whether an observed situation is a reflection of earlier conditions, the present one, or a dynamic "mixture" of both. This is important for data comparability between repeated surveys and to the design of transplant experiments.

Lichen accumulation mechanisms have been described under several situations (laboratory and field studies) but, having in mind accumulation is as a dynamic process, involving uptake and release processes until equilibrium with the surrounding environment is reached (Reis *et al.*, 1999), also important to understand lichen reflection characteristics is to observe the release process.

The present study focuses on the dynamics of accumulation and release of atmospheric elements in lichen thallus relating it to retention times. In the study, the rates of change in element concentrations in lichens are assessed after transplanting thalli of *Flavoparmelia* caperata into new ambient conditions in a long term field experiment: can these changes be detected in intervals of a few months?

7.2. Methods

7.2.1. *Collection of lichen material, site description, and exposure*

Flavoparmelia caperata (L.) Ach. samples were collected from olive trees in a clean rural zone in the centre of Portugal (39º30' N, 8º00' W), near Tomar in January 2004. Lichens were macroscopically cleaned and rinsed with distilled water (Garty *et al.*, 2001). Three replicate samples were taken to determine initial element concentration (time zero, January in figure 1). Then the lichens were transplanted to four different conditions (Figure 1) between two different sites Tomar and Sines. Tomar is considered a clean rural site that may show some impact of agriculture activity such as pesticides from vineyards (Freitas *et al.*, 1997). Sines is situated near an industrial complex which includes a refinery, a thermo-electrical power station and a petrochemical industry (Freitas *et al.*, 1997) but the samples were exposed in the campus of a thermo-electrical power station really close to the coal deposits, so local resuspension of the coal dust particles is to be expected as the main emission affecting the transplants. The meteorological conditions of both sites are compared in Godinho *et al.* (2004). Condition 1) All, 90 collected lichen thalli were transplanted into the same zone of initial collection (Line 1); 2) after 5 months of exposure in Tomar 42 lichen samples were taken to Sines polluted site (Line 2) while the remaining 30 rest in Tomar; 3) after 6 months of exposure in Sines 12 of the transplants were returned to Tomar (line 3); 4) while other 12 remained in Sines (Line 4).

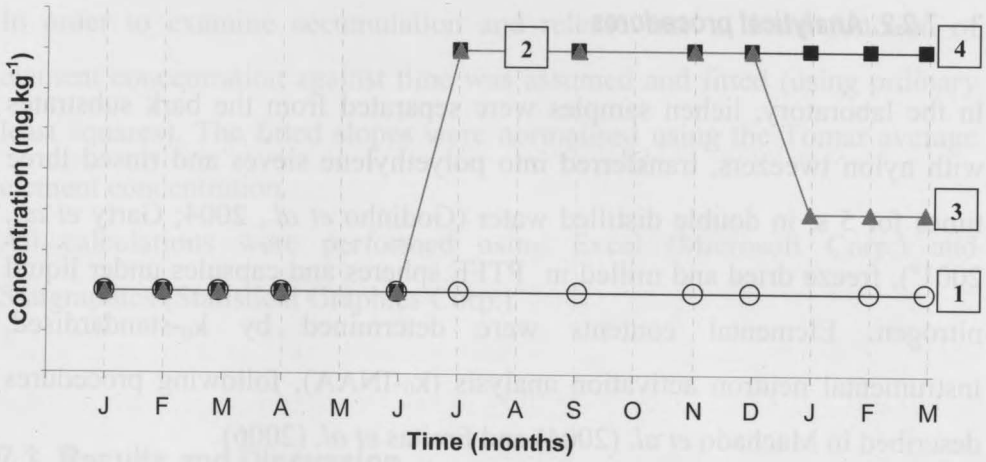

Figure 1. Set up of the exposition experiment. Line 1, 2, 3 and 4 represent the different exposure conditions: 1) Line 1, reference line, lichen transplanted to the clean site; 2) Line 2, after the 5 months of exposure at the clean site lichens were transferred to the polluted site; 3) Line 3, after 6 months of exposure at the polluted site, some lichens of the line 2 were brought back to the clean reference site; 4) Line 4, lichen of line 2 which remained exposed at the polluted site until the end of the experiment.

Samples were exposed keeping the substrate hanging, protected from direct rain, as described by Freitas *et al.* (2001). The whole experiment took 14 months. Every 1 or 2 months, 3 transplanted samples of each condition 1 to 4 were collected for analysis.

The foliose lichen *Flavoparmelia caperata* was chosen for this work because it is one of the most abundant species in Portugal and is often used as a biomonitor including by our research group.

7.2.2. *Analytical procedures*

In the laboratory, lichen samples were separated from the bark substrates with nylon tweezers, transferred into polyethylene sieves and rinsed three times for 5 s, in double distilled water (Godinho *et al.*, 2004; Garty *et al.*, 2001[a]), freeze dried and milled in PTFE spheres and capsules under liquid nitrogen. Elemental contents were determined by k_0-standardised, instrumental neutron activation analysis (k_0-INAA), following procedures described in Machado *et al.* (2004) and Freitas *et al.* (2006).

7.2.3. *Data analysis*

Concentration averages were calculated for: 1) Tomar for the period 6-14 months (N=11); 2) Sines for the period 6-13 months (N=15); 3) Sines-Tomar for the period 12-14 months (N=9); and 4) Sines for the period 12-13 months (N=5). The concentration averages were normalised to Tomar, aiming to compare elemental concentrations obtained for condition 1 with the ones for conditions 2 to 4.

A student-t test (at 95% significance level) was performed on the non normalised data to compare average element concentrations of condition 1 against condition 2 (Sines) and condition 3 (Sines-Tomar) against Sines from 12 months. Concentration data were checked for normal distribution by applying a Shapiro-Wilk test and a Chi-square test (at 95% significance level). Both test showed a normal distribution for all element concentrations except Br, Na and Zn, therefore the normal distribution was accepted for all elements.

In order to examine accumulation and release rate a linear function of element concentration against time was assumed and fitted (using ordinary least squares). The fitted slopes were normalised using the Tomar average element concentration.

All calculations were performed using Excel (Microsoft Corp.) and Statgraphics (Statistical Graphics Corp.).

7.3. Results and Discussion

Figure 2 shows the elemental concentration averages normalised to Tomar in order to compare the levels attained at the 4 conditions. Lichens from Tomar and Sines differed mainly in the concentrations of the elements: Ta, Cl, Ce, Hf, Ti, As, Sm, Eu, Th, Al, Fe, Sc, Cs, Yb, Tb, Dy, and Nd. Roughly speaking, normalised averages differed up to a factor 2.

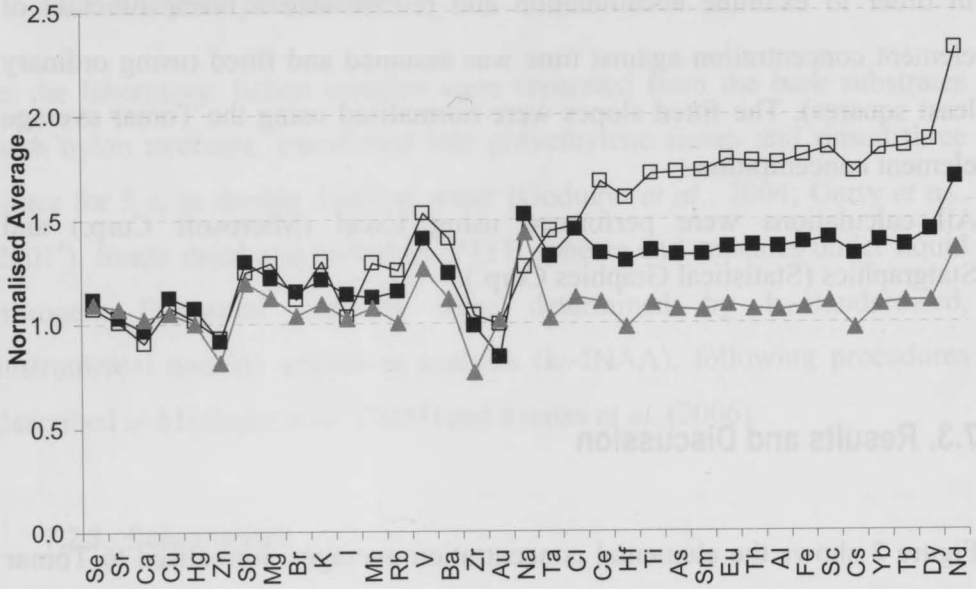

Figure 2. Average *F. caperata* elemental concentration normalised to the background values (Tomar from t= 6 months). Filled square, *F. caperata* exposed in Sines from t=6 months (line 2, Figure 1); Empty square, *F. caperata* exposed in Sines from t=12 months (line 4, Figure 1); Triangle, *F. caperata* exposed in Sines and brought back to Tomar, Sines-Tomar from t=12 months (line 3, Figure1).

Figure 3 illustrates two examples of variation in the lichen element concentration in the different exposure conditions: 1) Al and As, which show element accumulation/release phenomena between Tomar and Sines and 2) Se and Sr, which do not show such behaviour.

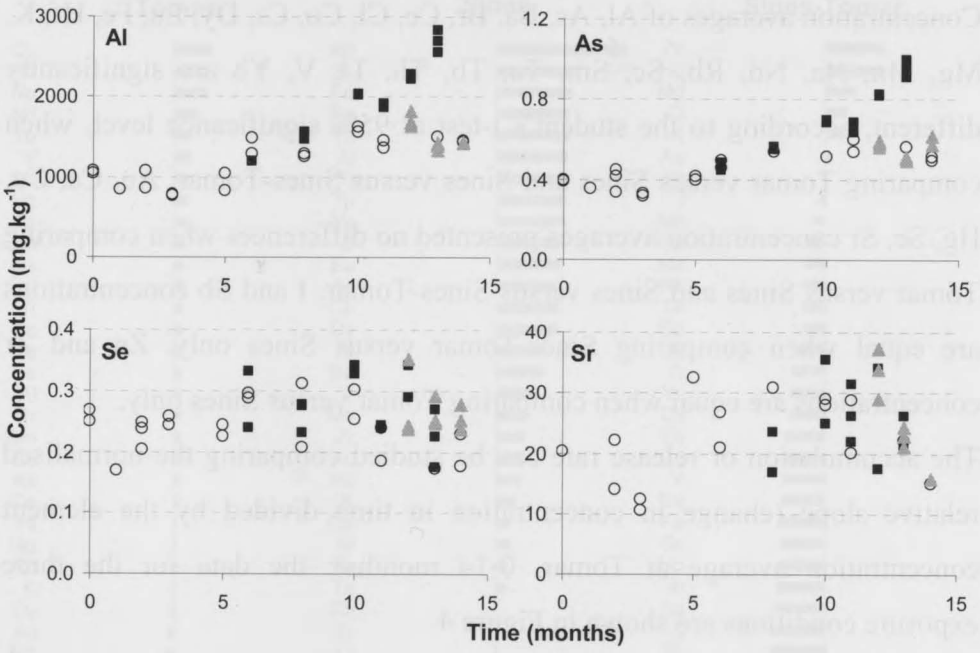

Figure 3. Elemental concentration of *F. caperata* under the various exposition conditions (Figure1). Circle, reference line, lichen transplanted to Tomar the clean site (line 1, Figure 1); Square, after the 5 months of exposure at the clean site lichens were transferred to Sines polluted site (line 2 and 4, Figure 1); Triangle, after 6 months of exposure at the polluted site, some lichens of the line 2 were brought back to the clean reference site (line 3, Figure 1);

The normalised concentration averages of the lichens exposed at Sines ranged 0.9-2.3 and 0.8-2.5 during the first 4 months and from 6 months till the end of the experiment respectively. In contrast, the lichens transplanted to Sines and brought back to Tomar presented concentrations between 0.7-1.4.

This experiment clearly shows the lichen capacity to reflect the current surrounding elemental concentrations.

Concentration averages of Al, As, Ba, Br, Ce, Cl, Co, Cs, Dy, Eu, Fe, Hf, K, Mg, Mn, Na, Nd, Rb, Sc, Sm, Ta, Tb, Th, Ti, V, Yb are significantly different, according to the student's t-test at 95% significance level, when comparing Tomar versus Sines and Sines versus Sines-Tomar. Au, Ca, Cu, Hg, Se, Sr concentration averages presented no differences when comparing Tomar versus Sines and Sines versus Sines-Tomar. I and Sb concentrations are equal when comparing Sines-Tomar versus Sines only. Zn and Zr concentrations are equal when comparing Tomar versus Sines only.

The accumulation or release rate can be studied comparing the normalised relative slope (change in concentration in time divided by the element concentration average at Tomar, 0-14 months): the data for the three exposure conditions are shown in Figure 4.

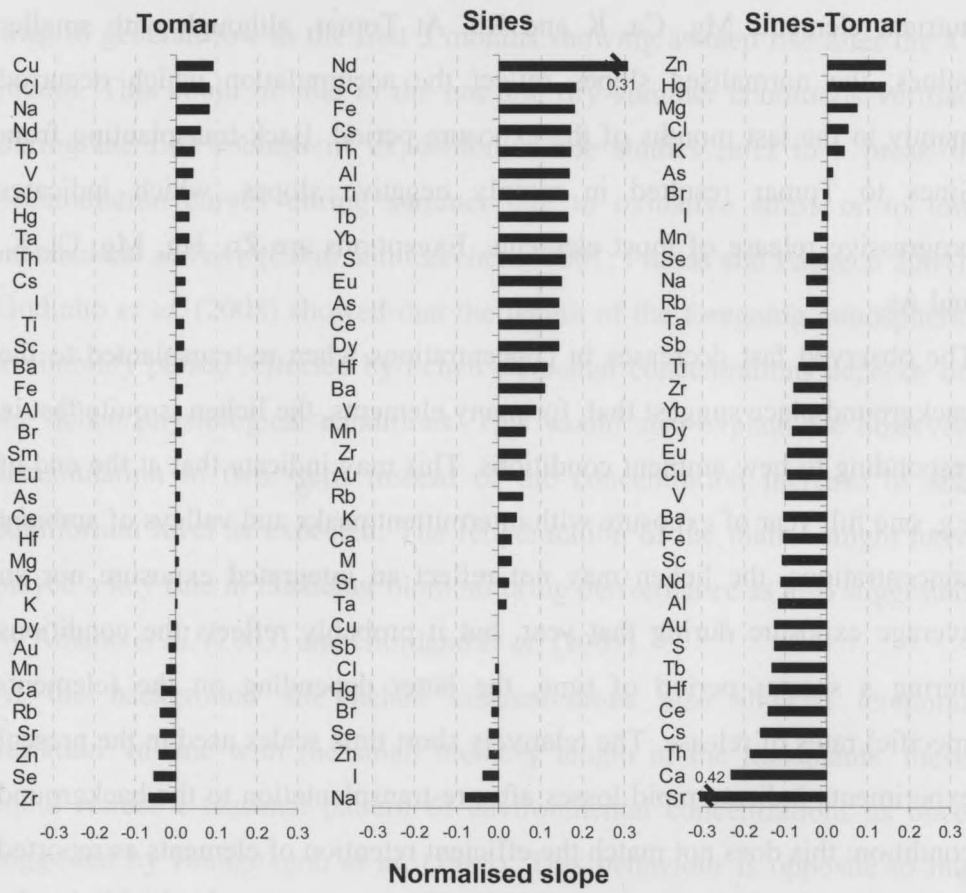

Figure 4. Slope of *F. caperata* concentrations as a function of time of exposition under the different conditions referred in Figure 1. Values are normalised to average element concentrations of Tomar background clean site. 1) *F. caperata* transplanted to Tomar clean site (line 1, Figure 1); 2) *F. caperata* transplanted to Sines polluted site (line 2 and 4, Figure 1); 3) *F. caperata* transplanted to Sines and brought back to Tomar (line 3, Figure1).

Sines presents the higher accumulation values for most elements. Three elemental groups can be identified: 1) relative slope > 0.1: lanthanides and soil elements (e.g. Fe, Sc, Th, Al, Hf) 2) relative slope < 0; volatile elements (e.g. Se, Hg, Sb) and sea spray elements (e.g. Na, I, Br, Cl). 3) A group of

nutrient elements Mg, Ca, K and Rb. At Tomar, although with smaller values, the normalised slopes reflect the accumulation which occurred mainly in the last months of the exposure period. Back-transplanting from Sines to Tomar resulted in mainly negative slopes, which indicates progressive release of most elements. Exceptions are Zn, Hg, Mg, Cl, K, and As.

The observed fast decreases in concentrations when re-transplanted to the background place suggest that, for many elements, the lichen is quite fast in responding to new ambient conditions. This may indicate that at the end of e.g. one full year of exposure with intermittent peaks and valleys of ambient concentrations, the lichen may not reflect an integrated exposure nor an average exposure during that year, but it probably reflects the conditions during a shorter period of time, the latter depending on the (element-specific) rates of release. The relatively short time scales used in the present experiments indicate rapid losses after re-transplantation to the background condition: this does not match the efficient retention of elements as reported for mosses by Tabors *et al.* (2004). The present results highlight the relevance of fast and reversible processes (such as binding of elements in the cell wall ion exchange, adsorption and/or desorption), as the uptake and/or release mechanisms of lichens, rather than non-reversible ones such as capture of solid particles. Exceptions are Hg and Zn

While passive reversible processes of uptake would suggest a fast kinetics of uptake and release, the increase of concentrations when transplanted to the polluted site was rather slow, when compared to concentration decrease when transplanted back to Tomar and to the rates of accumulation reported in previous works. At the polluted site (Sines), the level of bioconcentration

was, in general, low in the first 3 months showing a steep rise after the 4th month. This could be due to the hot and dry summer conditions verified during the first months of exposition. Some studies refer to a break in accumulation curves during summer due to oxidative stress or to low metabolical activity (Conti and Cecchetti, 2001; Freitas and Pacheco 2004). Godinho *et al.* (2008) showed that the length of the foregoing atmospheric availability period reflected by lichen elemental concentrations depends on the lichen physiological conditions. This would also explain the observed accumulation in two steps instead of the concentration increase to one equilibrium level as expected. The rehydratation of the thallus might have played a key role in the better biomonitoring performance as also suggested by Adamo *et al.* (2003) and Giordano *et al.* (2005).

At the background site lichen concentrations also showed temporal variation. In line with the small memory length of the transplants, these could reflect a seasonal pattern of environmental concentrations as once suggested by Boonpragob *et al.* (1988). This behaviour is opposite to the small variation in background moss element concentrations described by Fernandez and Carballeira (2000) and Tabors *et al.* (2004).

Longer periods of exposure may show further effects in the lichens than ongoing accumulation only. Assuming that accumulation and release will eventually lead to a steady concentration, the value depending on the ambient (steady) conditions, high ambient concentrations may cause toxic effects, changes in permeability, or cell death, water loss may cause membrane ruptures due to dehydration, and this all could expose more binding sites, and consequently increasing accumulation.

The rates of change were element specific, and may reflect differences in accumulation mechanisms. Cl and Na showed the faster changes. Many factors determine the rate of the processes of uptake and release in lichens, such as the chemical characteristics of the elements, the chemical composition and size of the particles associated with elements, and the influence of other elements present in the lichen.

7.4. Conclusions

The present experiments clearly show the lichen to respond fast (within months) to environmental changes. This implies that lichens most probably reflect a memory-dependent period of environmental exposure rather than show an integrated record of the full lichen's history.

The lichen's memory length is element specific, which says that the reflected exposure period is different for each element. An important question in future work is the possible effect of ambient conditions on the memory length of the lichen.

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Chapter 8

DISCUSSION, CONCLUSIONS AND OUTLOOK

The emphasis that is put on lichens as bioindicators and biomonitors has much to do with their abundance in both remote (background) and polluted sites. Lichens are one of the most valuable biomonitors of atmospheric pollution (Bargagli, 1989; Beeby, 2001; Conti and Cecchetti, 2001; Wolterbeek, 2002; Batzias and Siontorou, 2007): they are used as indicators to estimate the biological effects of pollutants, by measuring changes at the community of populations, and as accumulative monitors of persistent pollutants, the latter by assaying e.g. trace element content. Lichens may be useful for monitoring not only spatial patterns but also for temporal trends of trace element deposition (Zschau et al., 2003).

The present thesis focused on lichens as accumulative biomonitor organisms of atmospheric (trace) element pollution. The thesis acknowledges the use of lichens as quantitative or indicative monitors of deposition in both smaller- and larger-scaled surveys throughout the world.

Lichens grow slowly, consist of algal and fungal components (Nash III, 1996), may have species-specific or even ambience-specific morphology and physiology (Nash III, 1996), may accumulate elements by various

processes (Tyler, 1989; Richardson, 1995), and may be sensitive to both elemental and other than elemental pollution. Lichen components may specifically accumulate (Loppi et al., 1997; Nimis, 2001; Cercasov et al., 2002; Bergamaschi et al., 2007), lichen sensitivity may have effects on the dose-response relationships (Garty et al., 2001), and various accumulation processes may have characteristics such that accumulation and release are both operating simultaneously (Bargagli and Mikhailova, 2002).

The above makes that lichens should be studied in terms of the a) the distribution of accumulated elements, b) lichen parameters to be used in assessing and ensuring comparability of lichen response characteristics in space and time, and c) the dynamics of the elemental accumulation and release in lichens.

The present thesis focused on aspects a-c: chapters 2 and 3 deal with the distribution of elements in lichens, chapters 4 and 5 deal with comparability testing and chapters 6 and 7 are on studying lichens dynamics.

Chapter 2, in the context of comparability of lichen response characteristics, focused on thallus parts: the results indicate that central (older) thallus parts generally have higher concentrations than peripheral (younger) parts, at least when elements of less physiological significance are considered, while elements of more metabolic interest were more abundant in peripheral parts. The observed initial differences (in situ situation) related to age are consistent with literature data (Loppi et al., 1997), explained by longer exposure and/or higher ion exchange capacities (Nimis et al., 2001; Wolterbeek et al., 2002), but the higher rates of accumulation in younger tissues make that for both in-situ sampling and in transplant studies the to-be-sampled tissues should be selected very carefully. On the one hand this

may dictate that the element-specific differences determine an element-specific choice for sampling of either older or younger tissues, on the other hand, however, the suggested possible internal translocation (Chapter 2) indicates that sampling of the whole organism may be adopted in practical set-ups. More work should be carried out to increase insight in the fractional contribution of central and peripheral parts to total lichen mass.

Chapter 3 deals with the micro-PIXE-assessed micro-scale distribution of elements inside the lichen. Data indicate element-specific differences in distribution, not only in terms of "older" and "younger" parts, and of algal and fungal structures, but also in terms of dispersity. Results suggested e.g. Zn and Mn as uniformly distributed throughout the thallus whereas for e.g. Ca, Si and Ti spot-distributions were obtained. The data indicate that accumulation comprises particle entrapment and further passive accumulation (Wolterbeek et al. 2003), but also biologically-regulated internal concentrations. Again, this suggests that tissue-selection in monitoring should depend on the element of interest, and cannot be made into a generalized approach in survey set-ups.

In Chapters 4 and 5, lichens were studied with the aim to get more insight in the extent to which lichen monitor materials have compatible responses to ambient conditions under varying circumstances. Two approaches were followed: 1) lichen physiological vitality (Chapter 4) and 2) lichen ion exchange sites (Chapter 5), both chapters thus focusing on lichen (bio) physiology and physico-chemistry.

Many approaches are reported to track lichen physiological status quo (Branquinho et al., 1999; Garty et al., 2000; Mulgrew and Wiliams, 2000; Carreras and Pignata, 2001; Tretiach et al., 2007), but the thesis adopted a

practical and easy assessment by determining both membrane integrity via electrical conductivity measurements (Mulgrew and Wiliams 2000), and chlorophylls/phaeophytins. The transplant experiment (from background to polluted) indicated effects largely related to temperature and humidity. These data correspond to earlier results by Marques et al. (2005), and suggest that comparability of lichens in large geographical areas or areas showing large conditional variances may be limited: lichen's performance may be governed by the area's geographical or time-related variability in temperature and precipitation.

Chapter 5 is focused on lichen's exchange sites. The experimental approaches were by acid-base titrations of milled lichen materials in pre-set volume-to-mass ratio's (Van Elteren et al., 2004). The results indicate hardly any condition/site-influence or tissue-influence on pK-values and exchange-capacities. Although it was not possible to identify the composition of functional groups, pK values observed are compatible with the idea that usnic acid may largely contribute to lichen exchange capacities. Although Ravinskaya (1991) reports that light, temperature and humidity play an important role in the concentration of lichen acids, these relationships didn't show up with the presently used transplants, possibly because of the time-relations involved. More study should be carried out to assess differences between sites in in-situ lichens.

The overall results suggest that lichen's comparability may be mostly ensured for elements accumulated by physico-chemical processes.

In Chapters 6 and 7 lichen dynamics were studied. The line of thinking was that if the lichen not only accumulates elements but also releases elements, the observed concentrations are inevitably the result of simultaneous influx

and efflux. This reasoning makes that if the lichen releases elements rapidly, it will not reflect foregoing conditions of deposition for a long period of time: fast release means that the lichen has a short "memory" (Reis et al., 1999; Wolterbeek et al., 2002). Considering that many reports in the literature relate monitor data to deposition data, the above suggests that the period of deposition (exposure period) taken into that relationship very probably should be "memory-length" (and thus in many cases element)-specific.

The data in Chapter 6 clearly indicate that over a total exposure period of 6 months clear differences appear between elements as to the specifics of the relationship with the measured element concentrations in the transplanted lichens. In another approach (Chapter 7), the element specific "memory-length" of the lichen transplants was confirmed. Overall, the data indicate that the exposure period reflected by the lichen may be different for each element. Knowledge on these differences is of paramount importance in interpreting monitoring data.

Biomonitoring, large-scaled in terms of geography, time and/or ambient conditions, asks for more fundamental knowledge on comparability in behaviour, needs to be strictly defined in terms of tissues sampled and taken into elemental analysis. Furthermore, the "memory" of the lichen should be better understood: lichens may serve both as short-term and long-term deposition monitor, depending on the element of interest.

Gaining insight in aspects given above will further substantiate the value of the biomonitoring's approach in world-wide addressing of (elemental) air pollution issues.

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Summary

The present thesis addressed the use of lichens as biomonitor of trace element air deposition, with attention focused on the biomonitor's (accumulation) performance. The necessary quantitative assessment of elemental availability asks for well-defined dose–response relationships and knowledge on disturbances by impacts on the plant parameters on accumulation, retention and release processes.

The present work comprised three main parts, each addressing relevant issues on factors influencing bioaccumulation namely: *a)* distribution of accumulated elements, *b)* lichen parameters to be used in assessing and ensuring comparability of lichen response characteristics in space and time, and *c)* the dynamics of the elemental accumulation and release in lichens.

The study aimed at aspects *a-c* by chapters 2 and 3, which deal with intercellular distribution of elements in lichens, chapters 4 and 5, which deal with comparability testing and chapters 6 and 7, which are on studying lichens dynamics.

Chapter 1 (Introduction) presents the current state of the art of biomonitoring, and introduces relevant issues both generally and specifically recognized as needing attention, and finishes by giving the main objectives of the Thesis.

Chapter 2 (Accumulation of trace elements in the peripheral and central parts of two species of epiphytic lichens transplanted to a polluted site in Portugal) compares the dynamics, i.e. the rates of change in element concentrations of young and older lichen thallus parts, of one foliose and one fruticose lichen, during a transplant experiment to a polluted site. Both lichen parts respond to environmental changes. Here, differential accumulation suggests that differential constitution leads to differential uptake and release, and/or the overall behaviour is partly due to internal translocation and regulation mechanisms within the whole lichen. For thallus parts, internal translocation should be taken into account as one more factor affecting lichen ''memory length''. Young parts of the thallus presented higher rates of change, but different lichen parts accumulate different elements to different extents. Therefore tissue selection in monitoring may depend on the element of interest, and cannot be made into a generalized approach in survey set-ups: the choice depends on the element.

Chapter 3 (Micro-scale elemental distribution in the thallus of *Flavoparmelia caperata* transplanted to polluted site), addresses the elemental microdistributions of peripheral and central parts of the lichen *Flavoparmelia caperata* exposed to industrial pollution. Distribution patterns were analyzed in order to better understand the element distribution patterns in relation to the lichen's constitution, thereby increasing insight on uptake and release mechanisms. Nuclear microscopy techniques were used to visualize elemental distributions in sample transepts and associate their concentrations to sample morphology. The distribution data of the elements studied suggests a biological regulation of internal concentrations. Results

indicate that elements are not only entrapped but can be mobilized in the thallus suggesting an easy interchange with environment. Considering thallus parts, element-specific internal translocation should be taken into account as one more factor affecting lichen "memory length".

Chapter 4 (Assessment of lichen vitality during a transplantation experiment to a polluted site) evaluates the stress effects in two epiphytic lichen species with different thallus morphology, the foliose *Flavoparmelia caperata* and the fruticose *Evernia prunastri*, as resulting from transplanting from an unpolluted to an air-polluted area. Lichen samples were collected in Portugal in a clean area, during the spring 2003, and transplanted (1) to the same zone nearby and (2) to a polluted area as affected by an industrial complex. Transplant samples were taken periodically during four months in both places. At the same time lichen samples from the clean-air site native (in-situ) populations were also collected. For each sample the chlorophyll content, the chlorophyll degradation and the cell membrane damage were measured, the latter represented by leacheate conductivity. During the experiment the meteorological conditions were registered. The results indicate the absence of stress effects of transplanting as such, and suggest that leachate conductivity may be the more sensitive indicator of general lichen vitality.

Chapter 5 (Assessment of acid-base buffering properties of *Flavoparmelia caperata*. Influence of age and pollution exposure) focuses on the lichen's binding sites. The aim was to investigate the extracellular ionizable functional groups or binding sites which can play a (binding) ligand's role in young (peripheral) and older (central) lichen thallus parts of the epiphytic lichens *Flavoparmelia Caperata* collected under different pollution load

environments. Potentiometric acid-base titration was used for obtaining the capacity of lichens to retain extracellularly bound H^+ and pKa distributions describing the acid-base properties of lichen. Titration data were used to assess the proton-exchange properties of surface functional groups. The results indicate that for *F. caperata*, neither ambient conditions nor specific lichen thallus parts affect capacity and pK-values of ionizable groups. This suggests that *F. caperata* may be used in surveys comprising variable ambient conditions

Chapter 6 (Bioaccumulation behaviour of transplants of the lichen *Flavoparmelia Caperata* in relation to total deposition at a polluted location in Portugal) compares the short and long time element accumulation behaviour of transplants of *Flavoparmelia caperata* lichen thalli and total deposition in an atmospheric polluted area. It was found that lichens exposed for a short time behaved differently from lichens in longer-term cumulative exposure suggesting the presence of acclimatization behaviour. The lichen transplant elemental content does not unequivocally represent the average or cumulative environmental availability of the exposure period. Reflection characteristics depend on the element and the lichen physiological conditions. Good correlations between lichen elemental contents and total deposition were obtained when a physiological lichen parameter was introduced in a mathematical model, recommending that physiological status of the lichen may be assessed at sampling and considered when comparing lichen elemental concentrations.

Chapter 7 (Dynamics of element accumulation and release of *Flavoparmelia caperata* during a long term field transplant experiment) addresses the dynamics of accumulation and release of atmospheric elements in lichen

thallus relating it to retention times. The experiment assessed the rates of change in element concentrations in *Flavoparmelia caperata* collected from a clean site, transplanted to a polluted site, and then back transplanted to the clean site again. The whole experiment took 14 months. Every 1 or 2 months, 3 transplanted samples of each ambient condition were collected and lichen element concentrations determined. Transplanted to higher or lower environmental pollution level the lichen element concentration changed accordingly within a few months, proving the lichen to reflect the current ambient elemental concentrations. This implies that lichens most probably reflect a memory-dependent period. The lichen's memory length is element specific, which says that the reflected exposure period is different for each element. This experiment demonstrates that changes in the air pollution level, as expressed by the elemental concentration in thalli of *Flavoparmelia caperata*, can be detected at intervals of a few months.

Last but not least, Chapter 8 discusses, and resumes the principal work and conclusions, and future work is outlined.

Samenvatting

In dit proefschrift wordt het gebruik van korstmossen als biomonitor voor de depositie van sporenelementen uit de lucht behandeld, waarbij de aandacht zich concentreert op het (accumulatie) gedrag van de biomonitor. De relatie tussen dosis en respons moet goed bekend zijn om de benodigde kwantitatieve beschrijving te kunnen geven van de beschikbaarheid van de elementen, maar tevens is er inzicht nodig in hoe die relaties kunnen worden verstoord door de invloed van de plantaardige eigenschappen op de accumulatie, retentie en afgifte processen.

Dit onderzoek omvat drie delen waarbij in een ieder relevante factoren die de bioaccumulatie beïnvloeden zijn bestudeerd, te weten: a) de verdeling van de geaccumuleerde elementen, b) de eigenschappen van de korstmos die gebruikt zouden kunnen worden voor het bepalen en vergelijken van de mate van respons door de korstmos als functie van plaats en tijd, en c) de dynamiek van de accumulatie en afgifte van de elementen in korstmossen.

Het onderzoek aangaande deze aandachtspunten *a-c* is beschreven in de hoofdstukken 2 en 3 die betrekking hebben op de intercellulaire verdeling van elementen in korstmossen; in hoofdstukken 4 en 5 waarin de mate van vergelijkbaarheid is nagegaan; en in hoofdstukken 6 en 7 waarin het onderzoek naar de dynamiek van de korstmossen centraal staat.

Op de huidige stand van de wetenschap ten aanzien van biomonitoring wordt ingegaan in hoofdstuk 1 (Introduction), waarbij zowel algemene als

specifieke punten van aandacht worden besproken. Dit hoofdstuk wordt afgesloten met de voornaamste doelstellingen van het in dit proefschrift beschreven onderzoek.

In hoofdstuk 2 (Accumulation of trace elements in the peripheral and central parts of two species of epiphytic lichens transplanted to a polluted site in Portugal) wordt het onderzoek beschreven naar de snelheid waarmee de concentraties van de elementen veranderen in de jonge en oudere delen van de thallus van twee korstmos soorten (een foliose en een fruticose soort) met elkaar vergeleken na blootstelling van een transplant in aan omgeving met ernstige luchtverontreiniging. Het is gebleken dat beide onderdelen van de korstmos reageren op veranderingen in de omgevingsomstandigheden. De verschillen in accumulatie wijzen erop dat verschillen in samenstelling en structuur leiden tot verschillen in opname en afgifte, en/of dat het complete gedrag voor een deel moet worden toegeschreven aan het interne translocatie- en regel mechanisme binnen de gehele korstmos. De interne translocatie in de delen van de thallus zou beschouwd moeten worden als een additionele factor die invloed heeft op de duur van het geheugen ("memory length") van de korstmos. Hogere uitwisseling snelheden zijn waargenomen in de jonge delen van de thallus, maar er is een verschil in de mate van accumulatie van de elementen in verschillende onderdelen van de korstmos. De keuze van een onderdeel van de korstmos voor monitoring zou daarom kunnen afhangen van het element waar de belangstelling naar uitgaat en er kan daardoor geen algemene richtlijn gegeven worden voor de organisatie van het onderzoek: de keuze wordt bepaald door het element.

In hoofdstuk 3 (Micro-scale elemental distribution in the thallus of *Flavoparmelia caperata* transplanted to polluted site) wordt het onderzoek behandeld naar de microverdeling van elementen in de buitenste rand

(perifere gedeelte) en het centrale gedeelte van de korstmos *Flavoparmelia caperata* die was blootgesteld aan luchtverontreiniging door industriële activiteiten. De verdeling van elementen is bepaald om meer inzicht te verkrijgen in het verband tussen die verdeling, de structuur van de korstmos en de opname en afgifte mechanismes. Nucleaire microanalytische technieken en microscopie zijn gebruikt om de element verdelingen te bepalen over de dwarsdoorsnede van de korstmos preparaten, terwijl de hoeveelheden in verband zijn gebracht met de morfologie van het preparaat. De resultaten van die verdelingen wijzen erop dat er, voor de waargenomen elementen, de interne concentraties langs biologische weg geregeld worden. Verder blijkt uit de resultaten dat de elementen niet alleen zijn ingevangen maar ook mobiel zijn in de thallus. Dit wijst erop dat er een gemakkelijke uitwisseling met de omgeving zou kunnen zijn. Ook elementspecifieke interne translocatie in de delen van de thallus moet worden beschouwd als een additionele factor die invloed heeft op de "memory length" van de korstmos.

In hoofdstuk 4 (Assessment of lichen vitality during a transplantation experiment to a polluted site) wordt het onderzoek beschreven naar de effecten van stress in twee epifytische korstmossoorten met een verschillende morfologie van de thallus, de foliose *Flavoparmelia caperata* en de fruticose *Evernia prunastri* tengevolge van een verplaatsing vanuit een omgeving met niet-verontreinigde lucht naar een omgeving waar sprake was van luchtverontreiniging. De korstmos monsters zijn in Portugal verzameld in een schoon gebied in het voorjaar van 2003, en verplaatst naar (1) een nabij gelegen vergelijkbaar gebied en (2) een gebied waar sprake is van verontreiniging door een industrieel complex. Regelmatig zijn deze verplaatste korstmossen bemonsterd gedurende een periode van vier

maanden. Tegelijkertijd zijn ook korstmos monsters genomen uit het oorspronkelijke gebied van herkomst. Tijdens dit onderzoek zijn ook de weersomstandigheden vastgelegd. Het chlorofyl gehalte, de afbraak van het chlorofyl en de geleidbaarheid van de uitloogvloeistof –een maat voor de beschadiging van het cel membraam- zijn gemeten. De resultaten wijzen erop dat er geen stress effecten ten gevolge van verplaatsing waarneembaar zijn, en dat van de beproefde methoden de geleidbaarheid van de uitloogvloeistof de meest gevoelige indicator is voor de vitaliteit van de korstmos.

In hoofdstuk 5 (Assessment of acid-base buffering properties of *Flavoparmelia caperata*. Influence of age and pollution exposure) wordt de aandacht gericht op de bindingsplaatsen van de korstmos. Onderzoek is gedaan naar de extracellulaire en ioniseerbare functionele groepen en bindingsplaatsen die een rol kunnen hebben als (bindende) ligand in the jonge (rand gedeelte) en oudere (centraal gedeelte) van delen van de thallus van de epifytische korstmos *Flavoparmelia caperata* waarvan monsters zijn verzameld uit gebieden met verschillende mate van milieuverontreiniging. Een potentiometrische zuur-base titratie is gebruikt voor het bepalen van de capaciteit van de korstmos voor het vasthouden van extracellulair gebonden H^+ en voor de pKa verdelingen die de zuur-base eigenschap van de korstmos beschrijven. De uitkomsten van de titratie maken het ook mogelijk om de proton uitwisseling eigenschap van de functionele groepen aan het oppervlak te bepalen. De resultaten wijzen erop dat omgevingscondities noch door specifiek delen van de thallus invloed hebben op de capaciteit en pK-waarden van ioniseerbare groepen in *F.caperata*. Hieruit kan worden afgeleid dat F.caperata zich leent voor onderzoek waarbij variaties in omgevingscondities zich kunnen voordoen.

In hoofdstuk 6 (Bioaccumulation behaviour of transplants of the lichen *Flavoparmelia caperata* in relation to total deposition at a polluted location in Portugal) wordt het accumulatie gedrag voor elementen door de thalli van de korstmos *Flavoparmelia caperata* gedurende een korte en lange periode van blootstelling vergeleken met de totale depositie van elementen in een gebied met luchtverontreiniging. Daarbij is vastgesteld dat korstmossen die gedurende een korte periode worden blootgesteld zich anders gedragen dan korstmossen die gedurende een langere tijd cumulatief worden blootgesteld, wat wijst op een aanpassing eigenschap. De gemiddelde of cumulatieve beschikbaarheid van de elementen uit de omgeving tijdens de blootstellingperiode wordt niet weerspiegeld door de gehaltes van die elementen in de korstmos transplant. De mate van weerspiegeling hangt af van het element en van de fysiologische condities van de korstmos. Indien de fysiologie van de korstmos als parameter wordt ingevoerd in een wiskundig model worden goede correlaties gevonden tussen de gehaltes van elementen in de korstmos en de hoeveelheden in de totale depositie. Dat leidt tot de aanbeveling om de fysiologische status van de korstmos te bepalen tijdens de bemonstering, en in ogenschouw te nemen bij het vergelijk van de concentraties van elementen in korstmossen.

In hoofdstuk 7 (Dynamics of element accumulation and release of *Flavoparmelia caperata* during a long term field transplant experiment) wordt het onderzoek beschreven naar de dynamiek van de accumulatie van elementen uit de lucht en afgifte daarvan door de thallus van de korstmos, in relatie tot de duur van de retentie. De snelheid is bepaald waarmee de element concentraties veranderen in *Flavoparmelia caperata* dat verzameld is in een schoon gebied, verplaatst naar een verontreinigde omgeving en vervolgens weer teruggebracht naar het schone gebied. Dit experiment is

uitgevoerd in een periode van 14 maanden. Iedere 1-2 maanden zijn 3 getransplanteerde monsters uit ieder gebied genomen waarin de element gehaltes zijn bepaald. Daarbij is vastgesteld dat de element gehaltes in de korstmos binnen een paar maanden veranderd zijn, in overeenstemming met de hoogte van milieuverontreiniging. Dit wijst er op dat het zeer waarschijnlijk is er bij korstmossen sprake is van een vorm van 'geheugen' voor een bepaalde periode. De duur van dit geheugen van de korstmos is element specifiek, waardoor de weergegeven blootstellingperiode verschillend is voor ieder element. De resultaten van dit onderzoek tonen verder aan dat veranderingen in de mate van luchtverontreiniging, zoals kan worden afgeleid van de element gehaltes in de thalli van Flavoparmelia caperata, waargenomen kunnen worden binnen intervallen van enkele maanden.

Tenslotte worden in hoofdstuk 8 alle resultaten samengevat en bediscussieerd, wat leidt tot conclusies en omschrijving van mogelijkheden voor toekomstig aanvullend onderzoek.

Acknowledgements

I thank the institutions that have accepted me as a PhD student and gave me financial support, Instituto Tecnológico e Nuclear (ITN), Sacavém, Portugal, Faculty of Applied Science, Delft University of Technology, The Netherlands, and Portuguese Foundation for Science and Technology.

I am specially grateful to my supervisor Maria do Carmo Freitas who instate me to proceed the PhD in this area, received me in her workgroup, introduced me to the Neutron Activation Analysis and orientate me trough the whole work.

My very special thanks to my promoter, Bert Wolterbeek for accept me as a student and for the profitable discussions and debates that make this thesis enriched.

A very special Hugh to Tona Verburg who makes me feel at home when I am working at RID.

Special thanks to the ITN colleagues: To Susana Sarmento for her friendliness accommodation, for our nice talks and mutual support; to Ausenda Machado, for the company; to Bruno, always friendly and helpful; to Isabel Dionisio, Mané, Marta, Ana Paula, Márcia e Teresa Fernandes, who receive me very well in the work group and teach me how the things

work. Thanks to the entire reactor department at ITN, to the head of the department Dr. José Marques and the reactor operators.

Many thanks for all the people of the Radiation and Isotopes for Health group of the department of Radiation, Radionuclides and Reactors and all the persons I have contacted at RID where everyone is always ready to help and the atmosphere is very professional, encouraging and friendly. Thanks to Dr. Thea for the INAA analysis, to Dr. Olav M. Steinebach for the ICP-OES analysis and Dr. Volkers for the titration work performed at IRI laboratories. For all the assistance thanks to Yvonne and Folkert.

I would like to thank to Teresa Pinheiro for her friendly availability and Luis Alves for the analysis with the microprobe.

I would like to heartfelt thank my family for all the support!

To the *aunts* special thanks for the countless and so important little helps every time it is necessary.

For always being there no matter what, thanks to Mum and Dad.

To João, Leonor e Guilherme for making live so special.

Curriculum Vitae

Rita Pinto Eliseu Mendes Godinho was born on 26 September 1973 in Oeiras, Portugal. In 1997 she got her degree (*Licenciatura*) in Biology Applied to Animal resources attributed by Faculty of Sciences of University of Lisbon. Still during the university, she attended the last year of the Aquatic Ecology course of Queen Mary and Westfield College of London University, under the scope of Erasmus program, presenting the final thesis "Bicarbonate and trace Metal relations in two strains of *Emiliania huxleyi*", she completed the summer course "Physiological Ecology of Algae" at Laboratoire de Biologie Marine de Concarneau, taught jointly by the Birmingham, Brest and Lisbon Universities, and carry out the professional stage in water quality in fish farming working at the fish farm ViveiroVilaNova. During 1998 and 1999 she worked as technician in the Sanitary Hydraulics Department of the National Laboratory of Civil Engineering. From 2000-2004 she worked as trainee researcher at Oceanography Institute of the University of Lisbon participating in the projects EUFITOX (Dynamics of toxic phytoplankton and eutrophication in the Portuguese coast) and NICE (Nitrogen cycling in estuaries). In July 2000 she did the summer course "Marine Algae and Phytoplankton" at Friday Harbor Laboratory of University of Washington. In 2001 and 2002 she was monitor of the course "Microbiological diagnoses of Water quality" of the Science Faculty of University of Lisbon. In 2004 she got the Master

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List of Publications

GODINHO R.M., VERBURG T.G., FREITAS M.C., WOLTERBEEK H.TH., "Dynamics of element accumulation and release of *Flavoparmelia caperata* during a long term field transplant experiment", Submitted to the *International Journal of Environment and Health.*

GODINHO R.M., VERBURG T.G., FREITAS M.C., WOLTERBEEK H.TH., "Characterization of *Flavoparmelia caperata* as biomonitor of atmospheric pollution. Capacity, pK-values and metal-exchange behaviour before and after pollution exposure", Submitted to the *International Journal of Environment and Health.*

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ISBN 978-1-60750-482-5

ISBN 978-1-60750-482-5

TU Delft

Delft University of Technology

Delft University Press is an imprint of IOS Press

IOS Press