Positional Responses in Lichen Transplant Biomonitoring of Trace Element Air Pollution

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.

Positional Responses in Lichen Transplant Biomonitoring of Trace Element Air Pollution

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus, Prof.dr.ir. J.T. Fokkema,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen
op maandag 27 oktober om 15.00 uur
door

Ana Paula Valério MARQUES

Licentiate in Technological Chemistry of the
Faculty of Sciences of the University of Lisbon

geboren te Vila Franca de Xira, Portugal

Dit proefschrift is goedgekeurd door de promotor:
Prof.dr. H.Th. Wolterbeek

Samenstelling promotiecommissie:

Rector Magnificus voorzitter
Prof.dr. H.Th. Wolterbeek, Technische Universiteit Delft, promotor
Dr. M.C. Freitas, Instituto Tecnológico e Nuclear, supervisor
Prof.dr.ir. M. de Bruin, Technische Universiteit Delft
Prof. J. Garty, Tel Aviv University
Prof.dr. F.J. Beekman, Technische Universiteit Delft
Prof. K. Janssens, Universiteit Antwerpen
Dr.Ir. P.Bode, Technische Universiteit Delft

This thesis was developed with the financial support of the Portuguese Foundation for Science and Technology

ISBN .....

Para duas pessoas muito especiais, os meus Pais

(For two very special persons, my Parents)

Contents

Chapter 1

General Introduction

1.1 Introduction

Air pollution is a serious problem in many parts of the world. The significant impact of air pollution on the health of exposed human populations, on forest decline, loss of agricultural productivity, etc., has been a cause of increasing public concern throughout the world (Smodis and Bleise 2002). Increasing awareness of the potential hazards of large-scale contamination of ecosystems by pollutants has highlighted the need for continuous monitoring of the levels of these substances in the environment. Monitoring anthropogenic air pollution is complex 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 of a purely instrumental approach (Nimis, Lazzarin et al. 2000; Wolterbeek 2002). The problem of sampling density is particularly evident when it is necessary to establish and maintain region-wide monitoring systems and for retrospective studies (Ferretti, Brambilla et al. 2004; Frati, Brunialti et al. 2005).

Concern about atmospheric pollutants underlies the efforts to establish control programmes in many countries. The necessary (quantitative) information on chemical element air pollution is generally obtained by modelling of the dispersion (which is a source-oriented approach, making use of a priori known information on emission sources), or by field measurements of the immission (which is a receptor-oriented approach) (Wolterbeek, Garty et al. 2002). Immission measurements require long-term sampling at large numbers of sampling sites. Such measurements using technical equipment (such as particle sampling systems and deposition collectors) have been few, mainly due to high costs (including material, equipment,

electrical power, protection against vandalism, etc) and are person–power intensive. Also it is often not possible, due to logistic problems, to install instrumental equipment at all needed locations. It is here where biomonitoring comes in. Long-term biomonitoring may prove valuable in detecting trends and correlations of the main air-associated elements. It is important to focus on the possibility of (bio)monitoring a large study area completely lacking an instrumental network for detecting this kind of anthropogenic impact. This confirms that lichen biomonitoring can be useful in risk assessment for human health and it can be a powerful tool for administrators involved in environmental planning. (Brunialti and Frati 2007). In fact, in general terms, the use of living organisms as indicators for environmental stability has long been widely recognised. Plants, animals, fungi, and bacteria have been employed as bioindicators and biomonitors in air, soil and water pollution surveys over the past few decades (Garty, Galun et al. 1979; Nimis, Castello et al. 1990; Freitas and Nobre 1997; Bargagli 1998; Kirschbaum and Hanewald 1998; Beeby 2001; Conti and Cecchetti 2001; Wolterbeek 2002; Wannaz, Carreras et al. 2006; Batzias and Siontorou 2007).

1.2 Biomonitoring

Biomonitoring, in a general sense, may be defined as the use of bio-organisms (biomonitors) to obtain quantitative information on certain characteristics of the biosphere (Wolterbeek, Garty et al. 2002). In general, bioindicators are organisms that can be used for the identification and qualitative determination of (human-generated) environmental factors, while biomonitors are organisms used for the quantitative determination of contaminants (Conti and Cecchetti 2001). Biomonitors are organisms or communities of organisms whose content of certain elements or compounds and/or whose morphological, histological or cellular structure, metabolic-biochemical processes, behaviour or population structure(s), including changes in parameters, supply quantitative information on aspects of the quality of the environment or changes in the environment. A biomonitor is always a bioindicator as well, but a bioindicator does not necessarily meet the requirements for a biomonitor (Markert, Breure et al. 2003). The aim of monitoring is to determine spatial and temporal trends in levels and effects of pollutants that,

relative to their sources, are deposited or transported locally, regionally or continentally (Wolterbeek, Garty et al. 2002). For trace elements, the main advantages of biomonitoring are related primarily to the permanent and common occurrence in the field, the ease of sampling and the degree of trace element accumulation (Sloof 1993). Furthermore, biomonitoring provides a measure of integrated exposure over a certain amount of time, the monitor is present also in remote areas and no expensive technical equipment is involved (Steinnes 1989, Sloof 1993). However, results from biomonitoring may be difficult to interpret due to the lack of specificity of the response and the virtual absence of standard procedures: only recently standard approaches are started to be developed and approaches harmonized. Biomonitoring is therefore generally seen as a complementary tool to physical systems rather than a substitute (Figueira 2002). However, methods are being developed to calibrate lichen responses (Reis 2001), thereby linking biomonitors, air particulate matter and deposition. This linking could make biomonitoring to develop into a standard tool for quantitative monitoring and assessment of trace elements in the atmosphere. It is therefore extremely important to understand and to define uniform procedures for biomonitoring and more work needs to be done in this area.

The main and generally discussed characteristics of bioaccumulators (biomonitors) are that they 1) accumulate the pollutant without, however, being killed by the levels with which they come in contact, 2) have a wide geographical distribution, 3) are abundant, sedentary, or of scarce mobility, as well being representative of the collection area, 4) are available all year round and allow for the collection of sufficient tissues for analysis, 5) are easy to collect and resistant to laboratory conditions, as well as being usable in laboratory studies of contaminant absorption, if necessary, 6) have a high concentration factor for the contaminant under study, and thus allowing direct analysis with no prior increase in concentration, 7) have a simple correlation between the quantity of contaminant contained in the organism and the average contaminant concentration in the surrounding environment, and 8) have the same contaminant content level correlation with the surrounding environment in every site studied and under any condition (Sloof 1993; Conti and Cecchetti 2001). This must be true for all organisms examined. The most widely

used biomonitors for air pollution studies are mosses and lichens (Wolterbeek 2002).

1.3 Mosses and lichens

Mosses and lichens are completely unrelated groups of cryptogamic organisms but they have a number of features in common. They both occur in almost all terrestrial ecosystems and, by virtue of their ability to tolerate long periods of desiccation, may even colonize areas having extreme environmental conditions. Lichen thalli and carpets of living mosses usually lack root systems or protective waxy cuticles and are built up over extended periods of time – often years. They depend largely on atmospheric depositions for their nutrient supply and may show elemental compositions which, in an integrated way, reflect the gaseous, dissolved or particulate elements in the atmosphere (Bargagli 1998) and they have been used, for some time, to describe regional distribution patterns of several elemental concentrations (Ruhling and Tyler 1973, Pakarinen and Tolonen 1976, Groet 1976, Pilegaard, Rasmussen et al. 1979, Steinnes, Rambaek et al. 1992). Mosses and lichens have been compared, fo some time, for air pollution biomonitoring (Kansanen and Venetvaara 1991).

Some authors (Szczepaniak and Biziuk 2003) state that in spite all disadvantages, mosses and lichens are good tools for air pollution monitoring, but best results could be achieved while using both of them together, because of differences in their chemical element uptake and retention. The researcher dealing with biomonitoring faces many difficulties; for example, in the use of lichens, those of similar composition are not easy to find, because of the differences caused by the tree species on which the lichens are growing. Also, terrestrial moss is not always a good choice because of variations in its composition caused by the area it is growing on. Adamo *et al.* (Adamo, Giordano et al. 2003) compared the moss *Sphagnum capilifolium* to the lichen *Pseudevernia furfuracea* after 17 weeks of exposure and found that the moss was a more efficient accumulator than the lichen. Moss accumulation is seemingly not affected by meteorological conditions. By contrast, lichen accumulation capacity improves with wet conditions.

However the choice of the best and suitable biomonitor depends on many factors. For instance, in northern countries it is easy to find moss carpets with mosses of appropriate size for biomonitoring studies while in southern countries (especially the Mediterranean area), where the weather is drier, mosses usually are very small. Here, sample preparation and handling becomes much more difficult.

1.4 Lichens

1.4.1 What is a lichen?

Lichens first appeared about 400 million years ago (www.earthlife.net/lichens/intro.html). It has been estimated that there are approximately 20 000 lichen species in our planet (Nash III 1996). They are symbiotic organisms composed by 1) a fungus called mycobiont and 2) one or more algae and/or cyanobacteria called photobiont (Nash III 1996). Nearly forty genera of algae and cyanobacteria have been reported as photobionts in lichens; three genera, *Trebouxia, Trentepohlia* and *Nostoc* are the most frequent photobionts (Nash III 1996). The genera *Trebouxia* and *Trentepohlia* are of eukaryotic structure and belong to the green algae, the genus *Nostoc* belongs to the oxygenic photosynthetic bacteria (cyanobacteria) (Nash III 1996). The name of the lichen species is given by the fungal partner. The fungal partner benefits by getting sugars, its only nutrient from the algae which being green synthesises sugars through photosynthesis. The algae partner gets protection as the fungi normally forms the outer surface. This protection is against weather mostly, it results in the algae having a more stable and constant environment in, allowing it to grow better. The fungus collects the sugars by means of special hyphae called appessoria or haustoria which contact the wall of the algal cells. The fungi may produce a substance which increases the permeability of the algal cell walls, such that the algal loses (by diffusion) as much as 80% of the sugars it produces (Nash III 1996). In general, about 90% of the biomass is made up by the fungal partner (Tyler 1989).

Lichens can be classified according to their substrate and shape. There are lichen species growing on trees (epiphytic), on litter layers and dead moss carpets

(epigeic), on rocks (epilithic) and roofs (epigeic and epilithic). Concerning shape, the majority of lichens species are crustose (forms crusts), while the foliose (leaflike) and fruticose (shrubby) lichens are less abundant.

Morphologically, lichens are made up of a few distinct characters. The most obvious is the thallus. The form of the thallus is a result of the fungal species involved. The thallus is the body of the lichen and together with reproductive structures form the major part of the lichen. The fungal hyphae (filaments), branch and then fuse together (anastomose) to form a mesh of hair-like threads. The top surface is normally a layer of tightly packed hyphae called a cortex. Below this is the algal layer where the photobiont lives. Below this is the medulla, an area of loose hyphae in which nutrients are stored. Sometimes a lower cortex exists, in others the medulla rests on the surface. In crustose lichens there is no lower cortex. In foliose lichens there is a lower cortex and in fruticose lichens the lower cortex is replaced by a central cone (Nash III 1996).

Unlike higher plants, lichens have no roots or a well developed cuticle and they strongly depend on deposited material from the atmosphere to obtain their mineral nutrients. On the other hand, the lichen surface, structure and roughness facilitate the interception and retention of particles. Not withstanding extensive availability of literature on the use of epiphytic lichens as biomonitors of trace element air pollution, no consensus exists regarding the contribution of substrate-derived fractions of the lichen elemental contents. Element uptake by lichens from the substrate is likely to occur (Goyal and Seaward 1981), but may vary among lichen species, substrata and elements (Wolterbeek and Bode 1995).

In the particular case of the present Thesis, epiphytic lichens were used throughout; foliose lichen (*Parmelia sulcata*) attached to olive trees and fruticose lichen *Evernia prusnastri* harvested from both *Cistus ladanifer* and *Quercus* species of tree. *Parmelia sulcata* is a foliose lichen with a thicker upper and lower cortex and a diffuse medulla, it has sturdier, latterly branching rhizines. The algal layer has a haustoria where a fungal hyphae intrudes into the algal cell to take nutrients from it. It can be found in both hemispheres; from artic to temperate regions being tolerant to relatively high air pollution can also be found in some urban environments (Bargagli 1998). *Evernia prusnastri* is typical fruticose lichen with

thallus lobes hair-like and shrubby. It is attached to the substrate by a single point and dangle from this. It has dorsiventrally arranged thalli. It is a circumboreal-temperate species restricted to the northen hemisphere; very wide ecological range, usually found on bark, rarely on rocks, in stunted forms in regions of moderate pollution (Bargagli 1998). Both lichens have as the algal photobiont *Trouboxia.*

1.4.2 Use of lichens in air pollution studies

Lichens were recognized as potential indicators of air pollution as early as the 1860's in Britain and Europe. Since then, lichens have played prominent roles in air pollution studies throughout the world because of their sensitivity to different gaseous pollutants, particularly sulphur dioxide. They have also been found to act as accumulators of elements, such as trace and radioactive elements. Lichens have been used often as receptor-based biomonitors in air quality studies. Lichen characteristics measured in air pollution studies include morphological, physiological, and population data. Historically, lichens have been used in a qualitative way, with observations of population changes and morphological effects serving as indicators of pollutants. In the last few decades quantitative measurements of the chemical content of lichens and sensitive physiological processes have been used increasingly to indicate pollutants. Possible stress responses to air pollution include chlorophyll degradation, changes in photosynthesis and respiration, alterations in nitrogen fixation, membrane leakage, accumulation of toxic elements, and possible changes in spectral reflectance, lichen cover, morphology, community structure, and reproduction.

1.4.3 Mechanisms of chemical element uptake by lichens

The mechanisms of chemical element uptake in lichens have been reviewed by several authors (Puckett 1988; Tyler 1989; Brown and Brown 1991) and can be summarised as follows:

1) *Extracellular ion exchange*. Early studies on chemical element uptake by lichens emphasize the fact that uptake mainly occurs to extracellular sites through cation

exchange processes. These extracellular sites are presumed to be in the cell walls and on the outer surface of the cell membrane (Brown 1976; Nieboer, Richardson et al. 1978) and the similarity of the lichen thallus with an ion-exchange resin was proposed (Nieboer, Lavoie et al. 1976; Nieboer, Puckett et al. 1976). The lichen wall binding sites are probably carboxylic acid in nature, and are part of the protein component of the fungal cell walls and membranes (Richardson, Kiang et al. 1985). This process is physico-chemically regulated, rapid and reversible.

2) *Intracellular uptake.* The intracellular uptake requires the passage of an element across the plasma membrane, using an appropriate carrier system. The uptake is slower than by ion exchange and the rate of uptake remains approximately linear for a longer period than for extracellular uptake.

3) *Particle entrapment.* Trapping of particles contributes significantly to the elemental levels found in lichens (Nieboer, Richardson et al. 1978). Particles are accumulated by trapping onto and within the lichen, and may later be solubilized to some extent by secondary lichen products. The occurrence of chemical elements in particles in lichen thalli has been demonstrated by Garty et al. (Garty, Galun et al. 1979).

1.5 Diversity of particle deposition modes

Atmospheric deposition of particles to ecosystems takes place via both wet and dry processes, through three major routes: (1) wet deposition, by precipitation scavenging in which particles are deposited in rain and snow; (2) the much slower dry deposition; and (3) occult deposition, hidden from measurements that determine wet deposition, by fog, cloud-water, and mist interception (Table 1.1).

1.5.1 Wet deposition

Wet deposition results either from the incorporation of atmospheric particles and gases into cloud droplets by nucleation, and their subsequent precipitation as rain or snow (rainout), or from below-cloud scavenging of particles and gases by impaction (washout) as raindrops or snowflakes fall (Grantz, Garner et al. 2003).

1.5.2 Dry deposition

Dry deposition can be understood in a more general sense as all processes leading to the removal of airborne particles from the atmosphere due to impact against trapping surfaces. Dry deposition of atmospheric particles to plant and soil is a much slower process than wet or occult deposition, but it acts nearly continuously and affects all exposure surfaces (Grantz, Garner et al. 2003).

1.5.3 Occult deposition

Gaseous pollutant species may dissolve in the suspended water droplets of fog and clouds. Aqueous condensation may occur onto pre-existing fine particles and such particles may coalesce or dissolve in fog or cloud droplets. The stability of the atmosphere and the persistence of the droplets often allow a condition of gas/liquid phase equilibrium to develop. This permits knowledge of air mass history or ambient concentrations. Further, estimates of the deposition velocity of the pollutant droplets allow calculation of depositional fluxes. Unfortunately, interception of fog and cloud droplets by plant parts or other receptor surfaces remains difficult both to predict and to measure (Grantz, Garner et al. 2003).

Topography and vegetation characteristics influence the deposition modes differently and in general, dry deposition is the most sensitive and wet deposition the less sensitive to features of the vegetal surface (Grantz, Garner et al. 2003). The distribution of deposition of individual constituents and of total particulate matter between wet, dry and occult modes varies substantially between locations (Grantz, Garner et al. 2003). Rainfall and snowfall directly determine the magnitude of wet deposition. Precipitation events clean the air, so that dry deposition is eliminated or reduced during subsequent periods. Occult deposition depends upon landscape interception of the cloud base. This may occur at high elevation sites, in coastal areas subjected to onshore advection, or in low-lying interior areas subjected to radiation fogs. Thus, ecosystem exposure determines the mode, and to some extent the magnitude, of deposition. Total deposition among mountain sites was strongly related to the magnitude of the occult deposition (Grantz, Garner et al. 2003).

Table 1.1: Types and determinants of particle deposition and impact to vegetation (Grantz, Garner et al. 2003).

Types of deposition	Determinant of deposition	Quantifiable factors
Dry deposition	Ambient concentration	Distance from source
		Emission strength
	Atmospheric conditions	Wind speed
		Stability
		Mixing height
		Temperature
		Humidity
		Dew formation
	Aerosol properties	Chemical reactivity
		Particle solubility
		Aerodynamic diameter
		Biological availability
		Hygroscopicity
	Surface roughness	Terrain discontinuity
		Leaf pubescence
		Plant density
		Leaf shape
		Plant density
		Branch spacing
		Tissue flexibility
	Vegetation condition	Surface wetness
		Salt exudates
		Organic exudates
		Insect excreta
Wet deposition	Ambient concentration	Distance from source
		Emission strength
	Atmospheric conditions	Mixing height
		Timing of precipitation
		Intensity of precipitation
		Duration of precipitation
	Aerosol properties	Chemical reactivity
		Particle solubility
		Biological availability
	Aerosol properties	Chemical reactivity
		Particle solubility
		Biological availability
	Surface roughness	Terrain discontinuity
		Leaf pubescence
		Leaf area index
		Nature of exposed
		Bark or stem
Occult deposition	As above	Combination of above factors

1.6 Biomonitoring methods

Lichens may be used as bioindicators and/or biomonitors of air pollution in two different ways (Conti and Cecchetti 2001): 1) by mapping all species present in a specific area (distribution or frequency mapping using naturally occurring lichens), and 2) by the individual sampling of lichen species and measurement of the pollutant-specific response (such as the pollutants that accumulate in the thallus). Gradient studies may be undertaken using naturally occurring lichens (in-situ), or lichens may be transplanted from an uncontaminated area to a contaminated one, then measuring the morphological changes in the lichen thallus and/or evaluating the physiological parameters and/or evaluating the bioaccumulation of the pollutants (air pollution studies using lichen transplants).

The above indicates that a consideration of major importance is about deciding to use indigenous species (*in situ* lichens) or transplanted species (lichen transplants). This affects the selection of the species and may also be of relevance for the chemical analytical techniques employed. Factors which should be considered in methodology include finances and other resources, the desired accuracy and precision, time-scales, size of the study area, extend and type of pollution (Mulgrew and Williams 2000). In the present thesis, the focus is on trace elements in lichen transplants.

1.6.1 Gradient studies

Gradient studies correlate gradients (time, space,……) in pollutants with variabilities (over time, space,…..) in biomonitors responses (visible injury, species richness, species abundance, trace elements…..). These studies assume that the selected responses (exclusively) represent the environmental gradients. Many of these studies are done around existing or projected sources of contaminants, with pollutant loadings varying with distance from the source. Selected pollution response variables may be of biological nature (e.g. integrity of cell membranes, chlorophyll content and integrity, photosynthesis and respiration, potential

quantum yield of photosystem II (PSII), stress-ethylene production, MDA and ATP levels (Garty 2001), and may also comprise levels of the pollutants of interest.

Although appropriate in many cases, the use of non-specific responses implies the possibility that the observed effects reflect variabilities in other than the environmental parameters of interest.

1.6.2 Studies using lichen transplants

Lichen transplants are used in areas where lichens are absence or sparse, and/or when selected time periods of exposure are intended. Lichens or bark discs with lichen thalli attached can be placed at different distances from a pollution source, or distributed throughout a selected area. Richardson (Richardson 1991): reviews the use of transplants to assess air quality in urban environments and to monitor contaminants in air and water. (Pearson 1993) discusses advantages and limitations of transplant methods. Ideally, healthy lichens are transferred from an area where they occur naturally to a test area. Changes in physiology or element accumulation as a result of exposure are then studied. Physiological studies are most likely to be successful when using species within their normal range of adaptation both at the source and at the test area.

1.7 Species selection

In chemical element deposition biomonitoring, species selection criteria include the availability of the species, its tolerance, its bioaccumulation characteristics and ease of sampling (Wolterbeek and Bode 1995). The species to be used may depend on the element(s) of interest (Mulgrew and Williams 2000).

The nature and form of the chemical elements under study is important in the selection of species in that this often determines whether the lichen will die, show (toxicity) symptoms or accumulate without apparent harm (Richardson 1991). The chemical properties of an element, and those of the particle it may be associated to, may affect its accumulation by a biomonitor. The sensitivity of lichens to elevated

tissue concentrations of chemical elements (toxicity) varies greatly between species, populations and elements (Tyler 1989).

Table 1.2: Names and features of the most common species of lichens used for trace element biomonitoring (partially taken from (Bargagli, 1998)).

Species	**Ecology and distribution**
Hypogymnia physodes	Very wide distribution and ecological range; common on acid substrates (bark, siliceous rocks, soil and bryophytes); rather tolerant to air pollutants
Parmelia sulcata	In both hemispheres, from Artic to temperate regions; tolerant to relatively high air pollution in some urban environments
Parmelia caperata	Pantemperate species, widespread especially in the southern part of Europe, also occurring in the southern hemisphere; also found on well-lit rocks and overgrowing mosses
Evernia prunastri	Circumboreal-temperate species restricted to the northern hemisphere; very wide ecological range, usually found on bark, rarely on rocks; may be found in stunted forms in regions of moderate pollution
Pseudevernia furfuracea	Common in Europe, also known from Central America, Bolivia, eastern Africa; on different kinds of acid substrates (barks, siliceous rocks); occurring, with reduced vitality, in moderately polluted areas
Umbilicaria sp.	Widely distributed, cosmopolitan genus; most species are saxicolous and grow on acid substrates in alpine and polar habitats
Cladonia sp.	Cosmopolitan; considerable species variation in both morphology and chemistry

Species with the ability to bioaccumulate high chemical element concentrations without apparent symptoms may be used in "accumulation mode" rather than sensitive species: the latter may be used in studies where responses are followed such as abundance or behaviour. In general, studies on the chemical element sensitivities of lichens show that lichens containing cyanobacteria as the phycobiont are much more sensitive than lichens containing green algae (Bargagli 1998). In terms of sampling, fructose lichens (shrub-like) are easier to separate from substrate than foliose (leaf-like) and crustose (crust-forming) lichens. Table 1.2 shows the most common species of lichens used for trace element biomonitoring.

1.8 Standardised methods

Sampling and sample treatment protocols should be well documented (Zimmermann, Wagner et al. 2000). In moss biomonitoring, much work has been performed by Ake Rülhing (Sweden) and Eiliv Steinnes (Norway) who initiated the Europe-wide program of Atmospheric Heavy Metal Deposition in Europe – Estimation Based on Moss Analysis (Ruhling 1987; Ruhling 1994). For lichens, it is extremely important to developed more work in this area although some authors are starting to present the first protocols on trace elements air pollution biomonitoring studies using lichens (Garty 2002).

1.8.1 Sample collection strategies

In field studies, the quality assurance and quality control in the analytical laboratory can only be meaningful if also the sampling, as the first step of environmental analysis, is carried out with the same care and competence (Wagner 1995). Points to consider in sampling are the heterogeneous distribution of individual organisms in the overall system, and the intrinsic dynamics of living organisms in respect to their chemical composition as function of space and time (Markert 1995a). Standardisation of collecting methodologies would allow more appropriate comparisons to be made since sampling is one of the most important

steps in biomonitoring. Also, exposure, health status of lichens, the bark morphology and microclimatic conditions must be taken under consideration.

1.8.2 Sampling for in situ lichen studies (native or naturally occurring lichens)

Studies involving lichen thalli from homogeneous populations and collected at the same site usually show that element concentrations in areas affected by airborne particulate pollutants are highly variable. The frequency distribution of measured values tends to approach a bell-shape curve, so in order to improve sampling representativeness and to measure concentrations lying within the central part of this curve, 6-12 whole thalli from at least 3-6 tree trunks (possibly of the same species and similar diameters) should be taken at 1.5-2.0 m above the ground for each site. Wolterbeek and Bode (Wolterbeek and Bode 1995; Wolterbeek, Bode et al. 1996) discussed the possible substrate contributions to lichen elemental concentrations. This may vary depending on the species, substrate type and element. The Netherlands comparisons were carried out with (pH neutral) poplar, oak, willow and elm bark substrata and no significant effects of tree species on concentrations or local variations in elemental composition of *Parmelia sulcata* could be found (Wolterbeek and Bode 1995). Several healthy lichen thalli of different sizes were collected between 1.0 to 2.5 m above the ground and if possible, from 3 to 7 nearby trees in open light habitats, not exposed to extra nutrient supply (e.g. under fresh cut wounds), within a distance of 500 m of farms and motorways (Sloof 1993). Where possible, the lichen material was taken from all around the tree to reduce the influence of relative source positions (Sloof 1993) or also reducing influences from prevailing wind directions (Sloof 1995). Some other authors have adopted the same procedure for the same purpose (Jeran, Jacimovic et al. 1996). In a survey held in Portugal in 1993, a similar method was used; the same lichen *Parmelia sulcata* was collected from at least five different trees at a height of 1 to 2 m above the ground and to reduce the number of variables, lichens were collected from olive trees only (Freitas, Reis et al. 2000; Reis 2001) A similar procedure was performed (Sensen and Richardson 2002) where a composite lichen sample was collected from a group of three or more trees of the same tree species, starting at a minimum height of 1m. Material was

collected from both trunk and branches due to the scarcity of material. Others have collected from holm oak branches with inclinations lower than 45º (Rodrigo, Avila et al. 1999). Loppi et al. (Loppi and Bonini 2000) have collected lichen samples at 1.5 – 2 m above the ground only from trunks of isolated oaks (*Quercus pubescens*) and chestnut (*Castanea sativa*) not affected by steamflow. The authors have chosen these trees since the trace elements contents of lichens growing on oak and chestnut trees do not differ significantly. Some authors do not specify the substrate since soil is also collected (Rizzio, Bergamaschi et al. 2001). Figueira et al. have collected *Ramalina calicaris* from olive trees (*Olea europaea*) and *Usnea spp*. from cork oaks (*Quercus suber*) (Figueira, Pacheco et al. 2002). In some cases collection of lichen material was performed by several people on a rotational basis to avoid sampling bias (Chiarenzelli, Aspler et al. 1997).

The different age of the different parts of the lichen thallus have hardly been considered in biomonitoring of trace elements. However, it has been shown that in foliose lichens the content of some elements may increase in older parts of the same thallus and higher concentrations of trace elements have also been found in the lower, older podetium of terricolous fruticose lichens (Bargagli 1998). Carignan et al. has collected lichens only from the extremities of small tree branches in order to obtain samples that had been exposed to the atmospheric signal for a period of only a few years (Carignan, Simonetti et al. 2002). Nimis et al. have measured 16 chemical elements only in the peripheral and central parts of the foliose epiphytic lichens *Parmelia caperata* and *Xanthoria parietina* and found that the central, older parts of the thalli contained significantly higher amounts of most chemical elements in both species (Nimis, Andreussi et al. 2001).

One of the most complete sampling descriptions is the one performed by Nimis (Nimis, Lazzarin et al. 2000) where lichens were collected at each station from at least five different trees with: (a) inclination of the trunks less than 10º; (b) circumference >70 cm; (c) cover of bryophytes <30%. Peripheral parts (maximum 2 mm) of the lichen thalli, corresponding to the last year of growth were collected from at least six individuals from three different trees, 120 cm above the ground. Similar procedure was adopted by Loppi *et al.* (Loppi, Ivanov et al. 2002).

Storage of collected lichens or mosses material is normally into plastic bags (Branquinho, Brown et al. 1997; Figueira, Sousa et al. 1999; Capelão, Máguas et al. 2000; Rizzio, Bergamaschi et al. 2001) or strong brown paper bags (Bargagli 1995; Dilman 1996; Rodrigo, Avila et al. 1999; Coccaro, Saiki et al. 2000; Freitas, Reis et al. 2000; Sensen and Richardson 2002). Samples can be left in the latter bags for a few days without fungal overgrowth and the tops can be easily opened to allow the contents to air dry (Richardson, Shore et al. 1995).

In areas where lichens are not present it is possible to make biomonitoring studies by transplanting lichen thallus from an unpolluted area to the study area. Basically all sampling procedures described above, are valid and applicable for the collection of lichen material to be transplanted.

Once more, the need for exact protocols to be followed by all researchers is imperative not only for *in situ* but also for transplant studies.

1.9 Lichens and stress symptoms

A key lichen parameter is the lichen physiological vitality, sometimes analysed by determination of the lichen membrane permeability (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty 2001; Garty, Weissman et al. 2001). Certain procedures relate to the entire thallus, others refer to the photobiont part of the thallus while the exclusive response of the mycobiont is less documented (Wolterbeek, Garty et al. 2002). Electric conductivity was pointed out as the most sensitive parameter for physiological response to environmental stress, when compared to NDVI (normalised difference vegetation index) (Garty, Weissman et al. 2000) and chlorophyll degradation, being also related to the whole lichen and not to just the photobiont as are many other parameters (Mulgrew and Williams 2000). Damage of the plasma membrane permeability may be brought about by both the oxidation and crosslinking of membrane protein sulphydryls and by the introduction of lipid peroxydation (Wolterbeek, Garty et al. 2002). Previous studies have shown a link between the content of airborne elements accumulated in lichen transplants and the degradation of cell membranes. Most of these studies indicated a severe leakage of K from lichen thalli displaying a high degree of electric

conductivity due to cell membrane debility (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty 2001; Garty, Weissman et al. 2001).

However, K release may also be related to wet-dry cycles. Desiccation of lichens may cause disruption of the plasma membrane, but when water is added to a dry sample it may bring about the loss of soluble intracellular ions (Bargagli 1998).

Other studies (Calvelo, Baccalá et al. 2002) have analysed the effects of both transplantation to the original place and transplantation to an urban environment on the elemental composition of thalli of *Protousnea magellanica*, epiphytic, fruticose lichen. Results showed that, all transplanted collections are well differentiated from non-transplanted thalli. Those transplanted within the same site showed enough distortion in their elemental composition to group them in a cluster of their own, even when the transplanted method used placed them in a natural position. Another study (Godinho, Freitas et al. 2004) evaluated the stress effects in two epiphytic lichen species with different thallus morphology, the foliose *Parmelia caperata* and the fruticose *Evernia prunastri*, as resulting from transplanting from an unpolluted to an area nearby and to an air-polluted area. The results indicate the absence of stress effects of transplanting as such.

1.10 Exposure strategies using lichen transplants

Usually lichens are collected from a clean region and hanged on trees (Jeran, Smodis et al. 1993; Horvat, Jeran et al. 2000; Pla, Moreno et al. 2000; Vidergar-Gorjup, Sircelj et al. 2001). Figueira et al. have hanged the original phorophyte branches to a wood stand using a nylon thread (Figueira, Pacheco et al. 2002). Adamo *et al.* (Adamo, Giordano et al. 2003) has exposed lichen bags on house balconies far away from rain pipes, fixed on plastic sticks. The majority of the lichen transplants are used attached to the substrate, mostly bark (Sloof 1995; Reis, Alves et al. 1999). Some researchers specifically collect tree branches covered with lichens (Vidergar-Gorjup, Sircelj et al. 2001; Garty, Tomer et al. 2003) instead of pieces of lichen with bark attached (Jeran, Smodis et al. 1993; Horvat, Jeran et al. 2000; Haffner, Lomsky et al. 2001) while others put them in nylon nets or bags (Gonzalez and Pignata 1997; Carreras and Pignata 2001; Reis, Alves et al. 2002)

sometimes specifying the lichen exposed surface area (Sloof 1995) or sample weight (Carreras and Pignata 2001). Some authors defend that the bag construction should be made to keep the humidity of the sample stable to avoid its drying (Szczepaniak and Biziuk 2003). Generally lichen transplants are exposed 1.5 - 2 m above the ground (Jeran, Smodis et al. 1993; Horvat, Jeran et al. 2000; Haffner, Lomsky et al. 2001) but others use other heights of 2, 3 meters or more (Sloof 1995; Carreras and Pignata 2001; Adamo, Giordano et al. 2003; Garty, Tomer et al. 2003; Gonzalez, Pignata et al. 2003). Lichen transplants are exposed for a certain period of time depending on the purposes of the study and the general "health" status of the lichen.

Exposure procedures are highly variable. Bennet *et al.* (Bennet, Dibben et al. 1996) may serve as an example: they installed lichens on an artificial tree constructed of PVC pipe. Each tree consisted of an upright axis, with four horizontal branches, each carrying eight smaller branches,. To these latter branches, tree branches containing lichens were attached by fishing line in a horizontal position. To protect the lichens from direct sunlight, an umbrella-like cover was placed on top of the artificial tree. Each artificial tree was aligned north to south with each branch facing the four cardinal compass directions for easy reference.

Another example comes from the work of Haffner et. al.: Trying to investigate the effects of SO_2 on four lichens species thought to differ in sensitivity under field conditions in a long-term transplant experiment using an SO_2 gradient from only slightly polluted control to a very polluted area, some authors (Haffner, Lomsky et al. 2001) have exposed the lichens at the different experimental sites in PVC-coated wire-netting cages (30×20×20 cm) of 1 cm mesh-width which allowed free air circulation as well as direct fog and rain wetting under natural illumination. The lichens were fixed in the cages according to their natural growth position. The cages were fixed to wooden stakes 1.5 – 2m above the ground, facing southward in unshaded places.

Much work has been carried out in this area but it is concluded that more study is nedded concerning lichens transplants exposure.

1.11 Transplant studies (new developments)

In comparative evaluations of lichen transplants, (Cercasov, Pantelica et al. 2002) considered the initial elemental contents, the "accumulation factors" relative to the bulk deposition, the interspecies "calibration factors", and the "retention efficiencies", the latter defined as ratios of the lichen enrichment to the sum of the enrichment and the content in the lichen throughfall water.

Reis *et al*. presented a lichen response calibration experiment where several calibration methods were tested (Reis, Alves et al. 2002). The hypothesis was that lichens present a memory for their exposure history which fades out in time, permitted the establishing of calibration methods with better performance than linear regression calibration. The results obtained have shown that, for many elements available in the atmospheric environment, lichens do not act like a measuring instrument. Instead they present information on availability, already biased by biological effects. The modified availability was named Equivalent Constant Availability (ECA), which represents a group property similar to the Equivalent Aerodynamic Diameter for airborne particle sizes. The importance of this statistical description is that by using it, a calibration method was achieved which allows the determination of average, maxima and standard deviation of availability variables out of a lichen survey data. For this to be possible, a significant number of time separated samplings (say three or four sampling campaigns within a 1-year period) are necessary (see also Reis 2001 (Reis 2001)).

Some works have aimed at finding materials that could be used as alternatives to lichens as air quality monitors since the decreasing natural abundance and the large amount of lichen collected are two drawbacks of the use of these organisms. Freitas *et al.* focused on the evaluation of ectohydric bryophytes and bark from *Cryptomeria japonica* as an alternative to epiphytic lichens for air-monitoring purposes. The authors came up to the conclusion that, all things considered – including material availability and ecological concern – bark stands for a sensible choice for biomonitoring in the Azores (Freitas, Pacheco et al. 2006). Lichen transplants have also been used to find complementary/alternative tools to biomonitoring such as the use of non biological monitors. A field study was carried out in the spring and summer of 2003 (2 months each) in Portugal, to assess the

efficiency of alternative exposure modes of biological monitors – lichen biomass and tree-bark biomass – together with prospective, non-biological monitors – cellulose acetate and Chelex-100™ resin – versus conventional transplants of the same species (Machado, Freitas et al. 2004). In another study (Baptista, Vasconcelos et al. 2006), lichens were exposed in three different forms (transplant, detached from the substratum and as a biomass - ground and homogenized) and compared to the tree bark, exposed as a biomass, and two organic synthetic materials (Chelex-100™ resin and cellulose acetate). The results showed that the airborne accumulations of Cu, Ni, Pb and Sr were partially dependent on the meteorological conditions but mainly dependent on the nature of the exposed material. The standard deviations of the synthetic materials or homogenized biomass were the same or greater than lichen transplants or detached. The accumulation by biological materials, of the four studied elements, was comparable to the lichen transplant accumulation. The replacement of the traditional transplants by the biomass was not considered advantageous, since their preparation is time-consuming. Therefore lichens remained the most suitable in biomonitoring studies. The exposure of detached lichen allows the accurate measurement of the exposed area/volume so it can be useful to relate atmospheric deposition rates with the lichen chemical element content (Freitas, Pacheco et al. 2007).

Recently, arsenic speciation was studied in lichens. Farinha *et al.* (Farinha, Slejkovec et al. 2004) made a three-step sequential extraction procedure for extraction of arsenic species in lichen transplants and airborne particulate matter. Inorganic forms of arsenic (arsenite and arsenate) were present in significant amounts in most of the samples. Only in lichens also organic forms of arsenic (monomethyl arsonic acid and dimethyl arsinic acid) were identified which may indicate biotransformation of inorganic arsenic. In another study, arsenic and its chemical species were determined in transplanted lichens (*Parmelia caperata*) and tree bark (*Platanus hybrida*), in order to get a better understanding of their atmospheric cycling and the suitability of these materials for biomonitoring purposes. Various strategies were used (discontinuous and cumulative exposure of transplants) to biomonitor two highly industrialised areas in Portugal (Machado, Slejkovec et al. 2006).

The speciation data may facilitate more close fingerprinting of possible sources and/or help explaining biomonitor (vitality) responses to ambient pollutants.

Generally, when lichens are used in biomonitoring studies to indicate geographical- or time variances in trace-element air pollution (Conti and Cecchetti 2001; Garty 2001; Jacquiot and Daillant 2002; Wolterbeek, Garty et al. 2002), authors assume that the lichen response behaviour can be compared throughout the whole investigated area or time period, irrespective of variances in ambient conditions. For transplants, recent studies also focus on the effects of the transplanting (Calvelo, Baccalá et al. 2002; Godinho, Freitas et al. 2004).

1.12 Sample Preparation for Analysis

The (instrumental) element analysis of plant samples includes cleaning, drying and homogenization of the collected material. Cleaning may include purely mechanical steps such as the use of dry or moistened tissues, shaking, blowing and brushing of the sample material, or various washing techniques. Especially with cleaning, solvents leaching should be considered (Markert 1995b). After cleaning, the sample should be prepared for analysis. The non-homogeneity presents a serious sampling dilemma: how to represent a large, often non-uniform total mass by a small analytical sub-sample. For solid samples, often the best approach is first to take a quantity of material large enough to be compositionally representative and reduce it by selected grinding approaches to a fine powder. Then the sample can be adapted to a particular analytical technique, pressed into a sample disc, fused, dissolved, etc. This is the most vulnerable stage of an analysis with regard to contamination (Hamilton 1995).

For the assessment of element pollution levels and identification of their sources, which are a prerequisite for studying effects of contaminants on the environment and human health, following Smodis (2007) (Smodis 2007), a multivariate data base containing as many pollutant elements should be generated (Bode and Wolterbeek 1990; Sloof 1993; Kuik and Wolterbeek 1995; Wolterbeek and Bode 1995; Reis 2001; Freitas, Pacheco et al. 2006). Measuring a wide range of pollutants allows the assessment of source profiles (correlated abundances) and

offers the opportunity to investigate whether it is a specific pollutant or a source profile (with possibly unknown additional components) which might be responsible for any (human) health effects observed in correlative air pollution studies (Sarmento, Wolterbeek et al. 2008). Therefore, multielement methods are usually applied for such studies. In practise, the determination of the elemental content of samples is governed by sample type, the elements of interest, the asked-for sensitivity, precision and accuracy, and the availability of (or access to) the technique.

1.13 Multi-elemental analytical Techniques for Lichen Sample Analysis

Table 1.3 – Classification of bulk elemental analysis methods for plant samples.

Bulk Elemental Analysis Techniques			
Instrumental X-ray / γ-ray Techniques	**Optical Absorption / Emission Techniques**	**Mass Spectrometric Methods**	**Miscellaneous Techniques**
INAA; RNAA	AAS	GDMS	Wet Chemical
PIXE/PIGE	GFAAS; ETAAS	SSMS	ISE's
XRF	ICP/AES	ICP/MS	Ion Chromatography
SXRF	OES	Other	Colorimetric
Other	Other		Other

INAA - Instrumental Neutron Activation Analysis
RNAA - Radiochemical Neutron Activation Analysis
PIXE - Particle Induced X-Ray Emission
PIGE - Particle Induced γ-Ray Emission
XRF – X-Ray Fluorescence Analysis
SXRF - Synchrotron X-Ray Fluorescence
AAS - Atomic Absorption Spectrometry
GFAAS - Graphite-Furnace Atomic Absorption Spectrometry
ETAAS - Electrothermal Atomic Absorption Spectrometry
ICP/AES - Inductively Coupled/Plasma Atomic Emission Spectrometry
OES - Optical Emission Spectroscopy
GDMS - Glow-Discharge Mass Spectrometry
SSMS - Spark-Source Mass Spectrometry
ICP/MS - Inductively Coupled Plasma/Mass Spectrometry
ISE's - Ion-Selective Electrodes

Huggins (Huggins 2002) recognizes four groups of multi-elemental techniques for (lichen) analysis (see also Table 1.3):

- Instrumental X-ray/γ-ray techniques, which depend on the generation, detection and measurement of characteristic X-rays and/or γ-rays for element determination;
- Optical absorption/emission techniques, which depend on the generation, detection and measurement of characteristic atomic transitions in the visible and near – visible regions of the electromagnetic spectrum for element determination.
- Mass spectrometric methods, which depend on total volatilization of the sample and its introduction in such a form into a mass spectrometer that the atomic masses can be discriminated quantitatively among the different elements (and isotopes).
- Miscellaneous techniques: such techniques generally determine one or a few elements that, for one reason or another, cannot be adequately determined by other more general methods; such techniques include wet chemical and various electroanalytical methods.

1.14 INAA and PIXE

Other than their inherent accuracy, analytical robustness and non-destructive features (Wolterbeek 2001), instrumental neutron activation analysis (INAA) and particle-induced X-ray emission (PIXE) are both multi-elemental techniques that complement – and partially overlap – each other with respect to a vast majority of elements. Considering environmental samples:

Major advantages of INAA are:

- Many elements of the periodic table, up to 40 elements determined in solids simultaneously;
- Simple sample preparation, no digestion necessary (less time consuming and potential contamination is minimized);
- Hardly any matrix effects, transparent to Si, C, O and N.

Major disadvantages of INAA are:

- Depends on a neutron source, mostly a nuclear reactor, limiting INAA-availability;
- Insensitive to elements such as B, Be, Pb and Tl and low detection limits or significant interferences for Cd, Cu, F, Hg, Mo, Ni, and Si;
- Long turn around time.

Major advantages of PIXE are:

- Many elements of the periodic table determined in solids simultaneously;
- Some elements determined like Cu, Ni, S, Si, P, Pb etc complementary to INAA;
- Well suited for very small samples;
- Short turn around time.

Major disadvantages of PIXE are:

- Depends on a particle beam, limiting PIXE-availability;
- Solid samples, but more complicated in preparation than with INAA;
- Small sample masses in actual analysis (surface technique): difficulties in representativeness;
- Large matrix corrections, especially in thick samples.

There are several studies reporting on the INAA-PIXE comparative outcomes for biological and environmental material (Randle, Aljundi et al. 1993; Freitas, Reis et al. 2000; IAEA-TECDOC-1295 2002). Descriptive statistics for olive trees and bark indicate comparable results by INAA and PIXE (Pacheco, Freitas et al. 2003), and was also suggested for selected Fe, Mn, and K in lichens (Pacheco, Freitas et al. 2004). For Rb, biases were reported (Pacheco, Freitas et al. 2004). The authors however, hypothesed that an improvement in chemical and physical homogeneity of PIXE samples may improve the comparability of both techniques (see also Reis 2001, for comparative Cl results in lichens).

Apparently, for complementary use of PIXE and INAA, comparative controls are asked for, for numerous elements and for any type of samples to be studied. In general terms, the analytical technique(s) to be used should be selected on basis of the elements to be determined, the physical states of the samples, the necessary sample preparations, and the logistics of the whole process (time, costs), set in the context of the project. For air pollution studies, comprising biomaterials, soils, dusts etc., both PIXE and INAA are considered as sufficiently sensitive and inexpensive (Wolterbeek, Garty et al. 2002).

1.15 Scope of the thesis

The topic of the present thesis is the (bio) monitoring of trace element air pollution, with the attention focused on used techniques and selected approaches. Lichens are used in all experiments, and elemental analyses are by nuclear multi-elements techniques. The thesis is focused on both survey aspects, thereby aiming predominantly at both transplant vitality and the effects of the positioning of the transplants used, and on analytical aspects, thereby concentrating on aspects of comparative PIXE and INAA. The thesis consists of four parts: the first part is dedicated to elemental analysis, the second is about viability and vitality of lichens, third is focused on possible set-ups in transplant monitoring, and the fourth is a transplant case study in an industrialised region, aimed at the recognition of emission source profiles of industrial sources and possible differentiation between selected positioning, but variable in wind-directional exposure. The thesis is divided in six chapters:

Chapter 1 (Introduction) presents the main issues of the thesis. Chapter 2 (Grain-size effects on PIXE and INAA analysis of iaea-336 lichen reference material) focuses on the complementary use of the nuclear analytical techniques INAA and PIXE, discusses accuracy and precision, and specifically addresses grain sizes of the initial bulk samples used for PIXE.. Chapter 3 (Cell-membrane damage and element leaching in transplanted Parmelia sulcata lichen related to ambient SO2, temperature, and precipitation), addresses viability and vitality: lichens were

transplanted from a clean background site, exposed in an industrial area and collected on a regular basis to verify membrane damage during a one-year exposure. The main objective was to relate variability in lichen vitality to variability's in ambient conditions as well as to time-related changes in accumulated trace elements, to gain insight into the possibilities to use and compare lichens throughout larger geographical areas. Data were gathered on lichen element content, elements in leachates, electrical conductivity of leachates, and of the ambient parameters temperature, precipitation and SO2 level. Chapter 4 (Transplants set-ups and positioning towards wind direction: element concentrations and relationships with atmospheric element deposition) addresses the effects of positioning, thereby considering both wind-directional issues, rain-shielding and physical positioning. The chapter comprises three transplant-positioning approaches (free, horizontal covering, and vertical covering), combined with a wind-directional adaptation in the first two set-ups. Chapter 5 (Biomonitoring study of Setúbal peninsula region) focuses on a case study on transplant positioning in a survey carried out in the Setúbal area that includes the Sado river estuary and a very industrial city. It addresses the wind-directional positioning of lichen transplants, both in terms of total element concentrations, time of exposure, and transplant-expression of possible emission source profiles (by application of Monte Carlo Added Target Transformation Factor Analysis – MCATTFA). The main objective was to test the possibility of using the wind-directional positioning as a tool for the enhancement of the detection strength of local and remote sources Chapter 6 presents the general discussion and conclusions.

References

Adamo, P., Giordano, S., Vingiani, S., Cobianchi, R.C., and Violante, P. (2003) Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy). *Environmental Pollution* **122**: 91 - 103.

Baptista, M.S., Vasconcelos, M.T., Cabral, J.P., Freitas, M.C., and Pacheco, A.M.G. (2006) The ability of biological and organic synthetic materials to accumulate atmospheric particulates containing copper, lead, nickel and strontium. *Journal of Environmental Monitoring* **8**: 147-152.

Bargagli, R. (1995) The elemental composition of vegetation and the possible incidence of soil contamination of samples. *The Science of the Total Environment* **176**: 121-128.

Bargagli, R. (1998) Lichens as biomonitors of airborne trace elements. In *Trace Elements in Terrestrial Plants: an Ecophysiological Approach to Biomonitoring and Biorecovery*. Berlin, Germany: Springer Verlag, pp. 179-206.

Batzias, F., and Siontorou, C. (2007) A novel system for environmental monitoring through a cooperative/synergistic scheme between bioindicators and biosensors. *Journal of Environmental Management* **82**: 221–239.

Beeby, A. (2001) What do sentinels stand for? *Environmental Pollution* **112**: 285–298.

Bennet, J.P., Dibben, J.M., and Lyman, K.J. (1996) Elemental concentrations in the lichen *Hypogymnia physodes* (L.) Nyl., after 3 years of transplanting along lake Michigan. *Environmental and Experimental Botany* **36**: 255-270.

Bode, P., and Wolterbeek, H.T. (1990) Environmental research and instrumental neutron activation analysis: aspects of high accuracy and multi-element capability. *Journal of Trace Microprobe Techniques* **8**: 121-138.

Branquinho, C., Brown, D.H., Máguas, C., and Catarino, F.M. (1997) Lead (Pb) uptake and its effects on membrane intigrity and chlorophyll fluorescence in different lichen species. *Environmental and Experimental Botany* **37**: 95-105.

Brown, D.H. (1976) Mineral uptake by lichens. In *Lichenology: Progress and Problems*. D. H. Brown, D.H.H.a.R.B. (ed). London, United Kingdom: Academic Press, pp. 419-439.

Brown, D.H., and Brown, R.M. (1991) Mineral cycling and lichens: the physiological basis. *Lichenologist* **23**: 293-307.

Brunialti, G., and Frati, L. (2007) Biomonitoring of nine elements by the lichen Xanthoria parietina in Adriatic Italy: A retrospective study over a 7-year time span. The *Science of the Total Environment* **387**: 289-300.

Calvelo, S., Baccalá, N., Bubach, D., Arribére, M., and Ribeiro, S. (2002) Statistical analysis of elemental composition of lichen thalli from pristine areas transplanted within their original habitat and to urban areas (Patagonia, Argentina). *Bibliotheca Lichenologica* **82**: 165-174.

Capelão, A.L., Máguas, C., Branquinho, C., Cruz, C., and Martins-Loução, M.A. (2000) Lichens as biomonitors of atmospheric ammonium/ammonia deposition in Portugal. In *Biomonitoring of Atmospheric Pollution (with Emphasis on Trace Elements) - BioMAP*. IAEA (ed). Lisbon, Portugal: IAEA, pp. 168-172.

Carignan, J., Simonetti, A., and Gariépy, C. (2002) Dispersal of atmospheric lead in northeastern North America as recorded by epiphytic lichens. *Atmospheric Environment* **36**: 3759-3766.

Carreras, H.A., and Pignata, M.L. (2001) Comparison among air pollutants, meteorological conditions and some chemical parameters in the transplanted lichen *Usnea amblyoclada*. *Environmental Pollution* **111**: 45 - 52.

Cercasov, V., Pantelica, A., Salagean, M., Caniglia, G., and Scarlat, A. (2002) Comparative study of the suitability of three lichen species to the trace-element air monitoring. *Environmental Pollution* **119**: 129-139.

Chiarenzelli, J.R., Aspler, L.B., Ozarko, D.L., Hall, G.E.M., Powis, K.B., and Donalson, J.A. (1997) Heavy metals in lichens, southern district of Keewatin, northwest territories, Canada. *Chemosphere* **35**: 1329-1341.

Coccaro, D.M.B., Saiki, M., Vasconcellos, M.B.A., and Marcelli, M.P. (2000) Analysis of *Canoparmelia texana* lichens collected in Brazil by neutron activation analysis. In *Biomonitoring of Atmospheric Pollution (with Emphasis on Trace Elements) - BioMAP*. IAEA (ed). Lisbon, Portugal: IAEA, pp. 143-148.

Conti, M.E., and Cecchetti, G. (2001) Biological monitoring: lichens as bioindicators of air pollution assessment - a review. *Environmental Pollution* **114**: 471-492.

Dilman, K.L. (1996) Use of the lichen *Rhyzoplaca melanophthalma* as a biomonitor in relation to phosphate refineries near Pocatello, Idaho. *Environmental Pollution* **92**: 91-96.

Farinha, M.M., Slejkovec, Z., Van Elteren, J.T., Wolterbeek, H.T., and Freitas, M.C. (2004) Arsenic speciation in Lichens and in coarse and fine airborne particulate matter by HPLC–UV–HG–AFS. *Journal of Atmospheric Chemistry* **49**: 343-353.

Ferretti, M., Brambilla, E., Brunialti, G., Fornasier, F., Mazzali, C., Giordani, P., and Nimis, P. (2004) Reliability of different sampling densities for estimating and mapping lichen diversity as a bioindicator of environmental quality. *Environmental Pollution* **127**: 249–256.

Figueira, R. (2002) *Desenvolvimento de um Sistema de Biomonitorização Ambiental*. PhD Thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal, pp. 51-107, in Portuguese.

Figueira, R., Pacheco, A.M.G., Sousa, A.J., and Catarino, F.M. (2002) Development and calibration of epiphytic lichens as saltfall biomonitors - dry-deposition modelling. *Environmental Pollution* **120**: 69-78.

Figueira, R., Sousa, A.J., Brown, D.H., Catarino, F.M., and Pacheco, A.M.G. (1999) Natural levels of saline elements in lichens: determination of cellular fractions and their importance as saline tracers. *Lichenologist* **31**: 183-196.

Frati, L., Brunialti, G., and Loppi, S. (2005) Problems related to lichen transplants to monitor trace element deposition in repeated surveys: a case study from central Italy. *Journal of Atmospheric Chemistry* **52**: 221–230.

Freitas, M.C., and Nobre, A.S. (1997) Bioaccumulation of heavy metals using *Parmelia sulcata* and *Parmelia caperata* for air pollution studies. *Journal Radioanalytical and Nuclear Chemistry* **217**: 17-20.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace Elements - their Distribution and Effects in the Environment*. Market and Friese (ed). Amsterdam, The Netherlands: Elsevier Science, pp. 187-213.

Freitas, M.C., Pacheco, A.M.G., Vieira, B.J., and Rodrigues, A.F. (2006) Neutron activation analysis of atmospheric biomonitors from the Azores: a comparative study of lower and higher plants. *Journal of Radionalytical and Nuclear Chemistry* **270**: 21-27.

Garty, J. (2002) Biomonitoring heavy metal pollution with lichens. In *Protocols in Lichenology, Culturing, biochemistry, Ecophysiology and use in Biomonitoring.* Kranner, Beckett and Varma (ed.). Berlin, Germany, Springer Lab Manual, pp. 458-482.

Garty, J. (2001) Biomonitoring atmospheric heavy metals with lichens: theory and application. *Critical reviews in plant sciences* **20**: 309-371.

Garty, J., Galun, M., and Kessel, M. (1979) Localization of heavy metals and other elements accumulated in the lichen thallus. *New Phytologist* **82**: 159-168.

Garty, J., Cohen, Y., and Kloog, N. (1998) Airborne elements, cell membranes, and chlorophyll in transplanted lichens. *Journal of Environmental Quality* **27**: 973-979.

Garty, J., Tomer, S., Levin, T., and Lehr, H. (2003) Lichens as biomonitors around a coal-fired power station in Israel. *Environmental Research* **91**: 186-198.

Garty, J., Weissman, L., Cohen, Y., Karnieli, A., and Orlovsky, L. (2001) Transplanted lichens in and around the Mount Carmel National Park and the Haifa Bay industrial region in Israel: physiological and chemical responses. *Environmental Research A* **85**: 159-176.

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A., and Orlovsky, L. (2000) Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiologia Plantarum* **109**: 410-418.

Godinho, R.M., Freitas, M.C., and Wolterbeek, H.T. (2004) Assessment of lichen vitality during a transplantation experiment to a polluted site. *Journal of Atmospheric Chemistry* **49**: 355-361.

Gonzalez, C.M., and Pignata, M.L. (1997) Chemical response of the lichen *Punctelia subrudecta* (Nyl.) Krog transplanted close to a power station in an urban-industrial environment. *Environmental Pollution* **97**: 195-203.

Gonzalez, C.M., Pignata, M.L., and Orellana, L. (2003) Applications of redundancy analysis for the detection of chemical response patterns to air pollution in lichen. *The Science of the Total Environment* **312**: 245-253.

Goyal, R., and Seaward, M.R.D. (1981) Metal uptake in terricolous lichens; I. Metal localization within the thallus. *New Phytology.* **89**: 631-645.

Grantz, D.A., Garner, J.H.B., and Johnson, D.W. (2003) Ecological effects of particulate matter. *Environment International* **29**: 213-239.

Groet, S.S. (1976) Regional and local variations in the heavy metal concentrations of bryophytes in the northeastern United stades. *Oikos* **27**: 445-456.

Haffner, E., Lomsky, B., Hynek, V., Hallgren, J., Batic, F., and Pfanz, H. (2001) Air pollution and lichen physiology - physiological responses of different lichens in a transplant experiment following an SO_2 - gradient. *Water Air and Soil Pollution* **131**: 185-201.

Hamilton, E.I. (1995) State of the art of trace element determination in plant matrices: Determination of the chemical elements in plant matrices, an overview. *The Science of the Total Environment* **176**: 3-14.

Horvat, M., Jeran, Z., Spiric, Z., Jacimovic, R., and Miklavcic, V. (2000) Mercury and other elements in lichens near the INA naftaplin gas treatment plant, Molve, Croatia. *Journal of Environmental Monitoring* **2**: 139-144.

Huggins, F.E. (2002) Overview of analytical methods for inorganic constituents in coal. *International Journal of Coal Geology* **50**: 169-214.

IAEA-TECDOC-1295 (2002) Reference materials for microanalytical nuclear techniques. Vienna, Austria, IAEA pp. 1-14.

Jacquiot, L., and Daillant, O. (2002) Bio-accumulation of heavy metals, trace elements and radioelements, review-part III. Mazille, France: Bulletin de l'Observatoire Mycologique, pp. 1-33.

Jeran, Z., Smodis, B., and Jacimovic, R. (1993) Multielemental analysis of transplanted lichens (*Hypogymnia physodes, L. Nyl.)* by instrumental neutron activation analysis. *Acta Chimica Slovenica* **40**: 289-299.

Jeran, Z., Jacimovic, R., Batic, F., Smodis, B., and Wolterbeek, H.T. (1996) Atmospheric heavy metal pollution in Slovenia derived from results for epiphytic lichens. *Fresenius Journal of Analytical Chemistry* **354**: 681-687.

Kansanen, P., and Venetvaara, J., (1991) Comparison of biological collectors of airborne heavy metals near ferrochrome and steel works. *Water, Air and Soil Pollution* **60**: 337-359.

Kirschbaum, U., and Hanewald, K. (1998) Biological indication of air pollution by lichen mapping in long time observation areas in Hesse/Germany. *Journal of Applied Botany* **72**: 212–227.

Kuik, P., and Wolterbeek, H.T. (1995) Factor analysis of atmospheric trace-element deposition data in The Netherlands obtained by moss monitoring. *Water, Air and Soil Pollution* **83**: 323-346.

Loppi, S., and Bonini, I. (2000) Lichens and mosses as biomonitors of trace elements in areas with thermal springs and fumarole activity (Mt. Amiata, central Italy). *Chemosphere* **41**: 1333-1336.

Loppi, S., Ivanov, D., and Boccardi, R. (2002) Biodiversity of epiphytic lichens and air pollution in the town of Siena (Central Italy). *Environmental Pollution* **116**: 123-128.

Machado, A., Freitas, M.C., and Pacheco, A.M.G. (2004) Relative response of biological and non-biological monitors in a coastal environment. *Journal of Atmospheric Chemistry*: **49**: 161-173.

Machado, A., Slejkovec, Z., Van Elteren, J.T., Freitas, M.C., and Baptista, M.S. (2006) Arsenic speciation in transplanted lichens and tree bark in the framework of a biomonitoring scenario. *Journal of Atmospheric Chemistry* **53**: 237-249.

Markert, B. (1995a) Quality assurance of plant sampling and storage. In *Quality Assurance in Environmental Monitoring - Sampling and Sample Pretreatment*. Quevauviller, P. (ed). Weinheim: VCH - Publisher, pp. 215-254.

Markert, B. (1995b) Sample preparation (cleaning, drying, homogenization) for trace elements analysis in plant matrices. *The Science of the Total Environment* **176**: 45-61.

Markert, B.A., Breure, A.M., and Zechmeister, H.G. (2003) Definitions, strategies and principles for bioindication/biomonitoring of the environment. In *Bioindicators and Biomonitors. Principles, Concepts and applications*. Markert, B., Breure, A. M., Zechmeister, H. G., (ed). Amsterdam, The Netherlands: Elsevier, pp. 3-39.

Mulgrew, A., and Williams, P. (2000) Biomonitoring of air quality using plants. In. Berlin, Germany: WHO Colaborating Centre for Air Quality Management and Air Pollution Control, Federal Environmental Agency, pp. 1-165.

Nash III, T.H. (1996) *Lichen Biology*. England: Cambridge University Press.

Nieboer, E., Lavoie, R., and Susseville, K. (1976a) Cation-exchange equilibrium and mass balance in the lichen *Umbilicaria muhlenbergii*. *Canadian Journal of Botany* **54**: 720-723.

Nieboer, E., Puckett, K.J., and Grace, B. (1976b) The uptake of nickel by *Umbilicaria muhlenbergii*: A physiological process. *Canadian Journal of Botany* **54**: 724-733.

Nieboer, E., Richardson, D.H.S., and Tomassini, F.D. (1978) Mineral uptake and release by lichens: an overview. *Bryologist* **81**: 226-246.

Nimis, P.L., Castello, M., and Perotti, M. (1990) Lichens as biomonitors of sulfur-dioxide pollution in La-Spezia (Northern Italy). *Lichenologist* **22**: 333–344.

Nimis, P.L., Andreussi, S., and Pittao, E. (2001) The performance of two lichen species as bioaccumulators of trace metals. *The Science of the Total Environment* **275**: 43-51.

Nimis, P.L., Lazzarin, G., Lazzarin, A., and Skert, N. (2000) Biomonitoring of trace elements with lichens in Veneto (NE Italy). *The Science of the Total Environment* **255**: 97-111.

Pacheco, A.M.G., Freitas, M.C., and Reis, M.A. (2003) Trace-element measurements in atmospheric biomonitors - a look at the relative performance of INAA and PIXE on olive-tree bark. *Nuclear Instruments and Methods in Physics Research A* **505**: 425-429.

Pacheco, A.M.G., Freitas, M.C., and Ventura, M.G. (2004) A nonparametric assessment of the relative output of INAA and PIXE on joint determinands in environmental samples (atmospheric biomonitors). *Nuclear Instruments and Methods in Physics Research B* **222**: 587-592.

Pakarinen, P. and Tolonen, K. (1976) Regional Survey of heavy metal in peat mosses (sphagnum). *Ambio* **5**: 38-40.

Pearson, L. Stolte, K., Mangis, D., Doty, R., Tonnessen, K., Huckaby, L. S., (1993) Active monitoring. In *Lichens as Bioindicators of Air Quality*. Fort Collins, Colorado: U.S. Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station, pp. 89-95.

Pla, R.R., Moreno, M.A., and Adler, M. (2000) The use of biomonitors and neutron activation analysis in the study of air pollution of Buenos Aires city. In *Biomonitoring of Atmospheric Pollution (with Emphasis on Trace Elements) - BioMAP*. IAEA (ed). Lisbon, Portugal: IAEA, pp. 122-128.

Puckett, K.J. (1988) Bryophytes and lichens as monitors of metal deposition. In *Lichens, Bryophytes and Air Quality*, pp. 231-267.

Randle, K., Aljundi, J., Mamas, C.J.V., Sokhi, R.S., and Earwaker, L.G. (1993) A comparative-study of neutron activation analysis and proton induced X-Ray emission analysis for the determination of heavy-metals in estuarine sediments. *Nuclear Instruments and Methods B* **79**: 568-570.

Rasmussen, L. (1978) Element concentrations of Hypnum cupressiforme related to element content of the bark of different species of phorophytes. *Lindbergia* **4**, 2009-218.

Reis, M.A. (2001) *Biomonitoring and Assessment of Atmospheric Trace Elements in Portugal - Methods, Response Modelling and Nuclear Analytical Techniques*. PhD Thesis, Delft University of Technology, Delft, The Netherlands: pp. 1-230.

Reis, M.A., Alves, L.C., Freitas, M.C., Van Os, B., and Wolterbeek, H.T. (1999) Lichens (*Parmelia sulcata*) time response model to environmental elemental availability. *The Science of the Total Environment* **232**: 105-115.

Reis, M.A., Alves, L.C., Freitas, M.C., Van Os, B., De Goeij, J.J.M., and Wolterbeek, H.T. (2002) Calibration of lichen transplants considering faint memory effects. *Environmental Pollution* **120**: 87-95.

Richardson, D.H.S. (1991) Lichens as biological indicators - recent developments. In *Bioindicators and Environmental Management*. Jeffrey, D. W., Madden, B., (ed). London, United Kingdom: Academic Press, pp. 263-272.

Richardson, D.H.S., Kiang, S., Ahmadjian, V., and Nieboer, E. (1985) Lead and Uranium uptake by lichens. In *Lichen Physiology and Cell Biology*. Brown, D.H. (ed). New York, USA: Plenum Publishing Corporation, pp. 227-246.

Richardson, D.H.S., Shore, M., Hartree, R., and Richardson, R.M. (1995) The use of X-ray fluorescence spectrometry for the analysis of plants, especially lichens, employed in biological monitoring. *The Science of the Total Environment* **176**: 97-105.

Rizzio, E., Bergamaschi, L., Valcuvia, M.G., Profumo, A., and Gallorini, M. (2001) Trace elements in lichens and in airborne particulate matter for the evaluation of the atmospheric pollution in a region of northern Italy. *Environment International* **26**: 543-549.

Rodrigo, A., Avila, A., and Gómez-Bolea, A. (1999) Trace metal contents in *Parmelia caperata* (L.) Ach. compared to bulk deposition, throughfall and leaf-wash fluxes in two holm oak forests in Montseny (NE Spain). *Atmospheric Environment* **33**: 359-367.

Ruhling, A., and Tyler, G. (1973) Heavy metal deposition in Scandinavia. *Water, Air and Soil Pollution* **2**: 445-455.

Ruhling, A. (1994) *Atmospheric Heavy Metal Deposition in Europe - Estimation Based on Moss Analysis*. Copenhagen: The Nordic Council of Ministers.

Sarmento, S., Wolterbeek , H.T., Verburg, T.G., and Freitas, M.C. (2008) Correlating element atmospheric deposition and cancer mortality in Portugal: Data handling and preliminary results. *Environmental Pollution* **151, (**2), 341-351.

Sensen, M., and Richardson, D.H.S. (2002) Mercury levels in lichens from different host trees around a chloralkali plant in New Brunswick, Canada. *The Science of the Total Environment* **293**: 31-45.

Sloof, J.E. (1993) *Environmental Lichenology: Biomonitoring Trace Element Air Pollution*. PhD Thesis, Delft University of Technology, Delft, The Netherlands, pp 1-191.

Sloof, J.E. (1995) Lichens as quantitative biomonitors for atmospheric trace-element deposition, using transplants. *Atmospheric Environment* **29**: 11-20.

Smodis, B., and Bleise, A. (2002) Internationally harmonized approach to biomonitoring trace element atmospheric deposition. *Environmental pollution* **120**: 3-10.

Smodis, B. (2007) Investigation of trace element atmospheric pollution by nuclear analytical techniques at a global scale: Harmonised approaches supported by the IAEA. *Journal of Environmental Management* **85**: 121-127.

Steinnes, E. (1989) *Biomonitors of air pollution by heavy metals. In Control and Fate of Atmospheric Trace Metals*. J.M. Pacyna and B. Ottar (Ed), Kluwer Academic Publishers, Dordrecht, 321-338.

Steinnes, E., Rambaek, J.P. and Hanssen, J.E. (1992) Large-scale multi-element survey of atmospheric deposition using naturally growing moss as biomonitor. *Chemosphere* **25** (5): 735-752.

Szczepaniak, K., and Biziuk, M. (2003) Aspects of the biomonitoring studies using mosses and lichens as indicators of metal pollution. *Environmental Research* **93**: 221-230.

Tyler, G. (1989) Uptake, retention and toxicity of heavy metals in lichens. A brief review. *Water, Air and Soil Pollution* **47**: 321-333.

Vidergar-Gorjup, N., Sircelj, H., Pfanz, H., and Batic, F. (2001) Some physiological effects of biocide treatment on the lichen *Pseudevernia furfuracea* (L.) Zopf. *Symbiosis* **31**: 123 - 140.

Wannaz, D., Carreras, H., Pérez, C., and Pignata, M. (2006) Assessment of heavy metal accumulation in two species of *Tillandsia* in relation to atmospheric emission sources in Argentina. *The Science of the Total Environment* **361**: 267–278.

Wagner, G. (1995) Basic approaches and methods for quality assurance and quality control in sample collection and storage for environmental monitoring. *The Science of the Total Environment* **176**: 63-71.

Wolterbeek, H.T. (2001) Large-scaled biomonitoring of trace element air pollution: goals and approaches. *Radiation Physics and Chemistry* **61**: 323-327.

Wolterbeek, H.T. (2002) Biomonitoring of trace element air pollution: principles, possibilities and perspectives. *Environmental Pollution* **120**: 11-21.

Wolterbeek, H.T., and Bode, P. (1995) Strategies in sampling and sample handling in the context of large-scale plant biomonitoring surveys of trace element air pollution. *The Science of the Total Environment* **176**: 33-43.

Wolterbeek, H.T., Bode, P., and Verburg, T.G. (1996) Assessing the quality of biomonitoring via signal-to-noise ratio analysis. *The Science of the Total Environment* **180**: 107-116.

Wolterbeek, H.T., Garty, J., Reis, M.A., and Freitas, M.C. (2002) Biomonitors in use: lichens and metal air pollution. In *Bioindicators and Biomonitors*. Markert, Breure and Zechmeister (ed). Amsterdam, Netherlands: Elsevier Science, pp. 377-419.

Zimmermann, R.-D., Wagner, G., and Finck, M. (2000) Methodological guidance for the drawing-up of biomonitoring guidelines (Plants). Berlin, Germany: WHO Colaborating Centre for Air Quality Management and Air Pollution Control, Federal Environmental Agency, pp. 1-7

Chapter 2

Grain-Size Effects on PIXE and INAA Analysis of IAEA-336 Lichen Reference Material[1]

2.1 Introduction

The nuclear analytical techniques INAA (Instrumental Neutron Activation Analysis) and PIXE (Particle Induced X-ray Emission) have been shown to be well suited for elemental analysis of lichens and their complementary characteristics have already been emphasised (Freitas, Reis et al. 2000). Both are multi-element techniques, have low detection limits (INAA and PIXE can achieve down to ng/kg and mg/kg detection limits, respectively) and sample preparation is considered as simple (Erdtmann and Petri 1986; Johansson, Campbell et al. 1995). However when dealing with biological samples, some discrepancies may be observed between data obtained by these techniques (Randle et al., 1993; Freitas et al., 2000; IAEA-TECDOC-1295, 2002).

A serious limitation of PIXE is the rather small sample volume "seen" in the analysis. The penetration depth - depending on the energy of the X-rays of interest - is small (in the order of <50μm), and the sample area covered by a typical proton beam is also small (generally <0.25 cm^2) (Huggins 2002). This may raise questions

[1] A.P. MARQUES, M.C. FREITAS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ Grain-size effects on PIXE and INAA analysis of IAEA-336 lichen reference material, Nuclear Instruments and Methods in Physics Research B, Vol. 255 (2007), 380–394.

about representativity and homogeneity of the sample volume analysed, with consequences for X-ray generation and attenuation (Randle, Aljundi et al. 1993). Furthermore, matrix corrections tend to be large for thick samples (Reis, Alves et al. 1996; Huggins 2002). INAA is a bulk technique and not sensitive for inhomogeneities in samples of 1 g or less.

Studies have shown that in most biological materials the variations found in element distribution suggest that sub-samples may not be representative for the whole, due to a lack of homogeneity (Spyrou, Farooqi et al. 1990). Homogeneity is a very important property, especially when a rather small sample mass is analysed as in PIXE. This may *a priori* induce differences between PIXE and INAA results. Being aware that there are no materials 100% homogeneous, are these differences relevant for biomonitoring studies using lichens? Can both (INAA and PIXE) data sets, be used together in a single data set for biomonitoring studies purposes and be considered complementary techniques?

Since grain size is an important property for material homogeneous, the main objective of this study was to verify if decreasing the grain size of the sample would improve PIXE results when compared with INAA results. This was made through the preparation and analysis of lichen material with different grain sizes. On the other hand, another objective was to determine how many replicates would be necessary for PIXE to mach INAA when dealing with a lichen material. "Real" samples (field samples, transplants and in situ samples) were also separated into two different grain sizes and analysed by INAA. The main objective was to determine if any difference in elemental content was observed between the two fractions and if both (transplants and in situ samples) behave similarly. It is also an attempt to determine the grain size that should be used to prepared lichen *Parmelia sulcata* samples for analysis by INAA and PIXE.

2.2 IAEA–336 Lichen Reference Material

The preparation of homogeneous samples for analysis is difficult, particularly if the particle size is variable (Randle, Aljundi et al. 1993). Certified reference materials are prepared carefully, with controlled grain-size material and tested for

homogeneity. Nevertheless, most biological reference materials are certified for use of sample masses over 100 mg (in principle not suitable for small sample analysis as PIXE). Some attempts are being made in the preparation of a lichen material suitable for small sample analysis, the IAEA–338 with a grain size less than 20 μm (IAEA-TECDOC-1295, 2002). Samples, for instance dealt with in biomonitoring, are routinely prepared and analysed and do not follow such careful and lengthy procedures. The consequence may be a deterioration of the accuracy of the analytical results, since replicates may have a different composition. If only a single sample is analysed, inaccurate results may appear. Their degree of individual inaccuracy cannot be assessed, only an estimate of the overall accuracy can be given (Reis, 2001; Freitas et al., 2003). So far, there is no information as to the effect of grain size in the PIXE analysis of IAEA-336, and this was the material used in this work. The reasons for choosing this material were 1) it is a lichen reference material, 2) availability since it was prepared at ITN facilities and there are still some bottles as stock and 3) low cost since certified reference materials are expensive. IAEA-336 was prepared with a grain size lower than 125 μm and during the preparation process all material with grain size higher than 125 μm was discarded. It had been prepared earlier from the lichen *Evernia prusnastri* collected in unpolluted regions of Portugal. The material had been washed in de-ionised water, oven-dried at 40 ºC for 24 h, milled in Teflon mill and finally sieved through mesh sizes of 500 μm, 200 μm and 125 μm. The fraction passing the 125 μm sieve (about 90%) had been homogenised at 2 rpm for 2 weeks (Freitas, Catarino et al. 1993). The material has been certified via an international intercomparison of 42 laboratories, located in 26 countries, providing data with various analytical techniques, including NAA (78% of the data), X-ray techniques (14%), ICP-MS (14%), AAS and AES (20%) (Heller-Zeisler, Zeisler et al. 1999). Homogeneity tests with INAA have been reported (Stone, Freitas et al. 1995; Heller-Zeisler, Zeisler et al. 1999) For some elements up to 50% of the overall uncertainty (which ranges from 3-10%) was attributed to sub-sampling uncertainties (K, Mn, As, and La) (Freitas et al., 1993). Inter-bottle and intra-bottle homogeneities were tested for some elements (Sc, Cr, Fe, Co, Zn, Rb, Sr, Sb, Cs, Ba, Ce, and Th) and differences were found for Sr and Ba (Freitas, Catarino et al. 1993).The uncertainties in the

measurements were about 10%. Additional homogeneity measurements were performed using X-ray fluorescence for Ca, Fe, Zn, Br, and Sr, with overall standard deviations up to 5%, and with atomic absorption spectrometry for Pb with an overall standard deviation of 17% (Stone, Freitas et al. 1995). An additional component of 5% relative uncertainty in the calculation of the confidence intervals of the information and certified values has been included. To evaluate the effect of grain size in PIXE analysis, an aliquot of IAEA-336 was separated in different grain-size fractions (<64μm, <41 μm and <20 μm). The material was characterized to determine the real grain size and ten replicates out of each fraction were analysed by PIXE and by INAA as a reference technique. Concerning "real" samples, ten lichen transplant samples and ten in situ lichen samples were also separated in two different fractions (<125 μm and >125 μm) and analysed by INAA.

2.3 Experimental Section

2.3.1 Sampling handling

Lichens are easy to grind in order to obtain a fine powder until a certain mesh size. Most of the body of lichens is thallus. The fungal hyphae (filaments) branch and than fuse together (anastomose) to form a mesh of hair-like threads. The top surface is normally a layer of tightly packed hyphae called cortex. Below this is the algal layer (Nash III 1996). These filaments act like rubber when grinding and so fractions with lower particle size are more difficult to obtain. Part of the material did not pass through the sieves and was therefore discarded. Possibly part of the composition of the lichen was discarded and lost. Also in the preparation of IAEA–336 the part that did not pass through the <125μm was discarded.

2.3.1.1 Preparation of different grain size fractions from a RM

IAEA-336 material (grain size <125 μm) taken out from several bottles randomly chosen, was sieved through Hidro-Bios Kiel nylon nets of 64 μm (NY 64 HC), 41

μm (NY 41 HC), and 20 μm (NY 20 HC). Whenever the amount passing the nets was insufficient, the IAEA-336 material was carefully ground in a teflon mill for 10 minutes after 2 minutes in liquid nitrogen (balls and capsule) to reduce the grain size. The grinding/sieving/grinding procedure was repeated once to obtain the <41-μm fraction and four times to obtain the <20-μm fraction. The material that was not passing through the sieves was discarded. All fractions were homogenised in a turbulent homogeniser for 40 h.

2.3.1.2 Separation on "real samples" in different grain size fractions

Ten lichen transplant samples and ten *in situ* lichen samples were separated in two different fractions, >125-μm and <125-μm, by sieving them using a Hidro-Bios Kiel nylon net of 125 μm (NY 125 HC).

2.3.2 Particle size determination

Particle size measurements for IAEA-336 lichen reference material (bottle Nº107) and the different fractions prepared from it by sieving were performed at IAEA Laboratories Seibersdorf. The analyses were performed by laser diffraction or low angle laser light scattering (LALLS) technique as it is more correctly known. A Mastersizer X (© Malvern Instruments, Ltd.) in connection with a powder feeder sampling unit was used. The samples are introduced into the measurement cell, dry powders are blown through the laser beam; the laser light is scattered on the particles and detected by one of the instrument's detector elements, the angle of diffraction being inversely proportional to the particle "diameter" (for more details see (Fajgelj and Zeisler 1998)). The fundamental size distribution derived from laser diffraction is volume based. This volume is expressed in terms of equivalent spheres. The analysed distribution is expressed in a set of size classes, which are optimised to match the detector geometry and optical configuration giving the best resolution. All parameters are derived from this fundamental distribution. Three replicates were performed for fractions <64μm and <41μm and two replicates for <20 μm fraction. One replicate was done for IAEA 336 (bottle 107) and results

were compared with the ones obtained in 1994 (bottle 602). Measurements on an internal quality control Malvern Difraction Reticle Nº 836 (traceable to SI Unit meter) were performed before and after the measurements. With the use of this reticle, the traceability and repeatability of the measurements performed are assured.

2.3.3 Analysis via INAA and PIXE

Ten replicates from IAEA-336 and ten replicates from the fractions prepared from it were analysed by INAA and PIXE. The increase in surface area during the milling process did not influence atmospheric water uptake and so, all results were corrected with the same factor for moisture content, which has been determined by drying at 100 ^{o}C to constant weight according to the instructions (attached to the Reference Material). Results were only listed for those elements for which certified or informative values are available for IAEA-336. One replicate of fractions >125-μm and <125-μm from lichen transplants samples and *in situ* lichen samples were analysed by INAA.

2.3.3.1 INAA analysis

For INAA (Hevesy and Levi 1936, Boyd 1949, Brown and Goldberg 1949) analysis, pellets of 500 mg (1.3 cm diameter, 0.3 cm height, 10 ton/cm^2 pressure for about 1 min) were prepared and irradiated in the Portuguese Nuclear Research Reactor together with 0.1% Au-Al wires (IRMM-530) as comparators. For short irradiations the pellets were irradiated separately (30 s, neutron fluence rate 2.6x10^{12} cm^{-2}s^{-1}), put into polyethylene containers and measured for 10 minutes after 15 to 20 min of decay. For long irradiations (5 h, neutron fluence rate 4.0x10^{12} cm^{-2}s^{-1}) the pellets were irradiated also in polyethylene containers in batches of tens with comparators placed perpendicularly on top and bottom of each pellet. For the fractions >125-μm and <125-μm of real samples the comparators were placed on top and bottom of the irradiation polyethylene containers each containing ten samples. For measurements, each pellet was put into a polyethylene

container and spectrum measurements were performed for 2 h and 7 to 15 h after 4 and 30 days respectively. A hyperpure germanium (HPGe) detector (1.85 keV FWHM and 30% efficiency at 1.33 MeV) was used, while keeping the dead time below 15%. Net peak areas were determined by GELI program (Op De Beeck 1972). Effective solid angles were calculated with SOLANG (De Corte 1987) and concentrations via the k_0 method with an in-house adaptation of the SINGCOMP program (Freitas 1993). For elements with long-lived radioisotopes, an average concentration was calculated for each sample based on the two comparators used (on top and bottom of the pellet).

2.3.3.2 PIXE analysis

For PIXE analysis about 20 to 50 mg of material was put into a filter paper, transposed to a pellet mould and pressed with an acrylic pestle. After removal of the pestle, a small amount of boric acid was added and the materials were pressed (10 ton/cm^2 for 1 min). Samples were irradiated in the Van de Graaff accelerator at ITN with the beam collimated to 5-mm diameter. A first spectrum was taken to measure major and minor elements with a low current (proton beam of 1.2 MeV, current of 8 nA and total charge of 2 μC). Thereafter, another spectrum with a higher current was obtained from which the trace elements can be determined (2.4 MeV protons with Mylar 350 as a X-ray filter, current of 180 nA and total charge of 60 μC). Finally, again a spectrum with a low current was taken (proton beam of 1.2 MeV, current of 8 nA and total charge of 1 μC). From the latter spectrum the change was calculated in concentration of high-Z elements due to loss of low-Z matrix elements induced by radiation damage of the target (Reis, Alves et al. 1996). The X-ray spectra were obtained with a Si(Li) detector (155 eV energy resolution) and analysed by the AXIL program (Van Espen 1990). Concentrations were obtained by DATTPIXE program (Reis and Alves 1992).

2.3.4 Chlorophyll content determination.

IAEA-336 lichen reference material and the fractions prepared from it were analysed for its chlorophyll content. 50 mg of lichen powder and about 50 mg of $CaCO_3$ were homogenised in ethanol 96% and pigments extracted in the dark (Pfeifhofer, Willfurth et al. 2002) during the night at 10 °C for a more complete extraction (Ronen and Galun 1984). The suspension was then centrifuged for 5 min at 13.700 rpm and 800 µl of supernatant were carefully taken out with a pipette to avoid re-suspension. The suspension was replenished with 800 µl ethanol 96% and centrifuge again. This procedure was repeated to remove all greenish supernatant (four times for every lichen fraction). Chlorophyll content in all the supernatants was measured at 649, 654 and 665 nm with a spectrophotometer. Measurements beyond 700 nm were also performed to verify residual turbidity (Barr and Crane 1971). Concentrations of chlorophyll were calculated on a dry basis (Wintermans and De Mots 1965). To ensure that grain size was not affecting total chlorophyll determination a second extraction was made for 18, 45 and 64h. Three replicates were performed for each fraction. Fractions >125 µm and <125-µm from lichen transplants (10 samples) and *in situ* lichens (10 samples) were also analysed for determination of their chlorophyll content by similar process (only 1 extraction over the night).

2.3.5 Data handling

The data obtained was statistically treated in three ways: using the z-scores, t-tests and material homogeneity determination.

2.3.5.1 z-scores

Standardised differences or z-scores were used to achieve an objective assessment of the accuracy of both techniques for IAEA-336 and to get information on the agreement between INAA and PIXE in the several fractions (Thompson and Wood 1993; Bode 1996; Coquery, Carvalho et al. 1999; Weiss 1999; Wu, Hayes et al.

2001; Besada, Fumega et al. 2002, Mellado, Llauradó et al. 2002, ISO 13528:2005). The z values or z-scores can be generally calculated as:

$$z = \frac{(x - \mu)}{\sigma}$$

where: z = z-value; x = concentration value; μ = mean concentration value; σ = standard deviation in the series of concentration values from replicate measurements. The z scores were presently used for comparisons involving higher number of observations.

2.3.5.2 t –test

The t-test is one of the most widely used statistical tests (GraphPad Software 1999, Weiss 1999, Woolson and Clark 2002). The t-test was used to compare the two different fractions prepared (> 125-μm and <125-μm) from "real" lichen transplant samples and *in situ* lichen samples. The comparisons were made to judge the deviations from unit value in ratios (one sample t-test), and to compare values between transplants and *in situ* lichens (two samples t-test – comparison of means).

2.3.5.3 Sampling constants

The most widely use process to characterize a material concerning its homogeneity degree (especially potential candidates to certified reference materials) is to calculate the sampling constants and/or homogeneity factors (IAEA-TECDOC-1295 2002, Rossbach and Zeiller 2003). Homogeneity determination was used to characterize IAEA-336 and the different fractions prepared concerning its homogeneity degree (Ingamells and Switzer 1973; Ingamells 1974; Chatt, Rao et al. 1990; Thompson and Wood 1993; Sonntag and Rossbach 1997; Rossbach, Ostapczuk et al. 1998; Zeisler 1998; Ro, Hoornaert et al. 1999; Rossbach and Grobecker 1999; Dybczynski, Danko et al. 2000; Kempenaers, Vincze et al. 2000; Weizhi, Bangfa et al. 2000; Zeisler 2000; Sha, Zhang et al. 2002; Rossbach and

Zeiller 2003). The sampling constant (KS) is expressed in the units of mass and is numerically equal to the sample mass necessary to limit the error due to sample inhomogeneity (sampling uncertainty) to 1% (with 68% confidence). According to Ingamells and Switzer (Ingamells and Switzer 1973) the sampling constant is defined by:

$$K_S = R_S^2 \times m$$

with $R_0^2 = R_a^2 + R_s^2$

R_0^2 = total variance of the observations (analytical results, %)

R_a^2 = variance of the analytical method (%)

R_s^2 = sampling variance from the heterogeneity of the study material (%)

m = sample mass

2.3.5.4 Horwitz function

Another statistical treatment commonly used is the Horwitz function (Horwitz, Kamps et al. 1980; Heydorn and Damsgaard 1987; Griepink 1990; Horwitz and Albert 1996; Thompson and Lowthian 1997; Thompson 2000; AOAC 2002; Thompson, Ellison et al. 2002). Horwitz is a generalization about reproducibility standard deviation expected in a collaborative trial (Horwitz, Kamps et al. 1980). Accounting for deviations to the Horwitz equation for low and high concentrations, A modification of the Horwitz equation was proposed (Thompson 2000), where:

$$\sigma = \begin{cases} 0.22\,\mathrm{c} & \text{if } \mathrm{c} < 1.2\times10^{-7} \\ 0.02\,\mathrm{c}^{0.8495} & \text{if } 1.2\times10^{-7} \le \mathrm{c} \le 0.138 \\ 0.01\,\mathrm{c}^{0.5} & \text{if } \mathrm{c} > 0.138 \end{cases}$$

In the equation, both the expected standard deviation (σ) and the concentration (c) are expressed in dimensionless mass ratios (for instance, 1 mg/kg $\equiv 10^{-6}$).
The modified Horwitz equation was not directly applied to the data obtained since it was not an interlaboratory comparison (only data from two different techniques).

It was used to calculate sampling constants (K_s) for IAEA 336 lichen reference material. Since $R_0^2=R_a^2+R^2_{interlaboratory}+ R_S^2$, the $R^2_{interlaboratory}$ value was calculated using the modified Horwitz equation.

2.4 Results and Discussion

2.4.1 Particle size distribution

One of the important physical characteristics of a natural matrix is particle sizes and their distribution. They may affect the chemical composition of the material in terms of homogeneity and representativeness of test portions taken from the material (Fajgelj and Zeisler 1998). Homogeneity is directly related to particle size (Dybczynski, Danko et al. 2001). Therefore, the samples were analysed to determine their particle sizes and these results and some other relevant information are summarized in Table 2.1. Laser light scattering technique is an absolute measurement method for particle size determination. The measurement uncertainty can only be estimated from the repeatability study on the internal quality control reticle. The uncertainty in the determination of the mean value (38.24 μm) is 0.01 μm absolute, or 0.02% relative (at 95% confidence interval). Quality control results are therefore in perfect agreement with the laboratory established control value, no deviation in position of the peak or repeatability.

The particle size of a material is presented by a mean particle size, defined as size at 50% volume below and above this size, and by the width of the distribution that corresponds to the full width at the half maximum (FWHM) of the highest peak in the particle size distribution curve. These values were obtained by graphical interpolation. IAEA-336 lichen reference material, bottle 107, was analysed and compared with IAEA-336 measurements from 1994 (bottle 602). Sample IAEA-336 lichen reference material shows uniform particle size, which has exactly the same mean particle size distribution as the one measured during the preparation of the sample in 1994 (see also Fajgelj and Zeisler 1998). Results indicate that the particle size after sieving follows the same pattern as might be observed when sieving other similar materials (Fajgelj and Zeisler 1998). Bimodal distribution is

observed, which is by fine sieving turning to be more and more monomodal (Fig. 2.1). One of the objectives of the work was to decrease the particle size in order to obtain a better agreement between INAA and PIXE techniques.

Table 2.1: Particle sizes for IAEA-336 Lichen Reference Material and the different fractions prepared from it. Mean particle size refers to the 50% crossover point in the cumulative size distribution curve; range is the with of the volume percent size distribution curve at half the value of the peak maximum.

Sample	Distribution mean (μm)	Range (μm)	Comment
IAEA 336 (Bottle Nº107 and 602)	27.18 30.79	1.8 to 500	Bimodal with peaks at 6 and 60 μm, homogeneous particle size distribution
Sieved fraction (<64 μm)	43.24 42.90 42.52	0.2 to 122	Three modal distributions observed, largest fraction between 20 and 120 μm
Milled and sieved fraction (<41 μm)	24.54 24.27 24.39	0.2 to 122	Three modal distributions with more particles below 15 μm
Milled and sieved fraction (<20 μm)	9.86 10.76	0.2 to 40	Cut of not clearly visible, distribution becoming more narrow
Internal quality control measurements			
Reticle 836	38.72	18.21 to 83.87	Laboratory control value is 38.24 ± 2 μm

According to Zeisler (Zeisler 1998), distribution of particles not larger than 10 μm will probably fulfil the requirements for homogeneity when smaller sample masses are required, such as the case of PIXE analysis. According to the results obtained, lichen material fraction prepared <20 μm has shown particle size distribution and particle diameter range that could lead to the expectation of good homogeneity of elemental composition for analytical techniques using small sample masses for

analysis. However, additional analytical tests should be applied to confirm the chemical appropriateness of the material for this purpose.

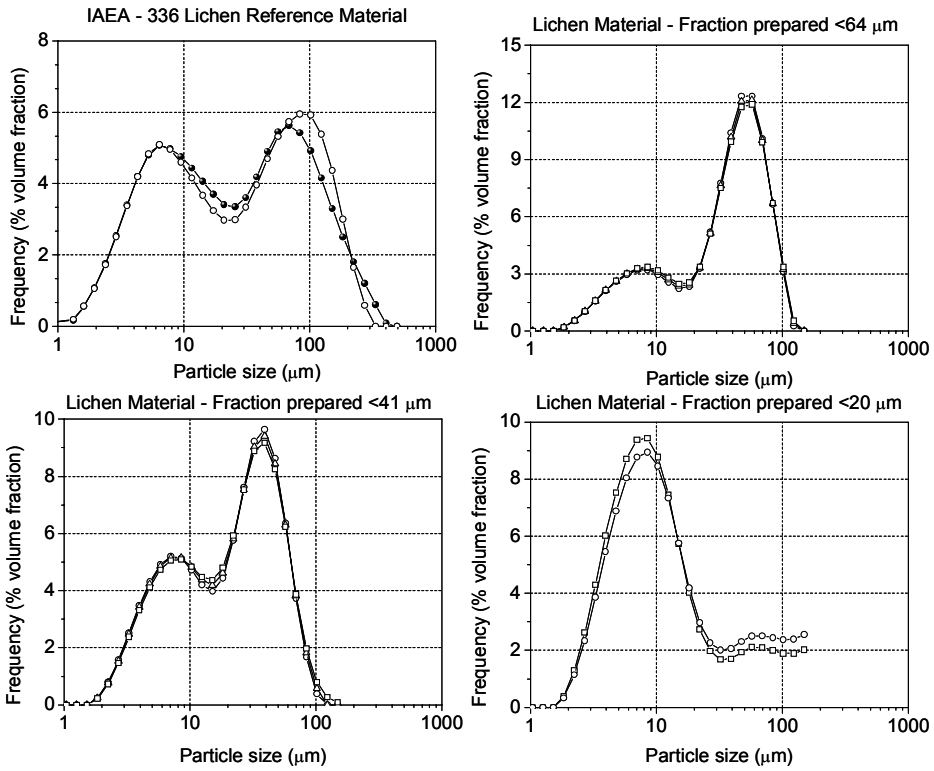

Fig. 2.1 - Characteristic particle size distributions in IAEA-336 lichen reference material and the different fractions prepared from it (<64 μm, <41 μm and <20 μm).

2.4.2 Tests of normality

The Gaussian distribution (also called normal distribution) has some mathematical properties that form the basis of many statistical tests (like t-tests, z-scores, etc). It is therefore important to know if the data are normally distributed or not far from being normally distributed. Normality tests check a given set of data for similarity

to the normal distribution. Table 2.2 shows average concentrations and associated standard deviation (including t-Student factor) obtained by INAA and PIXE for the ten replicates of IAEA-336 lichen reference material (<125μm) and the several fractions prepared from it (<64μm, <41μm and <20μm). Associated uncertainties (based on the mean of 10 replicates, 9 degrees of freedom) were calculated by multiplying the standard deviation by the t-value of 3.250 (for a confidence interval of 99.5%, 2-tailed test) (CETAMA 1986; Weiss 1999; Woolson and Clark 2002). Certified values, information values and confidence interval for IAEA-336 are also given. Five elements (Cl, Fe, K, Mn and Zn) well determined by INAA and PIXE, were tested to see whether the data followed a normal distribution (results not shown). A chi-square test was used, which divides the range of data into ten equally probable classes and compares the number of observations in each class to the number expected. Results showed that the data followed a normal distribution (95% probability level), except INAA values for K and Zn in the <20-μm fraction (results not shown). Both the z-value test and the t-test work reasonably well even when the variable under consideration is not normally distributed and the sample size is small or moderate, provided that it is not too far from being normally distributed. This means that the tests used are rather robust and may handle up to moderate violations of the normality assumption (Weiss 1999).

2.4.3 Assessment of the accuracy of PIXE and INAA by comparison with the IAEA-336 certified values

It is important to know the behaviour of the techniques used in this study, INAA and PIXE, in terms of the closeness of a measurement to its true or accepted value, in other words in terms of accuracy. In this case, the data obtained by both techniques for the analysis of IAEA-336 lichen reference material was compared with the certified values. The z-values were calculated for the replicates of IAEA-336 analysed via INAA and PIXE (only for Cl, K, Fe, Mn and Zn) versus the certified data (for Cl only an information value is available). The z-values were tested against the criterion $|z|<3$ for approval of the results at the 99.7% confidence level.

Table 2.2: Average concentrations and associated uncertainty (99.5% confidence level) for IAEA-336 and the different fractions prepared from it (columns 6, 7, 8 and 9) using PIXE or INAA (column 4). Certified values or information values (column 2) and confidence interval (column 3) for IAEA-336 are presented. The z-scores between IAEA-336 lichen reference values and results obtained via INAA or PIXE for Cl, K, Mn, Fe and Zn are given calculated either using σ-values obtained from the replicates or derived from the modified Horwitz function (column 5 and 6 respectively).

Element	Certified values (mg/kg) IAEA-336	Confidence interval IAEA-336	Method	z-score IAEA-336	Obtained value (mg/kg) IAEA-336	Obtained value (mg/kg) <64 μm	Obtained value (mg/kg) <41 μm	Obtained value (mg/kg) <20 μm
Al	*680	570-790	PIXE		570±52	505±38	444±52	406±43
As	0.63	0.55-0.71	INAA		0.771±0.022	0.851±0.025	0.853±0.018	0.881±0.021
Ba	6.4	5.3-7.5	INAA		5.76±0.86	5.81±0.86	6.0±1.4	6.3±2.0
Br	12.9	11.2-14.6	INAA		12.26±0.14	10.74±0.26	10.10±0.46	10.13±0.29
			PIXE		10.4±2.1	10.0±1.3	9.39±0.92	7.2±1.6
Cl	*1900	1600-2200	INAA	0.14	1878±34	1765±38	1519±61	1040±32
			PIXE	1.44	1676±40	1480±29	1259±37	892±59
Ce	1.28	1.11-1.45	INAA		1.245±0.031	1.237±0.040	1.194±0.031	1.201±0.020
Co	0.29	0.24-0.34	INAA		0.274±0.010	0.2413±0.0072	0.2201±0.0062	0.1806±0.0027
Cr	*1.06	0.89-1.23	INAA		1.237±0.057	1.220±0.053	1.165±0.047	1.211±0.029
Cs	0.110	0.097-0.123	INAA		0.1175±0.0075	0.113±0.011	0.1011±0.0143	0.0968±0.0122
Cu	3.6	3.1-4.1	PIXE		3.27±0.34	3.29±0.33	3.36±0.33	3.54±0.82
Eu	*0.023	0.019-0.027	INAA		0.0243±0.0057	0.0227±0.0052	0.0185±0.0041	0.0321±0.0095
Fe	430	380-480	INAA	0.21	424.7±5.2	420.1±7.9	403.2±5.5	412.4±6.6
			PIXE	0.98	396±25	382±22	356±16	372±26
K	1840	1640-2040	INAA	0.22	1817±31	1727±60	1611±24	1306±28
			PIXE	0.54	1780±48	1690±37	1497±77	1179±122
La	0.66	0.56-0.76	INAA		0.605±0.019	0.50±0.19	0.567±0.064	0.569±0.029
Lu	*0.0066	0.004-0.009	INAA		0.0055±0.0009	0.0055±0.0005	0.0054±0.0006	0.0059±0.0006
Mn	63	56-70	INAA	0.035	63.1±1.6	58.9±2.3	46.5±1.7	27.22±0.70
			PIXE	0.50	60.6±3.4	54.5±2.9	44.7±1.9	27.6±1.8
Na	320	280-360	INAA		340±13	263±13	205.8±7.1	191.1±6.6
P	*610	490-730	PIXE		414±19	422±16	426±23	427±23
Pb	*4.9	4.3-5.5	PIXE		5.5±4.1	5.6±3.5	6.2±4.8	6.2±4.2
Rb	*1.76	1.54-1.98	INAA		1.65±0.12	1.64±0.13	1.464±0.062	1.34±0.14
Sb	0.073	0.063-0.083	INAA		0.0691±0.0049	0.0686±0.0054	0.0677±0.0116	0.0793±0.0055
Sc	*0.17	0.15-0.19	INAA		0.1659±0.0041	0.1621±0.0029	0.1560±0.0021	0.1526±0.0025
Se	0.22	0.18-0.26	INAA		0.200±0.021	0.2106±0.0097	0.224±0.037	0.212±0.013
Sm	0.106	0.092-0.120	INAA		0.1094±0.0060	0.1127±0.0062	0.1157±0.0034	0.1223±0.0047
Sr	9.3	8.2-10.4	PIXE		8.8±1.3	7.3±1.9	6.97±0.84	5.05±0.80
Th	0.14	0.12-0.16	INAA		0.1488±0.0047	0.1518±0.0067	0.1464±0.0040	0.1518±0.0046
Zn	30.4	27.0-33.8	INAA	0.80	29.01±0.30	28.39±0.59	26.43±0.41	21.9±1.7
			PIXE	0.61	32.8±3.5	30.2±2.4	27.4±1.8	23.6±2.1

*IAEA-336 Information values

The results obtained showed that, within the error margins of the certified (or information) values and those of the measured values, no significant difference is present between the values by INAA or PIXE and the certified values of IAEA-336 (Table 2.2). It should be noted here that "good" z-values may be obtained for instance when large uncertainties are associated with the certified value. For the elements in this study the uncertainties associated with the certified values range from 11 to 16%.

2.4.4 Assessment of the agreement between INAA and PIXE values

Although it is known that both techniques used in this study are accurate within the uncertainties of the certified IAEA–336 lichen reference material, calculations were made to obtain a measurement of the level of comparability of INAA and PIXE analysis of lichen material at ITN facilities. Fig. 2.2 shows the comparison between INAA and PIXE results (only for Cl, K, Fe, Mn and Zn) for IAEA-336 and the fractions prepared from this material based on z-scores. The obtained z-values were tested against the criterion $|z|<3$ for approval of the results at the 99.7% confidence level. $|z|>3$ means that INAA and PIXE results are considered different. According to the criteria adopted for the z-scores, INAA and PIXE results showed no significant difference, except for Cl where only for the <20 μm fraction the z-value is < 3.

2.4.5 The chlorine case

Cl is the only element with a systematic difference (see Fig. 2.2) between INAA and PIXE. Also the z-values provide evidence that both populations are not comparable. The most probable explanation is that in the vacuum used in PIXE analysis volatilisation occurs during the proton bombardment. Similar losses of halogens (especially Cl) in PIXE analysis, have been reported and/or explained, *e.g.* from aerosols during sampling, storage and/or analysis (Salma, Maenhaut et al. 1994; Maenhaut and Cafmeyer 1998).

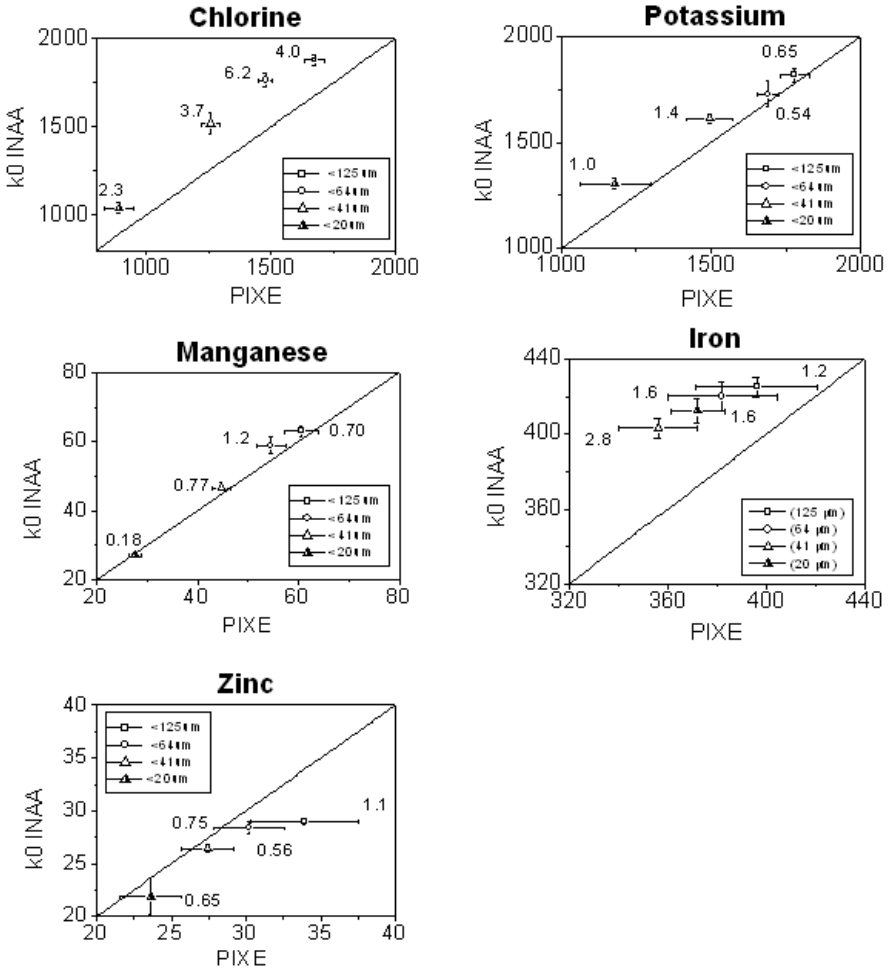

Fig. 2.2 - Results (in mg/kg) for the grain-size fractions prepared from IAEA-336 for Cl, K, Mn, Fe and Zn determined by INAA and PIXE. Bars are standard deviations of the mean values, (99.5% confidence intervals). Values of the z-scores, tested against the criterion $|z|<3$ for approval of the results at the 99.5% confidence level are given next to the error bars. The fraction <125 μm corresponds to the certified IAEA-336 lichen material itself.

2.4.6 Calculation of the necessary number of replicates for PIXE data to be comparable with INAA data

According to the criteria adopted for the z-scores, INAA and PIXE were considered not to give different results for Fe, K, Mn and Zn. However these z-scores calculations were made using the means of 10 replicates, and generally in routine analysis no replicates are used, only one single measurement. Therefore a simple exercise was made with the data obtained by INAA and PIXE (only for K, Fe, Mn and Zn). Here, the INAA mean values of each fraction were considered as the reference, because INAA is a bulk technique and the mass used was five times the minimum mass recommended for analysis. PIXE is a surface technique and the mass analysed ranges from 0.10 to 0.50 mg, much less than recommended.

Calculation of the necessary number of replicates for PIXE data to be comparable with INAA data was performed within the criterion of $|z|<3$. So, we calculated the number of PIXE replicates necessary for PIXE to match INAA results. For PIXE there are 10 samples per element and per fraction. From these 10 replicates we took randomly 2, 3, …10 samples with the possibility to take the same sample for the 2^{nd}, 3^{rd}, …10^{th} sample. An average value with an associated uncertainty and z-value was calculated. This procedure was repeated 500 times and an average z-value was obtained with an associated uncertainty. The probability of obtaining $|z|<3$ was then calculated using 2, 3, …10 replicates. The ideal number of replicates is reached when the probability equals 1. Figure 2.3 shows the results for K, Mn, Fe, and Zn. Cl was not included since INAA and PIXE data were not comparable when using z-value test.

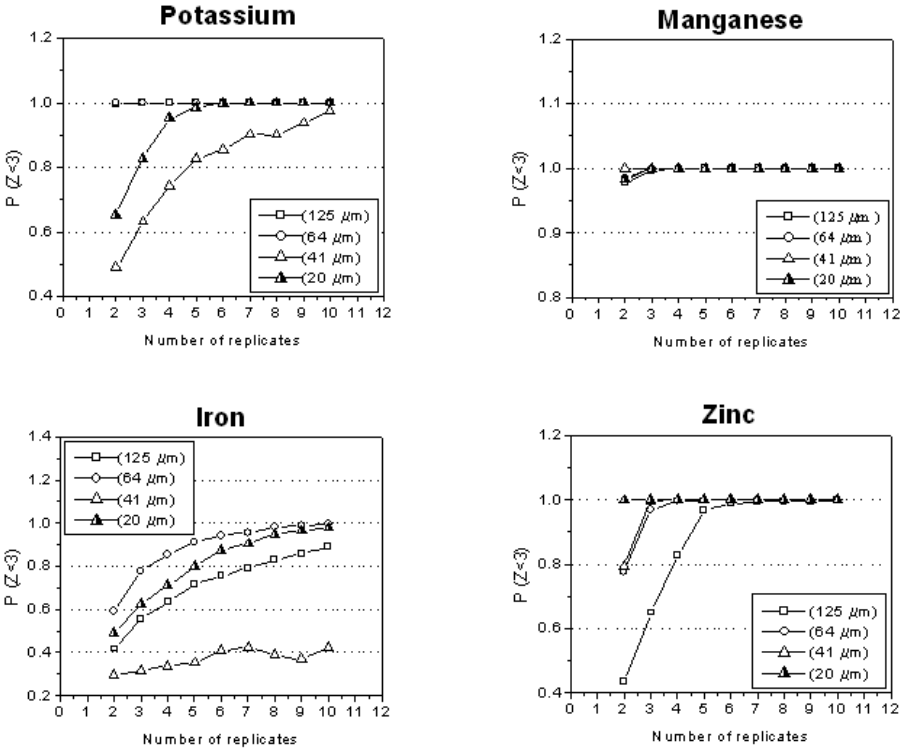

Fig. 2.3 - Number of replicates necessary for PIXE data to match the corresponding INAA data (based on |z|<3) criteria) for IAEA-336 and the fractions prepared from this material. The obtained z-values were tested against the criterion |z|<3 for approval of the results at the 99.5% confidence level.

The graph for K shows that this element does not follow a regular trend with decreasing grain size; for instance with 7 replicates a probability of 0.90 of having a |z| value lower than 3 is reached for the <41 μm fraction. The probability value of 1 is not reached, even with 10 replicates. For the <20 μm fraction, 4 to 5 replicates would be enough for INAA and PIXE data to be comparable. However for K, IAEA-336 (<125 μm) and the <64 μm fraction seem the most appropriate ones and so grinding and sieving to obtain lower grain size does not improve the results for this element. For Mn, 2 replicates are enough for the <41 μm fraction for PIXE to

match the INAA value. For the <64 μm and the <20 μm fractions, 3 is the number of replicates to be used, and for IAEA-336 it is advisable to use 4 replicates. However, it is also possible to observe that for 2 replicates the probability values for all fractions and IAEA-336 are above 0.98. For Fe in the <41 μm fraction, whatever the number of replicates used to compare PIXE and INAA data, will always end up with a probability lower than 0.4 of having $|z|<3$. Also the z-values on INAA and PIXE comparison for different lichen material fractions (Fig. 2.2) shows a high value (2.8 close to 3) for the <41 μm fraction. For IAEA-336, with 10 replicates we will have a probability of 0.86 for achieving $|z|<3$ and for <64 μm fraction that value is reached with 0.96 probability using 7 replicates. For the <20 μm fraction 8 replicates will be enough to guarantee that PIXE value agree with INAA value (probability of 0.95) for $|z|<3$. Also this element does not follow a regular trend since the appropriate fractions for PIXE to match INAA are the <64 μm and <20 μm fractions and the worst fraction is that of <41 μm. Zn shows a very regular trend following the decrease in particle size. Within the range used of particles (IAEA-336 with sizes <125 μm down to the <20 μm fraction), analysing 5 replicates whatever the grain size, makes PIXE values comparable with INAA values, obeying $|z|<3$ conditions for every grain size used. However, except for Zn, milling and sieving to a smaller grain size has not much influence on the results in the INAA and PIXE comparison.

2.4.7 Effect of particle size on element contents

IAEA-336 lichen reference material and the different fractions prepared from it were characterised for its particle sizes and their distribution. Another important characterisation of the material is trace element content, in this case analysed by INAA and PIXE which values are shown in Table 2.2. The authors expected, with the decreasing in particle size, to obtain a finer powder, more homogeneous but still maintaining the trace element content characteristics. Fractionation into smaller grain sizes resulted in lower concentrations of Na, Cl, K, Mn and Sr. For Al, Sc, Co, Zn, Br and Rb this effect is smaller, although still present. For P, Cr, Fe, Cu, As, Se, Sb, Cs, Ba, La, Ce, Sm, Eu, Lu and Th the contents are roughly

similar for all fractions. The element distribution in the different fractions does not follow a similar trend, which was an unexpected result.

Lichens are symbiotic organisms composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont (Nash III 1996). *Evernia prunastri* is fruticose lichen having pendulous thallus and *trebouxia* as photobiont. (http://www.botany.wisc.edu). Elemental locations within the lichen are not well known, nor the precise participation of the symbiotic algae and fungi. Some elements are ubiquitous distributed through the lichen thalli (Garty 2001). It might depend on the lichen species itself and can be influenced by competition effects, temperature or pH (Wolterbeek, Garty et al. 2002). For instance, according to Goyal and Seaward (Goyal and Seaward 1981) the chemical element accumulation capacity (mg/kg) of rhizinae was found to be maximal for Fe, Mn and Pb whereas the photobiont part of these lichen exhibited a maximal accumulation capacity for Cu, Ni and Zn. Flora and Nieboer (Flora and Nieboer 1980) have found that for *Umbilicaria muhlenbergii* a foliose (leaf-like) the Ni uptake capacity of the fungal layer was considerably smaller (640±50 mg/kg) than that of the algal zone (1430 mg/kg). Asta and Garrec (Asta and Garrec 1980) have published a study on the localization of Ca, K, Mn and P in different anatomical tissues of ten foliose and fructicose lichens.

In fungal tissues, the hyphae with thick wall are the richest in Ca. The algal layer shows abundant K and the presence of Mg and P. Scanning electron microscope (SEM) and X-ray emission microanalysis methods on *Cladonia cristatella* collected from a coal mine in Ohio demonstrated a consistent localization of Na and Cl in the mycobiont, and Fe in the phytobiont, whereas Si, P and K were ubiquitous throughout the lichen thallus (Garty and Delarea 1991).

Observations in ten mountain lichens (9 terricolous and 1 corticolous) with the electronic microscope showed the mineral location in lichens of Ca^{2+} in the fungal tissues and K^{+} and Mg^{2+} in the algal layer (Asta 1992). Clark et al (Clark, Mangelson et al. 1999) have made analysis of lichen (*Xanthoparmelia chlorochroa)* thin sections by PIXE and STIM using a proton microprobe and they have shown that concentrations ($\mu g/cm^2$) of the transition metals manganese, zinc

and copper have patterns similar to that of potassium. These elements seem to concentrate in the rhizine and the general region of the algal layer. Iron, titanium and silicon have shown large concentrations in the rhizine and on the lower cortex, with overall lower, uniform concentrations across the rest of the sample. It has been demonstrated that (Garty 2001) the binding of Pb cations was restricted to fungal hyphae (lead penetrated into the cortical cells and not into algal cells or into ascospores or medullary cells of *Ramalina lacera*). Other authors (Richardson, Kiang et al. 1985) also found that isolated symbionts incubated in a Pb solution, the photobiont cells of *Cladonia cristatella* accumulated a minor amount (μmol/g) of Pb compared with the amount bound to cell cultures of the mycobiont. Budka et al (Budka, Przybylowicz et al. 2002) found that Cl and K concentrations (mg/kg) were high near the algae layer whilst S concentrated mostly in the algal and lower cortex layers. The highest concentrations of P were found in the lower cortex. Mn and Zn were mostly concentrated in the algal layer and lower cortex while concentrations of Fe were noted in the lower cortex. The highest concentrations of Ca and Pb were found in the medullary layer. Some authors (Paul, Hauck et al. 2003) found that the photobiont partner of *Hypogymnia physodes* took up considerably less Mn than the mycobiont.

The grinding and sieving process may have changed the proportion of the symbiotic organisms in the lichen, changing therefore trace element content in the several fractions.

2.4.8 Chlorophyll results

Photosynthesis is driven by the energy of light, which is collected by the photosynthetic pigments. These pigments are predominantly chlorophyll a, chlorophyll b and carotenoids. Chlorophylls are the only pigments essential for photosynthesis because the photochemical reactions involve exclusively special types of chlorophyll a for electron transfer (Valladares, Sancho et al. 1996). Trying to interpret the results obtained concerning the decreasing on element content in the several IAEA-336 fractions, chlorophyll contents were determined in the several lichen fractions (Table 2.3). The results show that chlorophyll content and hence

algae fraction is increasing with decreasing grain size. Another extraction set was performed in parallel, only for IAEA-336 and 20 μm fraction, to determine if grain size was affecting chlorophyll extraction. The results have shown that after 45 and 64h of extraction chlorophyll content was very low and similar for both fractions indicating that 18, 19h of extraction more or less guaranties total extraction and so grain size is not a variable needed to account for. The grinding and sieving process has increased the algal fraction of the lichen and so the majority of the discarded material was in fact composed essentially of fungus. Elements like Na, Cl, K, Mn and Sr (Table 2.2) most probably are present in higher concentration in the fungal fraction of *Evernia prunastri* explaining therefore the decrease in concentrations for these elements although more work should be done in this area to properly explain the results observed.

2.4.9 Chlorophyll and Mg content

All cyclic tetrapyrrole pigments that are grouped under the general classification of chlorophylls (Chl's) contain Mg as the centrally chelated metal and a fifth, so called isocyclic ring (Horton, Moran et al. 2002). If the variations in concentrations are associated with the photobiont, could the Mg associated with chlorophyll be used as a marker?

In plants, the amount of Mg associated to chlorophyll is relatively small (10 to 20%) not exceeding 30% (Todd 1961). The molecular formula of chlorophyll a is $C_{55}H_{72}O_5N_4Mg$ with a molecular mass of 907.5 $gmol^{-1}$, and so this means 2.72% of Mg by mass of chlorophyll. According to Table 2.3, the maximum amount of extracted chlorophyll was about 0.4 mg/g in the <20 μm fraction, which makes approximately about 12 μg of Mg for each gram of chlorophyll.

The values obtain for Mg in the several fractions (below PIXE detection limit in half of the replicates of each fraction) have large associated uncertainty being respectively 269±140 for the <125 μm fraction, 266±76 for the <64 μm fraction, 183±118 for the <41 μm fraction, 159±120 for the <20 μm fraction (Table 2.2).

Despite this fact, it can be stated that Mg values are much too high to be able to relate them to chlorophyll.

Table 2.3: Average total chlorophyll concentrations and associated standard deviation (St. Dev.) for IAEA-336 (<125 μm), the different fractions prepared from it (<64 μm, <40 μm and <20μm) and three *in situ* lichen samples (<125 μm and >125μm). Results for different extraction periods used for IAEA-336 (<125μm and <20μm fractions) are also presented.

Sample	Extraction Time (h)	Total Chlorophyll (mg/g)	
		Average	St. Dev.
IAEA 336 <125μm	18	0.13007	0.00054
<64μm	18	0.203	0.012
<41μm	18	0.257	0.027
<20μm	18	0.321	0.043
IAEA 336 <125μm	19[a]	0.175	0.017
<20μm	19[a]	0.3656	0.0097
IAEA 336 <125μm	45[a]	0.0521	0.0064
<20μm	45[a]	0.0381	0.0078
IAEA 336 <125μm	64[a]	0.02677	0.00033
<20μm	64[a]	0.0258	0.0039
In situ sample 1 >125μm	18	0.0522	0.0038
In situ sample 1 <125μm	18	0.0578	0.0052
In situ sample 2 >125μm	18	0.0618	0.0015
In situ sample 2 <125μm	18	0.0897	0.0055
In situ sample 3 >125μm	18	0.0416	0.0021
In situ sample 3 <125μm	18	0.0527	0.0082

[a] Chlorophyll was extracted for 19h and analysed. The suspension (lichen powder used for the 19h extraction) was replenished with ethanol 96%, left for extraction for more 45h and analysed. The same procedure was adopted for 64h extraction.

2.4.10 Effect of particle size for" real" samples (in situ and transplanted lichens)

During this study, all the data presented and discussed concerned IAEA-336 lichen reference material and the different fractions prepared from it. However in biomonitoring studies using lichens, "real" samples (*in situ* or transplanted lichens) are gathered and reference materials are used only to generally estimate analytical quality. Trying to make the "bridge" to the samples routinely used in biomonitoring studies by our group at ITN facilities, ten transplants and ten *in situ* samples of the lichen *Parmelia sulcata* were separated in two different fractions, >125 μm and <125 μm. The first observation during the separation in these two different grain sizes, was that the powders obtained were completely different in terms of colour and texture (Fig. 2.4). The >125 μm fraction looked like smashed rubber or paper of colour grey with some black particles included. The <125 μm looked like a fine black powder.

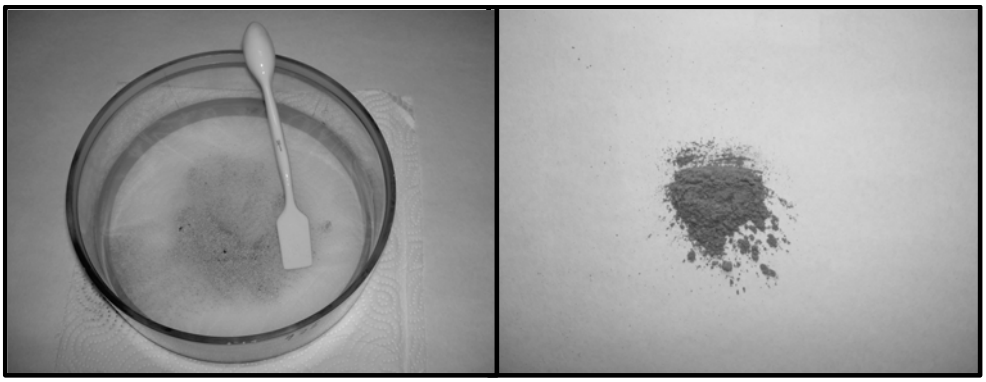

Fig. 2.4 - *Parmelia sulcata* samples separated in two different fractions; the > 125 μm fraction (right) looks like a greyish smashed rubber or paper with some black particles included and the <125 μm fraction looks like a fine black powder.

About 28 elements were determined by INAA in both fractions of transplants and *in situ* lichens. Mean ratios (<125 μm) /(>125 μm) and associated error for *in situ* lichens and lichen transplants were calculated (Table 2.4). Table 2.4 shows that, except for a few exceptions, the ratio of (<125 μm fraction)/(>125 μm fraction) it

is always higher than one, meaning that elemental contents are higher in the <125 μm fraction. Calcium in in situ lichen samples is the only exception, and so for this element concentration may be similar in both fractions of the sample. Chlorophyll content was also determined in both fractions (>125 μm and <125 μm) of three in situ lichens samples (Table 2.3) and results show that concentrations were always higher in the <125 μm fraction.

These results are not in line with the results observed for IAEA-336 and the different fractions prepared from it, where fractionation into smaller grain sizes resulted in lower concentrations of Na, Cl, K, Mn and Sr and in smaller degree for Al, Sc, Co, Zn, Br and Rb. Although the "real" samples were *Parmelia sulcata* lichens rather than the IAEA-336's *Evernia prusnastri*, differences in results may not be due to differences in species, but should probably be attributed to the >125 μm particles which was discarded in the preparation of the IAEA-336 reference material. This means that we do not know what was lost by preparing the IAEA material, but what we do know is that differences in element concentration over particle sizes should lead to the strictest protocols in sample preparations, if ever results from various groups are to be compared in multi-scaled surveys. All in all, the presented data may suggest that when dealing with lichen samples for elemental assessment the material may be reduced into a powder and passed all through a pore-standardized micro sieve.

Table 2.4: Mean ratios (<125μm)/(>125μm) and associated error (StDev) for *in situ* lichens and lichen transplants. P < 0.05 indicates significant differences from unit values ((<125μm)/(>125μm) or indicates that means are significantly different (mean *in situ* = mean transplants): these cases are marked in bold italic

Element	Mean *in situ* lichens	error	P ((<125μm)/(>125μm) =1)	Mean lichen transplants	error	P ((<125μm)/(>125μm) =1)	P (mean *in situ* = mean transplants)
Al	2.39	0.14	0.000	2.58	0.28	0.000	0.557
As	2.32	0.15	0.000	3.33	0.37	0.000	***0.020***
Ba	1.92	0.20	0.001				
Br	1.48	0.11	0.002	1.88	0.12	0.000	***0.023***
Ca	1.35	0.16	***0.058***				
Cl	1.53	0.09	0.000	1.19	0.08	0.039	***0.012***
Ce	2.30	0.15	0.000	2.46	0.15	0.000	0.456
Co	1.87	0.10	0.000	2.67	0.15	0.000	***0.000***
Cr	2.37	0.11	0.000	3.04	0.27	0.000	***0.033***
Cs	2.65	0.25	0.000	2.89	0.28	0.000	0.537
Eu	2.68	0.27	0.000				
Fe	2.36	0.11	0.000	2.91	0.28	0.000	0.085
Ga	8.55	1.55	0.003				
Hf	3.37	0.45	0.001	4.44	0.50	0.000	0.133
Hg	1.52	0.12	0.002	1.41	0.14	0.019	0.560
K	1.44	0.04	0.000	1.43	0.05	0.000	0.843
La	2.27	0.13	0.000	2.51	0.19	0.000	0.313
Lu	2.78	0.17	0.000	2.84	0.49	0.013	0.886
Mg	1.63	0.09	0.000	1.57	0.13	0.002	0.707
Mn	1.44	0.11	0.003	1.65	0.05	0.000	0.097
Na	1.83	0.13	0.000	1.33	0.06	0.000	***0.003***
Rb	1.89	0.09	0.000	2.18	0.12	0.000	0.076
Sb	1.82	0.09	0.000	2.24	0.31	0.003	0.208
Sc	2.74	0.29	0.000	2.96	0.30	0.000	0.592
Se	1.35	0.08	0.002	1.93	0.19	0.001	***0.011***
Sm	2.29	0.14	0.000	2.42	0.16	0.000	0.558
Ta	2.78	0.20	0.000				
Tb	2.22	0.17	0.000	2.53	0.33	0.002	0.398
Th	3.18	0.15	0.000	3.23	0.33	0.000	0.882
Ti	2.64	0.19	0.000	2.41	0.39	0.008	0.586
U	2.33	0.11	0.000				
V	1.84	0.17	0.001	2.21	0.16	0.000	0.125
W	2.93	0.29	0.000				
Zn	1.20	0.03	0.000	1.61	0.05	0.000	***0.000***

2.4.11 Homogeneity assessment with Ingamells constants

The distribution of chemical elements and compounds in natural matrix materials is of particular concern for analytical techniques using small sample mass (<10 mg) for investigation (Rossbach and Zeiller 2003). Ingamells "sampling constant" K_S, is defined as the weight of the subsample necessary to ensure a relative subsampling error of 1% at the 68% confidence level in a single determination (Chatt, Rao et al. 1990). Ingamells "sampling constant" is therefore an expression of the level of homogeneity of a certain material. As a simple exercise, sampling constants were calculated for IAEA-336 lichen reference material (particle size <125μm) and the different fractions prepared from it (<64μm, <41μm and <20μm) analysed by INAA and PIXE.

2.4.11.1 INAA

In neutron activation analysis, analytical uncertainty arises mainly from the standard deviations of counting statistics (R_c), neutron fluence rate variations (R_f), counting geometry (R_g) and weighing (R_w) (Chatt, Rao et al. 1990; IAEA-TECDOC-1295 2002). Analytical uncertainty given for elements determined by k_0-INAA at ITN facilities is roughly 4 to 5%. In order to be able to perform this exercise and calculate K_S values, analytical uncertainties determined by IRI-TU Delft Radiochemistry Department were used.

Sampling constants determined in this work by INAA are presented in Table 2.5. Sampling constant values found in literature for other biological materials are also presented together with sample mass analysed. For Ba, Br, Cl, Cs, La and Rb the sample mass necessary to limit the error due to sample inhomogeinity down to 1% (with 68% confidence) increases with the smaller grain size of the materials analysed in this work. For every IAEA-336 fraction, the lowest sampling constants were obtained for (<125μm) – Br, Cl, La, Mn, Rb, (<64μm) – Lu, (<41μm) – Eu, Sm, and (<20μm) – Na.

2.4.11.2 PIXE

For X-ray emission techniques, errors can be grouped as i) counting statistics including the spectrum fit errors (R_c); ii) total exposure by incoming radiation including the dead time (R_N), and iii) quantification procedure uncertainty (R_Q) – consisting from uncertainties in calculations or calibrations of detection efficiency (for particular X-ray energy) and X-ray yield (for particular geometry, sample matrix and X-ray line) (IAEA-TECDOC-1295 2002). The analytical uncertainty given for elements determined by PIXE at ITN facilities is roughly 5%. In order to be able to calculate K_S values, analytical uncertainties for lichen candidate reference material IAEA-338 taken from IAEA-TECDOC-1295 (IAEA-TECDOC-1295 2002) were used.

Sampling constants determined in this work by PIXE, are presented in Table 2.6. Sampling constant values found in literature for other biological materials are presented, together with sample masses. For PIXE with the IAEA-336 material, lowest sampling constants were obtained for (<125μm) – Ti (similar to <64μm fraction), (<64μm) – Cl, K, Rb, S, Si (similar to <20μm fraction), (<41μm) – Br, Cu, Fe, Mn, Zn

Considering Ingamells sampling constants for IAEA-336 and the fractions prepared from it, the presented data suggest that for INAA there is no significant improvement by milling and sieving IAEA-336 into smaller grain size fractions. For PIXE, <64μm and <41μm fractions presented the lowest sampling constants for several elements.

Table 2.5 – INAA Ingamells sampling constants taken from literature for lichen candidate reference material IAEA-338, for some biological reference materials, for reference material IAEA-336 analysed and the different fractions prepared from it. Sample mass used for analysis is also shown.

	K_S (mg)											
	Different biological materials							IAEA	IAEA-336 and fractions prepared from it			
Element	**IAEA 338[a] (Lichen)**	**IAEA 338[a] (Lichen)**	**IAEA 338[b] (Lichen)**	**IAEA 338[a] (Lichen)**	**IAEA 338[a] (Lichen)**	**CTA-VTL-2[b] (Virginia Tobacco Leaves)**	**RMF II [b] (Spruce Shoots)**	**336[c] (Lichen)**	**<125um[d]**	**<64um[d]**	**<41um[d]**	**<20um[d]**
As				1580	7912			3572	234			
Ba								7156			86847	136699
Br		26.3	80	222	1049	19		4113	5201	28788	40337	37096
Cl	1.11	13		265	843			6191	191	893	6700	3159
Cs										8361	45053	38832
Eu					1512				259846	254913	195665	417391
K		4.69	93			45		2912		1123		
La			2806			2200		5286	3886	5636	63309	11531
Lu					31610				123195	21553	47807	36206
Mn	0.144		50	378	229			2971	2123	6868	5321	2288
Na	4.18	1.47	70	149	232	380		3836	7320	10758	5454	5277
Rb					266	61	288	3569	6838	13184		11678
Sc		648	110		232	95	4760	2778	2970	1585	850	1269
Sm			70		3345	1910	1640		5247	5367		
Zn		26.3			315			2984				27803
Mass (mg)	1.5	3	10	10	120	10	1	100	520	536	524	514

[a] Values from different laboratories taken from IAEA– TECDOC-1295 (2002)

[b] Values taken from Dybczenski et al. (2000)

[c] For certified IAEA-336, $R_0^2=R_a^2+R^2_{interlaboratory}+R_S^2$. Considering $R_S^2 >> R_a^2$ then $R_S^2 \approx R_0^2 + R^2_{interlaboratory}$. $R^2_{interlaboratory}$ was calculated using Horwitz.

[d] This work

Table 2.6 - PIXE and μ-PIXE Ingamells sampling constants taken from literature for IAEA 338, for IAEA-336 and and for IAEA 336 analysed and the different fractions prepared from it. Sample mass analysed is also shown.

Element	[a]IAEA 338 Lichen Material								[c]IAEA	[d]IAEA-336 and fractions prepared from it				
	Ks (mg)	[b]Mass (mg)	Ks (mg)	[b]Mass (mg)	Ks (mg)	Mass (mg)	Ks (mg)	[b]Mass (mg)	336 Lichen Reference Material	<125um	<64um	<41um	<20um	Mass (mg)
Br					11.4	0.6			4113	178	68	40	206	0.43
Cl	0.0013	1.64E-04	0.0086	3.94E-03			5.79	1.289	6191	0.83	0.5	1.4	7.6	0.19
Cu					36	0.6			4551	45	41	40	227	0.45
Fe	0.0077	2.43E-04	0.078	5.83E-03	3.66	0.6	0.91	1.907	3316	12	10	4.2	16	0.46
K	0.001	1.97E-04	0.107	4.74E-03					2912	1.2	0.72	4.8	20	0.2
Mn					17.8	0.6			2971	11	10	5	15	0.47
Rb					30.3	0.6			3569	227	39	942	177	0.42
Zn					6.73	0.6			2984	48	24	16	33	0.45
Ca					62.5	0.6	3.4	1.64		25		6.6	7.5	0.45
S					366	0.6	16.1	1.096		0.82	0.65	1.6	2.6	0.17
Si	0.0026	6.20E-05	0.038	1.48E-03			2.65	0.484		2.7	2.6	3.8	2.8	0.13
Ti					2.5	0.6				3.2	3.2	5.6	7.5	0.2

[a] Values from diferent laboratories taken from IAEA– TECDOC-1295 (2002)

[b] μ-PIXE analysis

[c] For certified IAEA-336, $R_0^2=R_a^2+R^2_{interlaboratory}+ R_S^2$. Considering $R_S^2>> R_a^2$ then $R_S^2\approx R_0^2+R^2_{interlaboratory}$. $R^2_{interlaboratory}$ was calculated using Horwitz. Reference mass for IAEA-336 is 100 mg

[d] This work

2.5 Conclusions

- Based on z-scores outcomes, analysis via INAA and PIXE at ITN facilities showed no significant differences for Cl, K, Mn, Fe and Zn, when compared with the certified or information values of IAEA-336;
- For K, Mn, Fe and Zn there is no significant difference between the ten average replicates given by INAA and PIXE for IAEA-336 (grain size <125 μm) and the grain-size fractions <64 μm, <41 μm and <20 μm prepared from it. Thus, the limited amount of material "seen" in the PIXE analysis and the grain-size distribution have no measurable influence on the outcomes compared to those by INAA. The deviating results for Cl are explained by a loss of this volatile element in the vacuum during PIXE analysis;
- Milling and sieving to a smaller grain size has not much influence to reduce the number of replicates necessary for PIXE to match INAA for elements like Fe, Mn and K, under the z-scores approach. The number of replicates necessary for PIXE to match INAA, decreased with decreasing particle size in the case of Zn;
- In the set of reference material IAEA-336 and grain-size fractions prepared from it, elemental content changes with grain size, which has not been anticipated before. The contents of Na, Cl, K, Mn and Sr decreased progressively with declining grain size, up to a factor of 2. For Al, Sc, Co, Zn, Br and Rb this effect is much smaller and for P, Cr, Fe, Cu, As, Se, Sb, Cs, Ba, La, Ce, Sm, Eu, Lu and Th virtually absent;
- Chlorophyll content analysis has shown that the concentration of algae increased with decreasing grain size fraction. The decreasing percentage of fungus (most probably the major part discarded during fractions preparation) and a possible large accumulation capacity of certain elements (*e.g*. Na, Cl, K, Mn and Sr) on this part of the lichen might explain the observed phenomenon;
- Concerning algae content in different particle size fractions, results found for "real samples" of *Parmelia sulcata (in situ* and transplanted, separated

in >125 μm and <125 μm fractions) showed that the concentration of algae was higher in the lower particle size fraction (<125 μm);

- Except for a few exceptions, the element concentration ratio of (<125μm fraction)/(>125μm) fraction is always higher than unit value, meaning that elemental contents are higher in the <125μm fraction, a different result from data obtain from the several fractions prepared from IAEA-336.

The above results were obtained for a specific material, prepared from the lichen *Evernia prunastri* as reference material, which has been especially treated to guarantee homogeneity. Thus, the results are not necessarily representative for other biomonitoring materials (lichens included) and/or associated sample-preparation treatments. All in all, one conclusion can be drawn concerning preparation of lichen material: the sieving process and consequently the discard of part of the material, leads to a change of the properties of the original sample, namely algae/fungus percentage and elemental contents. Although not tested with all lichen materials, it is recommend that when dealing with lichen samples for elemental assessment the material may be reduced into a powder and passed all through a pore-standardized micro sieve. This means that if ever results from various groups are to be compared in multi-scaled surveys, strictest protocols in sample preparations should be made. These protocols should be obeyed very carefully for every study performed to ensure comparability of results.

References

AOAC, (2002) Appendix D: Guidelines for collaborative study procedures to validate characteristics of a method of analysis. In *Interlaboratory Collaborative Study, AOAC Official Methods of Analysis*: AOAC International, pp. 1-12.

Asta, J. (1992) Constitution minérale de quelques espèces de lichens. *Bulletin de la Société Botanique de France* **139**: 81-97.

Asta, J., and Garrec, J.P. (1980) Étude de la répartition du calcium, potassium, magnésium et phosphore dans les différentes couches anatomiques de dix lichens par analyse directe à la microsonde électronique. *Cryptogamie, Bryologie et Lichénologie* **1**: 3-20.

Barr, R., and Crane, F.L. (1971) Quinones in algae and higher plants. In *Methods in Enzymology*. Pietro, A.S. (ed). New York: Academic Press, pp. 473-474.

Besada, V., Fumega, J., and Vaamonde, A. (2002) Temporal trends of Cd, Cu, Hg, Pb and Zn (*Mytilus galloprovincialis*) from the Spanish North-Atlantic coast 1991-1999. *The Science of the Total Environment* **288**: 239-253.

Bode, P. (1996) *Instrumental and Organisational Aspects of a Neutron Activation Analysis Laboratory*. PhD Thesis, Delft University of Technology, Delft, The Netherlands pp. 1-251.

Boyd, G.E. (1949) Method of activation analysis. *Analytical Chemistry* **21:** 335-347.

Brown, H. and Goldberg, E. (1949) The neutron pile as a tool in quantitative analysis; the gallium and palladium content of iron meteorites. *Science* **109** 347-353.

Budka, D., Przybylowicz, J., Mesjasz-Przybylowicz, J., and Sawicka-Kapusta (2002) Elemental distribution in lichens transplanted to polluted forest sites near Krakow (Poland). *Nuclear Instruments and Methods in Physics Research B* **189**: 499-505.

CETAMA (1986) *Statistique Appliquée a l'Exploitation des measures*. S.A. Masson (ed). Paris, France: Commission d'établissement des méthodes d'analyses du Commissariat à l'énergie atomique (CETAMA), pp. 17-32.

Chatt, A., Rao, R., Jayawickreme, C., and McDowell, L. (1990) Determination of sampling constants for selenium in biological reference materials by neutron activation. *Fresenius Journal of Analytical Chemistry* **338**: 399-407.

Clark, B.M., Mangelson, N.F., St Clair, L.L., Gardner, J.S., Cooper, L.S., Rees, L.B. et al. (1999) Analysis of lichen thin sections by PIXE and STIM using a proton microprobe. *Nuclear Instruments and Methods in Physics Research B* **150**: 248-253.

Coquery, M., Carvalho, F.P., Azemard, S., and Horvat, M. (1999) The IAEA worldwide intercomparison exercises 1990-1997): determination of trace

elements in marine sediments and biological samples. *The Science of the Total Environment* **237**/**238**: 501-508.

De Corte, F. (1987) *The k0 - Standardization Method, a move to the optimisation of neutron activation analysis*. PhD thesis, Gent University, Gent, Belgium pp. 1-464.

De Bruin (1949) Instrumental Neutron Activation Analysis: a routine method.

Dybczynski, R., Danko, B., and Polkowska-Motrenko, H. (2000) NAA study on homogeneity of reference materials and their suitability for microanalytical techniques. *Journal of Radioanalytical and Nuclear Chemistry* **245**: 97-104.

Dybczynski, R., Danko, B., and Polkowska-Motrenko, H. (2001) Some difficult problems still existing in the preparation and certification of CRMs. *Fresenius Journal of Analytical Chemistry* **370**: 126-130.

Erdtmann, G., and Petri, H. (1986) *Nuclear activation analysis: fundamentals and techniques, Part 1.* Treatise on Analytical Chemistry. Elving, P.J. (ed). USA: John Wiley & Sons Inc., pp. 422-626.

Fajgelj, A., and Zeisler, E. (1998) Particle size dermination of some IAEA and NIST environmental and biological reference materials. *Fresenius Journal of Analytical Chemistry* **360**: 442 - 445.

Flora, C.J., and Nieboer, E. (1980) Determination of nickel by differential pulse polarography at a dropping mercury electrode. *Analytical Chemistry.* **52**: 1013-1020.

Freitas, M.C. (1993) *The development of k0 - Standardised neutron activation analysis with counting using a low energy photon detector*. PhD Thesis, Gent University, Gent, Belgium.

Freitas, M.C., Catarino, F.M., Branquinho, C., and Máguas, C. (1993) Preparation of a lichen reference material. *Journal of Radioanalytical and Nuclear Chemistry* **169**: 47-55.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace Elements - their Distribution and Effects in the Environment*. Markert and Friese (ed). Amsterdam, The Netherlands: Elsevier Science, pp. 187-213.

Freitas, M.C., Almeida, S.M., Reis, M.A., and Oliveira, O.R. (2003) Monitoring trace elements by nuclear techniques in PM10 and PM2.5. *Nuclear Instruments and Methods in Physics Research A* **525**: 430-430.

Garty, J. (2001) Biomonitoring atmospheric heavy metals with lichens: theory and application. *Critical Reviews in Plant Sciences* **20**: 309-371.

Garty, J., and Delarea, J. (1991) Localization of iron and other elements in the lichen *Nephoroma arcticum* (L) Torss. *Environmental and Experimental Botany* **3**: 367-375.

Goyal, R., and Seaward, M.R.D. (1981) Metal uptake in terricolous lichens; I. Metal localization within the thallus. *New Phytologist* **89**: 631-645.

GraphPad Software Inc. (1999) *Analysing Data with Graphpad Prism*. San Diego, USA.

Griepink, B. (1990) Quality and certified reference materials. In *Element Concentration Cadasters in Ecosystems-Methods of Assessment and Evaluation*. H. Lieth and B. Markert (ed). Weinheim, Germany: VCH, pp. 181-205.

Heller-Zeisler, S.F., Zeisler, R., Zeiller, E., Parr, R.M., Radecki, Z., Burns KI, and De Regge, P. (1999) *Report on the Intercomparison Run for the Determinations of Trace and Minor Elements in Lichen* Material IAEA-336.Vienna, Austria: International Atomic Energy Agency.

Hevesy, G. and Levi, H. (1936) The action of neutrons on the rare earth elements. Det.Kgl.Danske Videnskabernes selskab, Mathematisk-fysiske Meddelelser XIV, 5: 3-34.

Heydorn, K., and Damsgaard, E. (1987) The determination of sampling constants by neutron activation analysis. *Journal of Radianalytical and Nuclear Chemistry* **110**: 539-553.

Horton, H.R., Moran, L.A., Ochs, R.S., Rawn, J.D., and Scrimgeour, K.G. (2002) Photosynthesis. In *Principles of Biochemistry*. H. Robert Horton, Laurence A. Moran, Raymond S. Ochs, J. David Rawn, K. Gray Scrimgeour (ed). New York, USA: Prentice Hall.

Horwitz, W., and Albert, R. (1996) Reliability of the determinations of polychlorinated contaminants (biphenyls, dioxins, furans). *Journal of Association of Official Analytical Chemist* **79**: 589 - 621.

Horwitz, W., Kamps, L.R., and Boyer, K.W. (1980) Quality assurance in the analysis of foods for trace constituents. *Journal of Association of Official Analytical Chemist* **63**: 1344-1354.

Huggins, F.E. (2002) Overview of analytical methods for inorganic constituents in coal. *International Journal of Coal Geology* **50**: 169-214.

IAEA-TECDOC-1295 (2002) Reference Materials for Microanalytical Nuclear Techniques. Vienna, Austria, pp. 1-14.

Ingamells, C. (1974) New approaches to geochemical analysis and sampling. *Talanta* **21**: 141-155.

Ingamells, C., and Switzer, P. (1973) A proposed sampling constant for use in geochemical analysis. *Talanta* **20**: 547-568.

ISO 13528:2005, Statistical methods for use in proficiency testing by interlaboratory comparisons by ISO/TC, 69/SC 6 N459.

Johansson, S.A.E., Campbell, J.L., and Malmqvist, K.G. (1995) *Particle Induced X-ray Emisison Spectrometry (PIXE) in Chemical Analysis*. USA: John Wiley & Sons.

Kempenaers, L., Vincze, L., and Janssens, K. (2000) The use of synchrotron micro-XRF for characterization of the micro-heterogeneity of heavy metals in low-Z reference materials. *Spectrochimica Acta Part B* **55**: 651-669.

Maenhaut, W., and Cafmeyer, J. (1998) Long-term atmospheric aerosol study at urban and rural sites in Belgium using multi-elemental analysis by particle-induced X-ray emission spectrometry and short-irradiation instrumental neutron activation analysis. *X-Ray Spectrometry* **27**: 236-246.

Mellado, j., Llauradó, M., and Rauret, G. (2002) Determination of actinides and strontium in fish samples by extraction chromatography. *Analytica Chimica Acta* **458**: 367-374.

Nash III, T.H. (1996) *Lichen biology*. England: Cambridge University Press.

Op De Beeck, J. (1972) GELIAN program. Gent, Belgium.: Institute for Nuclear Sciences, University of Gent.

Paul, A., Hauck, M., and Fritz, E. (2003) Effects of manganese on element distribution and structure in thalli of the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*. *Environmental and Experimental Botany* **50**: 113-124.

Pfeifhofer, H.W., Willfurth, R., Zorn, M., and Kranner, I. (2002) Analysis of chlorophylls, caratenoids, and tocopherols in lichens. In *Protocols in Lichenology - Culturing, Biochemistry, Ecophysiology and use in Biomonitoring*. I. Kranner, R.P. Beckett, A.K. Varma (ed). Berlin, Germany: Springer-Verlag Lab Manual, pp. 363-378.

Randle, K., Aljundi, J., Mamas, C.J.V., Sokhi, R.S., and Earwaker, L.G. (1993) A comparative-study of Neutron-Activation Analysis and Proton-Induced X-Ray-emission analysis for the determination of heavy-metals in estuarine sediments. *Nuclear Instruments and Methods in Physics Research B* **79**: 568-570.

Reis, M.A. (2001) *Biomonitoring and Assessment of Atmospheric Trace Elements in Portugal - Methods, Response Modelling and Nuclear Analytical Techniques.* PhD Thesis, Delft University of Technology, Delft, Netherlands pp. 1-230.

Reis, M.A., and Alves, L.C. (1992) DATTPIXE- a computer package for TTPIXE data analysis. *Nuclear Instruments and Methods in Physics Research B* **68**: 300-304.

Reis, M.A., Alves, L.C., and Jesus, A.P. (1996) Matrix effects correction for quantitative TTPIXE analysis. *Nuclear Instruments and Methods in Physics Research B* **109/110**: 134-138.

Richardson, D.H.S., Kiang, S., Ahmadjian, V., and Nieboer, E. (1985) Lead and Uranium uptake by lichens. In *Lichen Physiology and Cell Biology*. D.H. Brown (ed). New York, USA: Plenum Publishing Corporation, pp. 227-246.

Ro, C., Hoornaert, S., and Grieken, R. (1999) Assessment of homogeneity of candidate reference material at the nanogram level and investigation on representativeness of single particle analysis using electron probe X-ray microanalysis. *Analytica Chimica Acta* **389**: 151-160.

Ronen, R., and Galun, M. (1984) Pigment extraction from lichens with dimethylsulfoxide (DMSO) and estimation of chlorophyll degradation. *Environmental and Experimental Botany* **24**: 239-245.

Rossbach, M., and Grobecker, K. (1999) Homogeneity studies of reference materials by solid sampling - AAS and INAA. *Accreditation Quality Assurance* **4**: 498-503.

Rossbach, M., and Zeiller, E. (2003) Assessment of element-specific homogeneity in reference materials using microanalytical techniques. *Analytical and Bioanalytical Chemistry* **377**: 334-339.

Rossbach, M., Ostapczuk, P., and Emons, H. (1998) Microhomogeneity of candidate reference materials: comparison of solid sampling Zeeman-AAS with INAA. *Fresenius Journal of Analytical chemistry* **360**: 380-383.

Salma, I., Maenhaut, W., Cafmeyer, J., Annegarn, H.J., and Andreae, M.O. (1994) PIXE Analysis of Cascade Impactor Samples Collected at The Kruger-National-Park, South-Africa. *Nuclear Instruments and Methods in Physics Research B* **85**: 849-855.

Sha, Y., Zhang, P., Wang, X., Liu, J., Huang, Y., and Li, G. (2002) Analysis of candidate micro-reference materials of lichen and algae by SRXRF and PIXE. *Nuclear Instruments and Methods in Physics Research B* **189**: 107-112.

Sonntag, T., and Rossbach, M. (1997) Micro-homogeneity of candidate reference materials characterized by particle size and homogeneity factor determination. *Analyst* **122**: 27-31.

Spyrou, N.M., Farooqi, A.S., Arshed, W., and Akandle, O.A. (1990) Determination of the sampling factor in biological standards using INAA and PIXE analysis. *Nuclear Instruments and Methods in Physics Research B* **299**: 589-592.

Stone, S.F., Freitas, M.C., Parr, R.M., and Zeisler, R. (1995) Elemental characterization of a candidate lichen research material-IAEA 336. *Fresenius Journal of Analytical Chemistry* **352**: 227-231.

Thompson, M. (2000) Recent trends in inter-laboratory precision at ppb and sub ppb concentrations in relation to fitness for purpose criteria in proficiency testing. *Analyst* **125**: 385-386.

Thompson, M., and Wood, R. (1993) The international harmonized protocol for proficiency testing of (chemical) analytical laboratories. *Pure & applied Chemistry* **65**: 2123-2144.

Thompson, M., Ellison, S.L.R., and Wood, R. (2002) Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Applied Chemistry* **74**: 835-855.

Thompson, M.T.A., and Lowthian, P.J. (1997) The Horwitz function revisited. *Journal of Association of Official Analytical Chemist International* **80**: 676 - 679.

Todd, J.R. (1961) Magnesium in forage plants. II. Magnesium distribution in grasses and clovers. *Journal of Agricultural Science* **57**: 35-38.

Valladares, F., Sancho, L.G., and Ascaso, C. (1996) Functional analysis of the intrathalline and intracellular chlorophyll concentrations in the lichen family *Umbilicariaceae*. *Annals of Botany* **78**: 471-477.

Van Espen, P. (1990) AXIL V3.0 Computer Code Manual.

Weiss, N.A. (1999) *Introductory Statistics*. Addison Wesley Longman, Inc., pp. 180-185.

Weizhi, T., Bangfa, N., Pingsheng, W., and Huiling, N. (2000) Suitability of NAA for certification of reference materials for multielements. *Journal of Radianalytical and Nuclear Chemistry* **245**: 51-56.

Wintermans, J.F., and De Mots, A. (1965) Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. *Biochimica et Biophysica Acta* **109**: 448-455.

Wolterbeek, H.T., Garty, J., Reis, M.A., and Freitas, M.C. (2002) Biomonitors in use: lichens and metal air pollution. In *Bioindicators and biomonitors*. Markert, Breure and Zechmeister (ed). Amsterdam, Netherlands: Elsevier Science, pp. 377-419.

Woolson, R.F., Clark, W.R. (2002) *Statistical Methods for the Analysis of Biomedical Data*: Wiley & Sons, Inc, USA.

Wu, H., Hayes, M., Weiss, A., and Hu, Q. (2001) An evaluation of the standardized precipitation index, the China-z and the statistical z-score. *International journal of Climatology* **21**: 745-758.

Zeisler, R. (2000) Investigations by INAA for the development of Natural Matrix Standard Reference Materials® (SRMs) suitable for small sample analysis. *Journal of Radianalytical and Nuclear Chemistry* **245**: 81-85.

Zeisler, R. (1998) Reference materials for small-sample analysis. *Fresenius Journal of Analytical Chemistry* **360**: 376-379.

Chapter 3[2]

Cell-membrane Damage and Element Leaching in Transplanted *Parmelia sulcata* Lichen Related to Ambient SO_2, Temperature, and Precipitation

3.1 Introduction

Lichens are often used in biomonitoring studies to indicate geographical variances in trace-element air pollution (Conti and Cecchetti 2001; Garty 2001; Jacquiot and Daillant 2002; Wolterbeek, Garty et al. 2002). Trace elements may be retained in the lichen by particulate entrapment, physiochemical processes and passive and active intracellular uptake (Godinho, Wolterbeek et al. 2008). Accumulation may be viewed as a dynamic process, involving uptake and release processes until equilibrium with the surrounding environment is reached (Reis, Alves et al. 1999). Chemical element release may occur by biological regulation, rain run-off, blow-off by wind, and cell leakage under oxidative stress (Ayrault, Clochiatti et al. 2007). The main implicit assumption in biomonitoring studies is that the lichen response behaviour can be compared throughout the whole investigated area, irrespective of variances in ambient conditions. When exposing lichen transplants

[2] A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ, "Cell-membrane damage in transplanted *Parmelia sulcata* lichen related to ambient SO2, temperature and precipitation", Environmental Science & Technology, Vol. 39, (2005), 2624 – 2630.

(or *in situ* sampled lichens), apart from the elemental information, lichen performance should be taken into account, both in a geographical set-up and in time-series. The ambient climatic conditions are repeatedly reported as of importance for the chemical element content of the lichen (Carreras and Pignata 2001; Baptista, Vasconcelos et al. 2008). Also, the vitality of the lichen is regarded as an important parameter for general lichen performance (Branquinho, Catarino et al. 1999; Garty, Weissman et al. 2000; Godinho, Freitas et al. 2004).

A key lichen parameter is the lichen physiological vitality, sometimes analysed by determination of the lichen membrane permeability (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty 2001; Garty, Weissman et al. 2001). Although there are several experimental procedures to test the impact of environmental pollution on lichen vitality (Mulgrew and Williams 2000) measuring either the conductivity or the K^+ content of a leachate with an appropriate electrode is the easiest way of monitoring membrane integrity. Electric conductivity was pointed out as the most sensitive parameter for physiological response to environmental stress, when compared to NDVI (normalised difference vegetation index) (Garty, Weissman et al. 2000) and chlorophyll degradation, being also related to the whole lichen and not to just the photobiont as are many other parameters (Mulgrew and Williams 2000).

Membranes organise the interior of cells into different compartments and by means of pore size, pumps and carriers they control uptake, release and relocation of molecules and ions. One main effect of pollution is disturbance of this organisation and thereby changing the membrane permeability to ions with an accompanying loss of electrolytes, particularly of K and Mg (Nash III 1996). Previous studies have shown a link between the content of airborne elements accumulated in lichen transplants and the degradation of cell membranes. Most of these studies indicated a severe leakage of K from lichen thalli displaying a high degree of electric conductivity due to cell membrane debility (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty 2001; Garty, Weissman et al. 2001). It was shown by Garty et al. (Garty, Cohen et al. 1998) that the impairment of cell membranes in the transplanted lichen thalli could be the result of the presence of either SO_2, indicated by the correlation of electric conductivity and the concentration of S and SO_4^{2-}, or

any of the elements and ions, such as B, Ba, Cl, Cr, Cu, Na, Ni, and NO_3^-, also found by positive correlations with electric conductivity. Pollutants such as SO_2, O_3, and NO_2, are powerful catalysts of lipid membrane peroxidation. This may lead to a decrease of phospholipids content and an increase of unsaturated fatty acids, having cell-membrane damage as major effect on lichens (Conti and Cecchetti 2001). Membrane integrity was also found to be highly correlated with Ca which is a macronutrient and has regulatory functions in sites of extracellular interchange on the surface level of alga cells walls or hyphae, and intracellular interchange with proteins (Conti and Cecchetti 2001).

However, K release may also be related to wet-dry cycles. Laboratory studies have shown that soluble intracellular chemicals are released when dry lichens are placed in water (Brown and Brown 1991). Desiccation of lichens may cause disruption of the plasma membrane, but when water is added to a dry sample it may bring about the loss of soluble intracellular ions (Bargagli 1998). Nash (Nash III 1996) has already pointed out that lichens are remarkable in their ability to re-hydrate and resume normal metabolic functions following periods of desiccation, and re-establishment of membrane integrity occurs after exposure to relatively short periods (minutes) of high humidity. Desiccation tolerance is the ability of cells to survive in an air-dried state even at water contents below 10% (w/w), which would be lethal to most vascular plants. In the desiccated state, tolerant organisms appear completely dry, but rapidly regain normal physiological characteristics during rehydration (Kranner 2002).

Garty and co-workers (Garty, Weissman et al. 2000), proposed the wet–dry cycles as a possibility for K loss from the lichen, but their results have shown that very low contents of K were found near industrial areas and consequently air pollution was pointed out as the major cause. A factor of approximately 2 in electric conductivity was found in lichens from industrial polluted sites compared with those from rural sites (Garty, Cohen et al. 1998).

In the present work, lichens were transplanted from a clean background site, exposed in an industrial area and collected on a regular basis to verify membrane damage during an up-to one-year exposure. The main objective was to relate

variability in lichen vitality to variability in ambient conditions as well as to time-related changes in accumulated trace elements, to gain insight into the possibilities to use and compare lichens throughout larger geographical areas. Data were gathered on lichen element contents, elements in leachates, electrical conductivity of leachates, and of the ambient parameters temperature, precipitation and SO_2 level.

3.2 Experimental section

3.2.1 Sampling and experiment

The procedures for collection, preparation and exposure of the lichens were similar to the approaches in previous Portuguese studies with lichen transplants (Reis, Freitas et al. 1999; Freitas, Reis et al. 2000a; Freitas, Reis et al. 2000b; Freitas, Reis et al. 2001; Reis 2001; Marques, Freitas et al. 2004; Marques, Freitas et al. 2004). Samples of the epiphytic lichen (*Parmelia sulcata*) with their substrate were collected from olive trees at about 1.5 meters above the soil in the Mafra/Ericeira region.

This region, considered clean from an air pollution point of view, is situated 30 km north-west from Lisbon and 8 km from the coast (Freitas, Reis et al. 2000). Samples of about 2 g of the lichen (still attached to their substrate olive bark) were put into nylon net bags and suspended, fixed to a nylon rope, at about 1.5 meters above the soil. A total of 15 lichen transplants (with 20 cm distance from each other) were exposed for a one-year period a the ITN campus (8 km north from Lisbon) starting in August 2001. One transplant was collected every week during the first month and from then onwards a new transplant was collected every month. Maximum and minimum thermometers were also applied at the exposure site: data were obtained at the same time as transplant collection.

The exposure site (ITN campus) is situated within an industrial and populated area, which is considered a polluted region (Freitas, Reis et al. 2000; Almeida 2004). ITN campus is situated near Tagus river (8 km north from Lisbon), and is characterised by a high population and traffic density (INE, 2001).

3.2.2 Exposure site

Information on elemental contents of in situ lichens in this region is reported elsewhere (Freitas, Reis et al. 2000). Principal component analysis performed on element data from aerosol filters collected within the same ITN campus during 2001 yielded three main groups of elements related to three different sources: soil elements (Al, Ca, Co, Fe, K, La, Mn, Sc, Si, Sm and Ti), sea elements (Na, Cl, K, Mg and S) and industrial elements (As, Ca, Co, Cu, Hg, K, Mn, Ni, S, Sb, Se, V and Zn) (Almeida 2004).

3.2.3 Conductivity measurements

To judge lichen vitality, exposed transplants were soaked in demineralised water after which the water conductivity was determined. The procedure essentially followed approaches by Garty and co-workers (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty, Weissman et al. 2001): lichen material was cleaned, rinsed rapidly with demineralised water, two to three times for 5 s. After air-drying, about 1 g was weighted and immersed in 100 ml of demineralised water for 1 h. After removal of the lichens the electric conductivity of the solution was measured in $mS.m^{-1}$ with a Microcomputer Conductometer K220. The electric conductivity of the demineralised water was always measured as a blank, before solution conductivity measurements, and always subtracted (0.160 ± 0.024 $mS.m^{-1}$ on average). Conductivity results of the solutions are given relatively to the dry mass of lichen used. Electric conductivity of lichens before exposure was measured as 2.822 ± 0.051 $mS.m^{-1}.g^{-1}$ (n=3). For the other conductivity measurements a single measurement was used. In all solutions used for determination of conductivity, pH was also measured, using a digital pH meter.

3.2.4 ICP-OES analysis

For the January to August 2002 period, all leachate solutions (used for conductivity measurements) were frozen and stored to be analysed by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES), (Montaser and Golighty 1992, Boss and Fredeen 2004), using a Perkin Elmer As-93Plus Autosampler (Perkin-Elmer, Shelton CT, USA), and, where

necessary, a Perkin Elmer Ultrasonic Nebulizer (USN 6000+). The instrumental conditions and operational parameters settings have been based upon the optical Signal-To-Background (SBR) of the Mn II 257.610 nm line, as the best single representative of the ICP-OES emission lines. A multi element solution containing 10.0 mg.L-1 Ba and 1.0 mg.L-1 Mg and Zn was used to optimize axially and radially viewed configurations and to obtain maximal signal-to-background ratios (SBR). Analytical calibration curves for all elements to be determined were used to assess the precision and accuracy of measurements performed in both axially and radially viewed configurations, as well as in Ultra Sonic Nebulizer analysis.The analyses were performed at the Interfaculty Reactor Institute at Delft, The Netherlands.

3.2.5 Instrumental Neutron Activation Analysis (INAA)

For the January to August 2002 period, lichens (both directly and after being leached in the conductivity testing) were prepared for INAA multi-elemental analysis (Erdtmann and Petri 1986), using the k_0 factor method (De Corte 1987). In short, the samples were cleaned, rinsed with distilled water, freeze-dried and carefully ground in a Teflon mill for 10 min after immersion for 2 min in liquid nitrogen. Pellets of 500 mg were irradiated together with 0.1% Au-Al foils with a diameter similar to the pellet diameter. Short irradiations (18 s, thermal-neutron fluence rate 2.8×10^{12} $cm^{-2}s^{-1}$) and long irradiations (5 h, thermal-neutron fluence rate 3×10^{12} $cm^{-2}s^{-1}$) were performed at the Portuguese Nuclear Research Reactor (RPI) at ITN. Gamma spectra were collected using HPGe semiconductor detectors. Quality control was pursued by analysing the IAEA-336 lichen material.

3.2.6 PIXE measurements

For the November and December 2001 period, lichens (not used in conductivity testing) were analysed by PIXE (Johansson, Campbell et al. 1995) mainly for sulphur determination. The samples were cleaned, rinsed with distilled water, freeze-dried and carefully ground in a Teflon mill for 10 min after 2 min in liquid nitrogen. Pellets of a thin layer of lichen powder in a boric acid support were

prepared. Samples were irradiated in the Van de Graaff accelerator at ITN with a 2.4 MeV proton beam collimated to a 5-mm diameter. The X-ray spectra were obtained with a Si(Li) detector. Matrix effects corrections for quantitative TTPIXE analysis were performed (Reis, Alves et al. 1996). Quality control was pursued by analysing the IAEA-336 lichen material.

3.2.7 Additional data

Additional data were taken from the Portuguese Meteorological Institute (IM) web page (www.meteo.pt), such as temperature and precipitation. Lisbon IM airport monitoring station is the one which is closest to the sampling site (about 4 km south): its general meteorological conditions are rather similar to the ones at ITN campus. It may be noted here, however, that the ITN campus is somewhat closer to the Tagus River and experiences more frequent fog periods. For SO_2, data were used from two monitoring stations, Bobadela and São João da Talha (about 2 km south-west and about 5 km north respectively from the ITN campus), as obtained from the urban waste incinerator VALORSUL (www.valorsul.pt).

3.3 Results and discussion

3.3.1 Conductivity and lichen-released ions

Electric conductivity is highly dependent of the ions present in solution and their equivalent ionic conductivities. The H^+ ion has the highest equivalent ionic conductivity value (349.65×10^{-4} $m^2.S.mol^{-1}$) and may have a major contribution to conductivity if the pH is low. Therefore, the solution pH was also measured. The pH of the leachates was on average 6.2±0.1. Table 3.1 presents the results from the ICP-OES elemental analysis: combined with data on equivalent ionic conductivities (Raton 1986-1987) the results indicate that conductivities obtained should be related to leached elements rather than to the pH. Table 3.1 also presents correlations between conductivity and leachate element content. The highest correlations were found for K, Na and Mg and a significant correlation was also obtained for Cs.

Table 3.1 – Element concentrations (µg/L) in leachate solutions, from transplants exposed from January till July 2002, obtained after soaking 1 g of lichen transplant in 100 ml of demineralised water for 1 h. Concentrations are given in averages, standard deviations (StDev), their minimum and maximum values. Correlations between conductivity ($mS.m^{-1}.g^{-1}$) and element contents in solution (µg/L) are presented by the correlation coefficients R. In addition, P values are presented for all correlations and significant ones (threshold $P < 0.05$) are given in bold italics.

	Al	Ba	Ca	Cr	Cs	Cu	Fe	K
Average	16.6	3.3	2400	1.20	2.06	0.034	12.4	1210
StDev	7.9	1.7	950	0.34	0.57	0.023	5.0	366
Min	8.0	1.5	1600	0.82	1.5	0.007	5.9	770
Max	31	6.5	3900	1.7	3.1	0.071	19	1700
R	0.65	0.11	0.57	0.13	***0.76***	0.70	0.66	***0.89***
P	0.11	0.82	0.18	0.79	***0.049***	0.08	0.11	***0.008***
	Mg	Mn	Na	Ni	Si	Sr	V	Zn
Average	498	2.83	2020	2.00	45.8	7.71	1.53	26.8
StDev	140	2.76	695	0.66	17.2	6.12	0.35	27.1
Min	330	0.15	1000	1.2	32	3.8	1.0	8.9
Max	750	7.6	3100	3.0	84	21	2.0	87
R	***0.84***	0.56	***0.93***	0.71	0.39	0.14	0.70	-0.28
P	***0.018***	0.20	***0.003***	0.07	0.39	0.76	0.08	0.54

Fig. 3.1 shows the similarity in variability between Na and K and conductivity for the period between January and July 2002. Most probably, Na and K are largely responsible for the conductivities obtained. This suggestion is underlined by the concentration levels of Na and K relative to those from Cs and Mg: their releases may coincide with those from Na and K, but their concentrations differ, especially in the case of Cs where concentrations differ by some orders of magnitude (Table 3.1). The general results support approaches by Garty et al. (Garty, Weissman et al. 2000), who implicitly associate conductivity to the release of K, but some more

discussion should be devoted to the simultaneous presence of Na and Cl (see further). It should also be noted here that none of the other leached elements showed any significant correlation with conductivity; apparently, their leaching did not depend on lichen vitality.

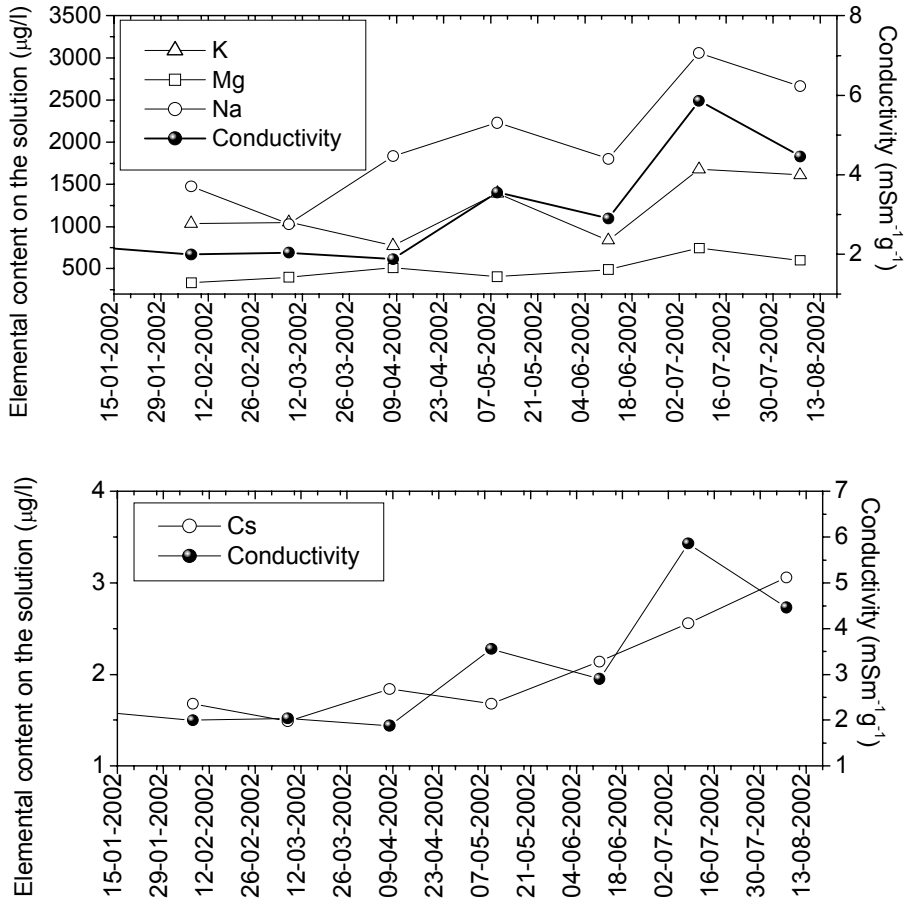

Fig. 3.1 – Electrical conductivity (expressed per unit lichen mass, $mS.m^{-1}.g^{-1}$) and Cs, K, Mg and Na contents of the leaching solution (in µg/L) after soaking of the transplants for 1 h in demineralised water. Transplants were exposed at the ITN campus and collected on a monthly basis.

3.3.2 Conductivity and element levels, ambient temperature, precipitation and SO_2

Fig. 3.2 shows conductivity values at the various days of transplant sampling (at the end of the exposure periods at the ITN sampling station) versus SO_2 values (Bobadela and S.J. Talha sampling stations). High conductivity values were obtained in the first month of exposure followed by a decrease, a certain stability period and an increase again at the end of the exposure period.

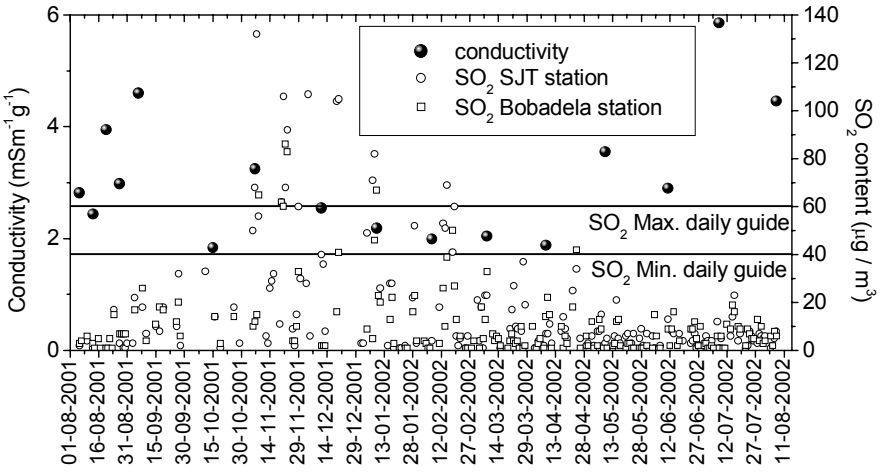

Fig. 3.2 – Electrical conductivity (expressed per unit lichen mass, $mS.m^{-1}.g^{-1}$) of leachates of transplanted lichen material exposed from August 2001 till August 2002 at the ITN campus, and corresponding ambient SO_2 concentrations (in $ug.m^{-3}$) for the São João da Talha (SJT) and Bobadela stations, obtained by VALORSUL (www.valorsul.pt).

During the first five weeks of exposure, SO_2 values were below the minimum daily guide value. The most probable explanation for the rise in conductivity at the beginning of the exposure period is the removal of the lichen material from its natural habitat, a clean environment (Freitas, Reis et al. 2000), to a polluted one (Almeida 2004), with higher temperatures and no precipitation at that time (Fig. 3.3). Although it cannot be claimed that all lichens exhibit similar degrees of stress

due to cold and heat, it is reasonable to assume that lichens are generally well adapted to the temperatures experienced in their particular microhabitats. Furthermore, if (suddenly) exposed to temperatures that are not within their operational temperature range, some species do exhibit adverse responses (Nash III 1996). Also as stated above, the background area is close to the coast, which may imply that Cl and Na show up as important in conductivity values after lichen leaching.

After two months of exposure, lower conductivity values were obtained possibly due to the precipitation observed at that time (Fig. 3.3). Most lichens are remarkable in their ability to re-hydrate and re-establish membrane integrity after short periods (minutes) of exposure to high humidity levels (Nash III 1996). After three months the conductivities raised again, coinciding with a decrease in the minimum temperature to values close to 0 ºC, but most likely more related to the increases in SO2 values (Fig. 3.2) from November till the end of January, detected in both S.J. Talha station (about 5 km north) and Bobadela station (about 2 km south-west). Most of the SO2 values from November 2001 till the middle of February 2002 were above the minimum daily guide value and the maximum daily guide value was exceeded sometimes. In the period of increased conductivity, two lichen samples corresponding to November and December were analysed directly by PIXE for S levels: the data were compared with results of *Parmelia sulcata* in previously performed studies (Reis, Freitas et al. 1999; Freitas, Reis et al. 2000; Freitas, Reis et al. 2001).

The sulphur contents observed (1860 and 1990 mg/kg in November and December respectively) were higher than the reference levels obtained in an unpolluted area of Portugal (1390±110 mg/kg) (Reis, Freitas et al. 1999) and similar to the values obtained for a three-month period exposure in an industrial area (2400±570 mg/kg) (Reis, Freitas et al. 1999). The low conductivity value in October 2001 was obtained again in February 2002 with the disappearance of the high SO_2 values and maintained while precipitation was relevant. In previous studies (Haffner, Lomsky et al. 2001), where membrane damage was determined by K concentrations in the lichen, *Parmelia sulcata* was able to recover, and even restored some physiological parameters suggesting that *Parmelia* may be one of the lichens most resistant to

pollution. It was the only one of the four lichen species studied capable of retaining more than 50% of its original K^+ concentration. In the study of Haffner (Haffner, Lomsky et al. 2001), the resistance of the lichen was correlated with thallus morphology, metabolic activity, fungal respiratory performance, and sulphur accumulation capacity.

At nine-month exposure the conductivity raised again reaching values higher than the zero-month value. For the eleven-month exposure the result obtained was twice the initial one. High temperatures and the absence of precipitation may be the main reasons for these rapid increases in conductivity (Fig. 3.3).

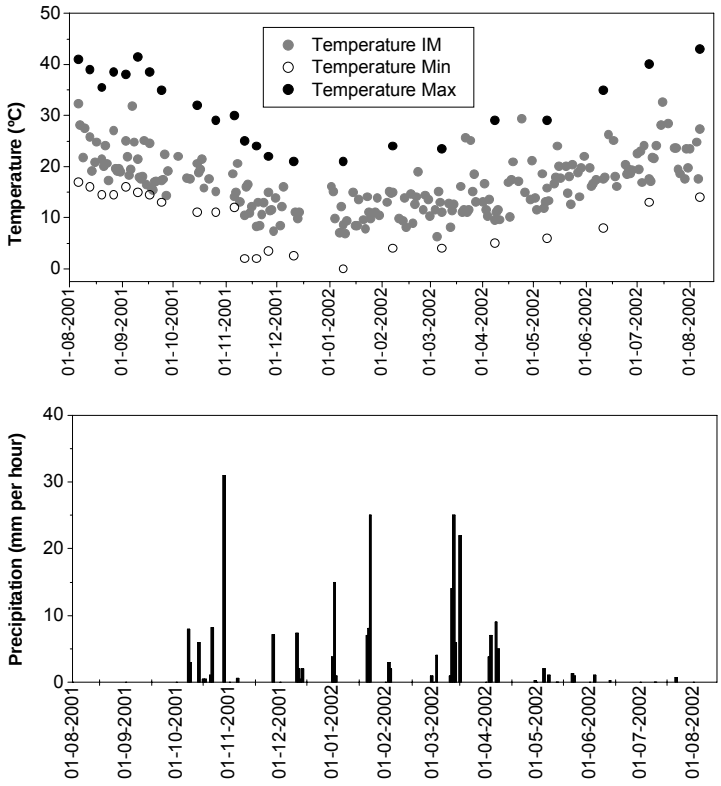

Fig.3.3 – Minimum temperature (Min) and maximum (Max) temperature taken on exposure site. Temperature (IM) and precipitation ($mm.h^{-1}$) at the airport station of Lisbon; data taken from the Meteorological Institute (IM) of Portugal (www.meteo.pt).

Table 3.2 shows the correlations between lichen element levels and conductivity. Significant correlations were found only for Na and Cl, which suggest the absence of any effect from other elements.

Table 3.2 - Element concentrations ($mg.kg^{-1}$) in lichen transplants (directly after exposure, not leached for conductivity determination), exposed from January till July 2002. Concentrations are given in averages, standard deviations (StDev), their minimum and maximum values. Correlations between conductivity ($mS.m^{-1}.g^{-1}$) and element concentrations are presented by the correlation coefficients R. In addition, P values are presented for all correlations and significant ones (threshold $P < 0.05$) are given in bold italics.

	Al	As	Ba	Br	Ca	Cl	Ce	Cr	Co	Cs
Average	3120	1.09	31.4	51.2	8030	1010	7.33	4.81	0.71	0.349
StDev	744	0.20	7.4	14.9	1530	297	2.31	0.98	0.14	0.069
Min	2200	0.84	20	26	660	70	4.6	3.3	0.54	0.25
Max	4500	1.3	40	75	11000	1600	11	6.5	0.99	0.45
R	0.06	0.41	0.63	-0.56	0.08	***0.78***	0.42	0.36	0.22	0.38
P	0.91	0.37	0.13	0.19	0.86	***0.039***	0.35	0.42	0.64	0.40
	Fe	Hf	K	La	Lu	Mg	Mn	Na	Rb	Sb
Average	1800	0.60	4840	3.15	0.031	1320	39.7	506	9.98	1.15
StDev	352	0.12	319	0.90	0.007	171	14.4	243	0.90	0.37
Min	1300	0.45	4500	2.0	0.021	1100	25	260	8.7	0.51
Max	2400	0.81	5300	4.3	0.039	1700	63	880	11	1.6
R	0.35	0.35	-0.01	0.47	-0.01	-0.33	-0.30	***0.91***	0.42	0.34
P	0.44	0.44	0.99	0.28	0.99	0.47	0.52	***0.005***	0.35	0.46
	Sc	Se	Sm	Ta	Tb	Th	Ti	V	Zn	
Average	0.49	0.635	0.62	0.078	0.075	1.12	296	9.16	99.7	
StDev	0.10	0.086	0.18	0.015	0.018	0.42	67	1.57	12.2	
Min	0.37	0.54	0.40	0.051	0.053	0.61	200	6.8	860	
Max	0.68	0.80	0.90	0.099	0.11	1.8	420	11	110	
R	0.26	-0.02	0.43	0.31	0.23	0.46	0.13	0.14	0.48	
P	0.57	0.97	0.33	0.50	0.63	0.30	0.79	0.77	0.28	

Taking the fractional element presence in leachates (Table 3.3), conductivity was again correlated only to Na and Cl: these results indicate that the rinsing procedure apparently induces Na and Cl losses which are of different origin or nature than the losses of K. The data in Table 3.3 indicates the leachability of the elements.

The highest leachability was shown for Na, but relatively high (month-specific) leachability was also evident for elements such as Ca, Cl, Hf, Lu and others. In discussing Na, Cl, Mg and K in leaching, it should be noted that the majority of Na^+ is generally reported as associated to surface and intercellular sites, Mg^{2+} is mostly associated to exchange groups of the cell wall and K^+ is regarded as present in intracellular fractions and the cell wall (Figueira 2002). Generally, the superficial and intercellular cell fractions show highest variability in element levels, which are highly affected by precipitation (Figueira 2002) or (in our experimental case) the direct immersion of the lichen in water. Further, as stated already, Na and Cl may be sea-salt related, whereas K may originate from soil-dusts and tree-stem flow.

Based on considerations on both origin (source, physico-chemical occurrence) and possible intra/inter-cellular distributions of the elements in the lichen transplant, the question may be raised to what extent elements may be grouped towards their leachability. As a first set-up, the present elements were subjected to Monte Carlo Assisted Target Transformation Factor Analysis (MCATTFA). MCATTFA (Kuik, Blaauw et al. 1993; Kuik, Sloof et al. 1993; Kuik and Wolterbeek 1995) is a multivariate statistical method, which is often used in environmental pollution studies to simplify large and complex data sets by grouping elements into a limited set of factors, composed of correlated (fractional) sets of elements. The method is mostly used to identify pollution sources (factors) and their relative elemental composition, but may also be used to group elements in their leaching behaviour.

Table 3.3 – Fractional element leaching from the lichen transplant (in %)*. R = correlation coefficient for the correlation between conductivity ($mS.m^{-1}.g^{-1}$) and the fractional element leaching (%). Significant correlations (threshold $P < 0.05$) are indicated in bold italics.

Month	Al	As	Ba	Br	Ca	Ce	Cl	Cr	Cs	Co
January	24	20	8	-21	24	25	16	20	16	22
February	21	19	32	6	37	13	11	22	23	22
March	17	16	17	-3	-14	13	2	12	18	16
April	21	21	18	36	5	42	31	29	35	25
May	51	31	39	29	63	50	32	44	46	45
June	-16	18	12	25	25	2	45	17	22	17
July	10	1	34	-3	8	3	57	17	26	16
R	0.67	0.34	0.05	0.46	0.01	0.35	***0.86***	0.10	0.15	0.23
P	0.10	0.46	0.92	0.31	0.98	0.44	***0.013***	0.83	0.75	0.62
Month	Cu	Fe	Hf	K	La	Lu	Mg	Mn	Na	Rb
January	-3	25	50	13	13	14	21	30	43	5
February	4	25	39	7	9	21	22	13	51	25
March	22	15	20	12	16	22	20	13	39	9
April	13	31	36	30	41	47	26	-15	65	34
May	32	41	41	26	43	45	43	47	63	28
June	-15	14	10	21	7	14	-6	-8	69	13
July	42	14	11	19	-7	18	23	-19	75	16
R	0.14	0.35	0.69	0.53	0.25	0.10	0.57	0.61	***0.85***	0.07
P	0.76	0.44	0.08	0.23	0.58	0.82	0.18	0.14	***0.016***	0.88
Month	Sb	Sc	Se	Sm	Ta	Tb	Th	Ti	V	Zn
January	55	25	23	23	39	34	25	26	15	15
February	3	26	27	14	15	23	10	28	18	14
March	14	15	17	18	19	19	15	37	13	14
April	6	34	30	41	50	43	45	32	5	14
May	23	42	34	49	38	45	54	34	36	16
June	23	16	0	6	13	0	0	1	-7	18
July	32	12	19	8	17	3	1	31	17	24
R	0.00	0.31	0.64	0.33	0.26	0.58	0.35	0.72	0.56	0.66
P	0.99	0.50	0.12	0.47	0.57	0.17	0.44	0.07	0.19	0.11

* The percentage of lichen leached was calculated by:

$$Leached\_lichen = 100\% - (\frac{Lichen\_soaked \times 100\%}{Lichen\_not\_soaked})$$

Table 3.4 illustrates the approach, for a sub-set of the six elements K, Sc, V, Cu, As and Sb. The number of elements was limited to approach Henry's reasoning (Henry 1991) towards a rule-of-thumb relationship between the number of observations and number of elements. Furthermore, the elements were selected to represent physiology (K), soil dust (Sc) and industries (V, Cu, As and Sb). It presents the average relative elemental contributions of each factor to the total relative release of a specific element and the total (90 – 101%) sums the factor statistical explanation of the actual release data. For instance, Table 3.4 indicates that Factor 1 contributes to 83% of the arsenic fractional release, which is explained by MCATTFA for 91%.

Table 3.4 – Average Factor (1-4) contributions to total relative element release by leaching, illustrated for K, Sc, V, Cu, As and Sb. Calculations by MCATTFA analysis of fractional element data (percentage of lichen element content released into the solution after soaking 1 g of lichen transplant material in 100 ml of distilled water for 1 h). Indicated factor contributions are marked with * ($P < 0.01$), + ($P < 0.05$), or P (highest significance = Pilot element). The totals sum the factors 1 to 4 and indicate the overall percent MCATTFA-explanation of the element release data

Element	Factor 1	Factor 2	Factor 3	Factor 4	Total
K	18+	2	71P	1	92
Sc	70*	0	21+	4	95
V	38+	13*	3	36P	90
Cu	0	53*	31*	13+	97
As	83P	1	4	3	91
Sb	2	99P	0	0	101

The outcomes indicate that the fractional release of K (71% in Factor 3) is not correlated with the fractional release of V, As or Sb, that the fractional release of As (83% Factor 1) is not correlated with that of Sb and Cu, that the fractional release of Sb (99% Factor 2) is not correlated with that of K, Sc and As, and that the fractional release of V (36% Factor 4) is not correlated to that of K, Sc, As and

Sb. Considering K to represent lichen vitality (Garty, Cohen et al. 1998; Garty, Weissman et al. 2000; Garty, Weissman et al. 2001), Sc to represent soil dust (Bowen 1979; Bargagli 1998), and V to represent oil combustion (Nriagu 1989), and As and Sb to represent volatile elements (Seiler, Sigel et al. 1994), the data again suggest the absence of any clear relationships between fractional release and vitality for many elements.

Table 3.4 also indicates possible similarities in origin (source, physico-chemical occurrence) between Sc and As (Factor 1), Sb and Cu (Factor 2), K and Cu (Factor 3) or V and Cu (Factor 4), all to varying degrees of significance. It should be noted here, however, that although grouping of element leachability may give insight in possible similar origin, occurrence or behaviour, the variable levels of explanation in Table 3.4 indicate that more study is needed to better explain differences in release. Here it shows that, although the lichen capacity to accumulate elements from the atmosphere has been widely studied, still little is known about chemical element location, retention and leaching mechanisms.

3.4 Conclusions

All in all, the data indicate that, apart from lichen Na and Cl levels, and for temperature and precipitation, no clear relationships with conductivity could be observed. Conductivity was mostly related to released Na, Cl, K, Mg and Cs. On basis of concentrations, Na, Cl and K could be considered as largely determining the conductivity. Parmelia sulcata was sensitive enough to reflect appreciable ambient rises in air SO2 and resistant enough to recover afterwards. Generally speaking, the present data suggest that the comparability of lichen vitality in large geographical areas may be limited and governed by the area's variability in temperature and precipitation rather than by variability in chemical element deposition rates. Currently, three major biochemical mechanisms for protection from desiccation-induced injury are known (Kranner 2002). One mechanism includes proteins such as the late embryogeneses abundant (LEA) proteins and LEA-related proteins like dehydrins and rehydrins. Second, nonreducing sugars have been suggested to facilitate tolerance to desiccation by protecting membranes

and inducing vitrification. Third, desiccation-tolerance is based on a capability to scavenge desiccation-induced free radicals. Desiccation-tolerance has been correlated with maintenance and/or synthesis of antioxidants such as reduced glutathione, ascorbic acid and tocopherols and/or enzymes scavenging reactive oxygen species during the desiccation/rehydration process.

The leaching data on all elements and element groups (Tables 3.1 to 3.4), however, strongly suggest that wet deposition may also severely affect lichen elemental levels (Tables 3.1 to 3.3). This latter observation makes that comparing outcomes for time- or spatial series of lichen samples should be accompanied by a comparably careful monitoring of (preceding) ambient conditions.

References

Almeida, S.M. (2004) *Composição Elementar do Aerossol Atmosférico em Zona Urbano-Industrial.* PhD Thesis, Universidade de Aveiro, Aveiro, Portugal, in portuguese.

Ayrault, S., Clochiatti, R., Carrot, F., Daudin, L., and Bennett, J. (2007) Factors to consider for trace element deposition biomonitoring surveys with lichen transplants. *The Science of the Total Environment* **372**: 717–727.

Baptista, M., Vasconcelos, M., Cabral, J., Freitas, M., and Pacheco, A. (2008) Copper, nickel and lead in lichen and tree bark transplants over different periods of time. *Environmental Pollution* **151**, (2): 408-413.

Bargagli, R. (1998) Lichens as biomonitors of airborne trace elements. In *Trace Elements in Terrestrial Plants: an Ecophysiological Approach to Biomonitoring and Biorecovery*. Berlin, Germany: Springer Verlag, pp. 179-206.

Branquinho, C., Catarino, F.M., Brown, D.H., Pereira, M.J., and Soares, A. (1999) Improving the use of lichens as biomonitors of atmospheric metal pollution. *The Science of the Total Environment* **232**: 67-77.

Boss C., Fredeen, K. (2004) *Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectrometry*, Perkin Elmer, USA.

Bowen, H.J.M. (1979) *Environmental Chemistry of the Elements*. London, United Kingdom: Academic Press.

Brown, D.H., and Brown, R.M. (1991) Mineral cycling and lichens: the physiological basis. *Lichenologist* **23**: 293-307.

Carreras, H.A., and Pignata, M.L. (2001) Comparison among air pollutants, meteorological conditions and some chemical parameters in the transplanted lichen *Usnea amblyoclada*. *Environmental Pollution* **111**: 45 - 52.

Conti, M.E., and Cecchetti, G. (2001) Biological monitoring: lichens as bioindicators of air pollution assessment - a review. *Environmental Pollution* **114**: 471-492.

De Corte, F. (1987) *The k0 - Standardization Method, a Move to the Optimisation of Neutron Activation Analysis*. PhD Thesis, Gent University, Gent, Belgium pp. 1-464.

Erdtmann, G., and Petri, H. (1986) Nuclear activation analysis: fundamentals and techniques, Part 1. In *Treatise on Analytical Chemistry*. Elving, P.J. (ed). USA: John Wiley & Sons Inc., pp. 422-626.

Figueira, R. (2002) *Desenvolvimento de um Sistema de Biomonitorização Ambiental.* PhD Thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal pp. 51-107, in Portuguese..

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000a) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace*

Elements - their Distribution and Effects in the Environment. Markert and Friese (ed). Amsterdam, Netherlands: Elsevier Science, pp. 187-213.

Freitas, M.C., Reis, M.A., Marques, A.P., and Wolterbeek, H.T. (2000b) Dispersion of chemical elements in an industrial environment studied by biomonitoring using *Parmelia sulcata*. *Journal of Radioanalytical and Nuclear Chemistry* **244**: 109-113.

Freitas, M.C., Reis, M.A., Marques, A.P., and Wolterbeek, H.T. (2001) Use of lichen transplants in heavy metal atmospheric deposition studies. *Journal of Radioanalytical and Nuclear Chemistry* **249**: 307-315.

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A., and Orlovsky, L. (2000) Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiology Plantarum.* **109**: 410-418.

Garty, J. (2001) Biomonitoring atmospheric heavy metals with lichens: theory and application. *Critical Reviews in Plant Sciences* **20**: 309-371.

Garty, J., Cohen, Y., and Kloog, N. (1998) Airborne elements, cell membranes, and chlorophyll in transplanted lichens. *Journal of Environmental Quality* **27**: 973-979.

Garty, J., Weissman, L., Cohen, Y., Karnieli, A., and Orlovsky, L. (2001) Transplanted lichens in and around the Mount Carmel National Park and the Haifa Bay industrial region in Israel: physiological and chemical responses. *Environmental Research A* **85**: 159-176.

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A., and Orlovsky, L. (2000) Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiologia. Plantarum.* **109**: 410-418.

Godinho, R.M., Freitas, M.C., and Wolterbeek, H.T. (2004) Assessment of lichen vitality during a transplantation experiment to a polluted site. *Journal of Atmospheric Chemistry* **49**: 355-361.

Godinho, R.M., Wolterbeek , H.T., Verburg, T.G., and Freitas, M.C. (2008) Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. *Environmental Pollution* **151, (**2), 318-325.

Haffner, E., Lomsky, B., Hynek, V., Hallgren, J., Batic, F., and Pfanz, H. (2001) Air pollution and lichen physiology - physiological responses of different lichens in a transplant experiment following an SO_2 - gradient. *Water Air and Soil Pollution* **131**: 185-201.

Henry, R.C. (1991) Multivariate receptor models. In *Receptor Modelling for Air Quality Management*. Hopke, P.K. (ed). Amsterdam, Netherlands: Elsevier Science, pp. 117-147.

INE (2001) Censos 2001. Lisboa, Portugal: *Instituto Nacional de Estatistica*.

Jacquiot, L., and Daillant, O. (2002) Bio-accumulation of heavy metals, trace elements and radioelements, review-part III. Mazille, France: *Bulletin de l'Observatoire Mycologique*, pp. 1-33.

Johansson, S.A.E., Campbell, J.L., and Malmqvist, K.G. (1995) *Particle Induced X-ray Emission Spectrometry (PIXE) in Chemical Analysis*. USA: John Wiley & Sons.

Kranner, I. (2002) Glutathione status correlates with different degrees of desiccation tolerance in three lichens. *New Phytologist* **154**: 451-460.

Kuik, P., and Wolterbeek, H.T. (1995) Factor analysis of atmospheric trace-element deposition data in the Netherlands obtained by moss monitoring. *Water, Air and Soil Pollution* **83**: 323-346.

Kuik, P., Sloof, J.E., and Wolterbeek, H.T. (1993a) Application of Monte Carlo-assisted Factor Analysis to large sets of environmental pollution data. *Atmospheric Environment* **13**: 1975-1983.

Kuik, P., Blaauw, M., Sloof, J.E., and Wolterbeek, H.T. (1993b) The use of Monte Carlo methods in factor analysis. *Atmospheric Environment* **13**: 1967-1974.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., and Verburg, T.G. (2004) MCTTFA applied to differential biomonitoring in Sado estuary region. *Journal of Radioanalytical and Nuclear Chemistry* **259**: 35-40.

Montaser, A., Golighty, D. W. (1992) *Inductively Coupled Plasmas in Analytical Atomic Spectrometry.*VCH Publishers: New York, USA.

Mulgrew, A., and Williams, P. (2000) *Biomonitoring of Air Quality using Plants*. Berlin, Germany: WHO Colaborating Centre for Air Quality Management and Air Pollution Control, Federal Environmental Agency, pp. 1-165.

Nash III, T.H. (1996) *Lichen biology*. England: Cambridge University Press.

Nriagu, J.O. (1989) Natural versus anthropogenic emissions of trace metals to the atmosphere. In *Control and Fate of Atmospheric Trace Metals*. J. M. Pacyna and B. Ottar (ed). Amsterdam, Netherlands: Kluwer Academic Publishers, pp. 3-13.

Raton, B. (ed) (1986-1987) *Handbook of Chemistry and Physics*. Florida, USA: CRC Press.

Reis, M.A., Alves, L.C., Freitas, M.C., Van Os, B., and Wolterbeek, H.T. (1999) Lichens (*Parmelia sulcata*) time response model to environmental elemental availability. *The Science of the Total Environment* **232**: 105-115.

Reis, M.A. (2001) *Biomonitoring and Assessment of Atmospheric Trace Elements in Portugal - Methods, Response Modelling and Nuclear Analytical Techniques.* PhD Thesis, Delft University of Technology, Delft, The Netherlands, pp. 1-230.

Reis, M.A., Alves, L.C., and Jesus, A.P. (1996) Matrix effects correction for quantitative TTPIXE analysis. *Nuclear Instruments and Methods in Physiscs Research B* **109/110**: 134-138.

Reis, M.A., Freitas, M.C., C., A.L., Marques, A.P., and Costa, C. (1999) Environmental assessment in an industrial area of Portugal. *Biological Trace Element Research* **71/72**: 471-479.

Seiler, H., Sigel, A., and Sigel, H. (eds) (1994) *Handbook on Metals in Clinical and Analytical Chemistry*. New York: Marcel Dekker Inc.

Wolterbeek, H.T., Garty, J., Reis, M.A., and Freitas, M.C. (2002) Biomonitors in use: lichens and metal air pollution. In *Bioindicators and Biomonitors*. Markert, Breure and Zechmeister (ed). Amsterdam, Netherlands: Elsevier Science, pp. 377-419.

Chapter 4

Transplants set-ups and positioning towards wind direction: element concentrations and relationships with atmospheric element deposition

4.1 Introduction

Biomonitoring studies are often performed through the determination of contaminants in the thalli of *in situ* lichens, but in some areas there are no lichens present due to high pollution levels; in these cases transplanted lichen thalli are used (Conti and Cecchetti 2001). Transplanting is using lichen thalli, which are collected in areas of low pollution level, and exposing them in the area of study. Mostly, transplants are sampled periodically and analysed. In both *in situ* studies and transplant studies the lichen accumulation of contaminants depends on factors such as contaminant availability, characteristics of the specific lichen used (anatomy, physiology), age, health status, temperature, available moisture, substratum characteristics, etc. (Conti and Cecchetti 2001). In many lichen surveys, epiphytic lichens (lichens growing on trees), are used (Jacquiot and Daillant 2002).

Under natural conditions epiphytic lichens are either distributed all around the circumference of tree stems or branches, or distributed preferentially in specific positions. They may or may not be shielded by stems or branches from wind or rain; they may grow horizontally, vertically or in any other positioning (Fig.4.1). In epiphytic lichen studies, some investigators collect only from one tree species, others from numerous tree species within a specified range. Some investigators specify collection height of lichens from trees e.g. 1.5 to 2 metres above ground. Regardless of this variability, or to rule out any specific influences, sampling is generally from all around the tree, although some investigators prefer other methodologies (Mulgrew and Williams 2000). Some samplers specify composite lichen volumes while some do not. With lichen transplants, the material is generally positioned without any pre-set fixed position (Bargagli 1998). Thus, in both cases, positioning of the lichen is not usually taken into account as a variable of importance.

However, results from a lichen transplant study on trace-element air pollution in Portugal (Reis 2001) suggested that both the positioning of the transplants towards the wind direction and the rate of precipitation were relevant factors in eventual data interpretation. In later Portuguese studies, the transplants were fixed towards the wind direction, and vertically covered (Reis, Freitas et al. 1999; Freitas, Reis et al. 2000; Freitas, Reis et al. 2001; Marques, Freitas et al. 2004a; Marques, Freitas et al. 2004b). The studies, however, did not give any clue as to what extent these measures affected the eventual results obtained.

Therefore, the present chapter focuses on transplant positioning in a comparative set-up of accumulation experiments. The study comprises three transplant-positioning approaches; free, horizontal covering, and vertical covering (newly introduced transplant positioning system). Furthermore, in a number of these set-ups, wind directional positioning (facing or shielded) was addressed. The results, which are considered as of importance for both transplant- and *in situ* approaches in surveys, should give further insights in the necessary protocols for lichen biomonitoring of trace-element air pollution.

Fig. 4.1 – Epiphytic lichens under natural conditions. Pictures were taken in Sintra and Mafra region.

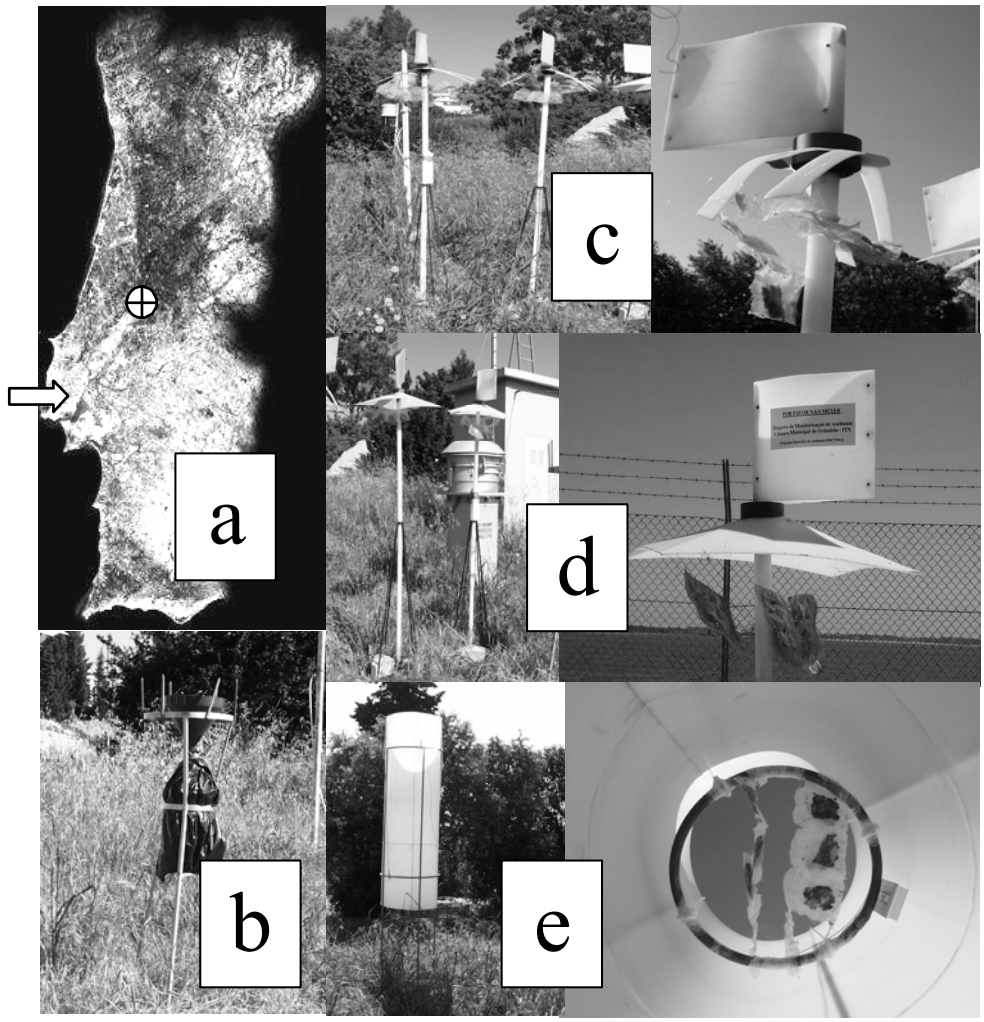

Fig. 4.2 - Geographical map of Portugal with collection site (⊕ - Fátima) and exposure site (⇨ - ITN campus, Sacavém) (a); Water collection (b); Hanging systems: Fi with detail (c), Hi with detail (d) and Vi with detail (e).

4.2 Experimental Section

4.2.1 Exposition set-ups and sampling

Parmelia sulcata was collected from a region of Portugal considered clean from the point of view of air pollution (Freitas, Reis et al. 2000a) (Fig. 4.2a). Preparation of the transplants followed previously reported procedures (Freitas, Catarino et al. 1993). Nine transplants were separated for determination of the 0-month exposure level functioning as reference levels (RL). A total of 50 transplants (of about 1 g each) were vertically positioned in a polluted area (Almeida 2004), at ITN campus on February 2001 in three different exposure systems. The transplants were exposed at a fixed height of 1.5 m above the soil. Fig. 4.2 shows the three systems: free influx (Fi), horizontal influx (Hi) and vertical influx (Vi). The Fi system (Fig. 4.2c) is allowing free influx, the Hi system (Fig. 4.2d) has a cover shielding transplants from direct vertical deposition and the Vi system (Fig. 4.2e) consists of a vertical white polyethylene tube (0.5 m diameter, 1.5 m high and 3 mm thick) placed over a metallic support to prevent any direct lateral element deposition on the lichen transplant. Both Fi and Hi systems rotate to be in line with the wind direction. Two Fi and two Hi systems were put in parallel, each having ten transplants: five facing (F) the wind (Fi_{F1}, Fi_{F2} on Fi system and Hi_{F1}, Hi_{F2} on Hi system) and five shielded (S) from the wind (Fi_{S1},Fi_{S2} on Fi system and Hi_{S1},Hi_{S2} on Hi system). Within the Vi set-up, two transplant sets (Vi_{1} and Vi_{2}) were put inside the tube, with five transplants each. For notations explanation, see Table 4.1.

4.2.2 Vi set-up characteristics

4.2.2.1 Light

The light was measured inside and outside the Vi hanging system before lichen transplants exposure, with a digital illuminance meter (TES–1330), having a spectral sensitivity of 400-700 nm and a range of 20–200 000 Lux. Measurements were made under various weather conditions (sunny, cloudy and rainy).

Table 4.1 - Transplants exposed at ITN campus from February till the beginning of July 2001 (5 months exposure) in three different hanging systems.

Hanging systems
$Fi_{\_F1}$ (free influx, facing the wind 1)
$Fi_{\_S1}$ (free influx, shielded from the wind 1)
$Fi_{\_F2}$ (free influx, facing the wind 2)
$Fi_{\_S2}$ (free influx, shielded from the wind 2)
$Hi_{\_F1}$ (horizontal influx, facing the wind 1)
$Hi_{\_S1}$ (horizontal influx, shielded from the wind 1)
$Hi_{\_F2}$ (horizontal influx, facing the wind 2)
$Hi_{\_S2}$ (horizontal influx, shielded from the wind 2)
$Vi_{\_1}$ (vertical influx 1)
$Vi_{\_2}$ (vertical influx 2)

4.2.2.2 Turbulence

The air turbulence, possibly generated within the Vi set-up by chimney-effects, was evaluated before lichen transplants exposure with an air-velocity meter (KM4007), calibrated in $m.s^{-1}$. The maximum operating air velocity was 30 $m.s^{-1}$.

4.2.2.3 Temperature

Thermometers indicating minimum and maximum temperatures were positioned within the Fi, Hi, and Vi hanging systems. The temperature was measured on a week basis.

4.2.2.4 Total element deposition

Total element deposition (Fig.4.2b) was monthly collected using a 25 cm diameter funnel on top of a 10 L polyethylene bucket. The bucket was covered with a non-transparent (black) plastic bag to avoid light interference. After collection the water volume was determined and all samples have passed through the 125 μm nylon net to retain possible small floating insects. Samples were acidified with 0.5 ml 67% HNO_3 for each 1 L of water collected, to maintain pH lower than 2, thereby avoiding formation of organic material. The samples were then frozen.

4.2.2.5 Transplant sample preparation and analyses

Samples were collected on a monthly basis and cleaned by rinsing with distilled water. They were freeze-dried and ground in a Teflon ball mill for 10 min, which together with the sample had been immersed before in liquid nitrogen for 2 minutes Element contents were determined by Instrumental Neutron Activation Analysis (INAA, k_0–standardisation, De Corte, 1987) and Particle Induced X-ray Emission (PIXE, Johansson, Campbell et al., 1995). INAA analysis was carried out using pellets of 500 mg irradiated at the Portuguese Research Reactor (RPI) together with 0.1% Au-Al foil (IRMM-530R) as comparators and a high-purity germanium detector for gamma spectra determination. Concentrations were obtained by the k_0-factor method. PIXE analysis was made using a pellet of a thin layer of lichen powder in a boric acid support. Samples were irradiated in ITN Van de Graaff accelerator. The X-ray spectra were obtained with a Si(Li) detector. For both techniques quality control was pursued by analysing the IAEA-336 lichen material.

4.2.2.6 Water sample preparation and analyses

The collected water samples were analysed by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES), (Montaser and Golighty 1992, Boss and Fredeen 2004), using a Perkin Elmer As-93Plus Autosampler (Perkin-Elmer, Shelton CT, USA), and, where necessary, a Perkin Elmer Ultrasonic Nebulizer (USN 6000+). The instrumental conditions and operational parameters settings have been based upon the optical Signal-To-Background (SBR) of the Mn II 257.610 nm line, as the best single representative of the ICP-OES emission lines. A

multi element solution containing 10.0 mg.L-1 Ba and 1.0 mg.L-1 Mg and Zn was used to optimize axially and radially viewed configurations and to obtain maximal signal-to-background ratios (SBR). Analytical calibration curves for all elements to be determined were used to assess the precision and accuracy of measurements performed in both axially and radially viewed configurations, as well as in Ultra Sonic Nebulizer analysis. Ten measurements per wavelength were made at a flow rate of 1.50 $mL.min^{-1}$. The analyses were performed at the Interfaculty Reactor Institute at Delft, The Netherlands.

4.3 Results and discussion

4.3.1 Vi set-up characteristics, relative to Fi and Hi set-ups

The Vi set-up was considered a set-up for which additional measurements were necessary to characterise this set-up relative to the Fi and Hi ones, for factors thought relevant for the general performance of the transplant lichens.

4.3.1.1 Light

The results (Table 4.2) showed a 50% reduction in illuminance inside the tube. It should be noted here that photosynthesis in plant leaves is powered by 1% of the sunlight that falls on the plant, 10% of the light is reflected, 10% passes through the leaf and about 80% is used for transpiration. Some of the light is re-radiated, while the fraction that remains is used for synthesising organics from CO_2, minerals and water (www.biocontrols.com). Because the photobionts comprise only 5-10% of the total biomass of many lichens, photosynthetic rates are necessarily lower than those of leaves (Nash III 1996). The light reaching the photobiont is reduced when compared with superficial light measurements by 54-79% when dry and 24-54% when saturated; lichens also tend to adjust photosynthetically when shifted to low light conditions (Nash III 1996). Based on the above, the reduced illuminance was assumed as not significantly affecting lichen photosynthetic rates in the Vi system, relative to the other systems.

Table 4.2 - Light measured inside and outside the Vi hanging system before lichen transplants exposure.

Outside (Lux × 10^2)	**Inside (Lux × 10^2)**
33	13
45	22
75	30
106	50
116	52
124	104
175	81
219	103
223	111
247	116
253	105
271	138
273	100
291	153
292	120
296	147
307	170
332	148
335	156
625	304
654	323
673	325
706	342
727	345
774	361

4.3.1.2 Turbulence

Results (Table 4.3) indicated that additional turbulence was negligible in the middle of the tube: transplants were put at that position within the tube, and the total tube position was adjusted to assure transplant positioning at a height of 1.5 m above soil level.

Table 4.3 - Turbulence measured inside the Vi hanging system before lichen transplants exposure at different heights.

Height (M)	Measurements (M/s)
2	0.4-0.5
1.8	0.3-0.4
1.6	0.1-0.2
1.4	0.0-0.1
1.2	0.0-0.1
1.0	0.0-0.1
0.8	0.0-0.1
0.6	0.0-0.1
0.4	0.0-0.1
0.2	0.1-0.2

4.3.1.3 Temperature

Minimum and maximum temperatures were recorded for each exposure system. Lichens are assumed as generally well adapted to the temperatures experienced in their particular microhabitats. However, if exposed to temperatures that are not within their operational temperature range, some species do exhibit adverse responses (Nash III 1996). Marques et al. (Marques, Freitas et al. 2005) have suggested from conductivity measurements that *Parmelia sulcata* may show some membrane damage when exposed to higher temperatures. Fig. 4.3 shows the minimum and maximum temperatures measured during the exposure period. Although for the Vi system higher minimum temperatures and lower maximum temperatures are recorded than for the two other systems, overall temperatures were regarded as similar for the three Fi, Hi and Vi systems: as such, all transplants were considered as subjected to similar temperature conditions.

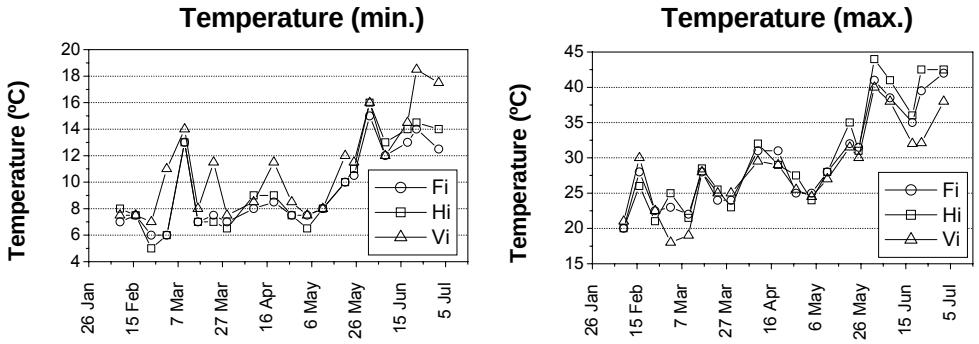

Fig. 4.3 - Maximum and minimum temperatures observed from 9th February till 2nd July 2001 (local measurements).

4.3.2 Lichen element content compared to the reference level (RL)

Student t-tests (GraphPad Software 1999, Weiss 1999, Woolson and Clark 2002) were used to test the significance ($P < 0.05$) of differences between the lichen element data from the three hanging systems and the initial levels, serving as reference levels (RL), all irrespective of wind-directional positioning. Figures 4.4 to 4.9 show the average element contents, with associated standard deviation, obtained in each hanging system and the probability values (presented only $P < 0.05$). The results indicate that transplant element contents should be considered as not significantly different from RL values for the full 5-month exposure period for Cs, Hf, K, P, Rb, Se, Si, and Sr (Fi system), Cr, Cs, Fe, Hf, La, P, Rb, Sc, Si, Sm, Sr, Th, and Zr (Hi system), and Al, As, Cr, Cs, Fe, K, Mg, Sb, Sc, Si, Sm, Sr, Ti, and Zn (Vi system). Differences from RL values were obtained for the full exposure period (starting with the first observation after a one-month exposure), for Co, Mn, Na, Ni, Pb, Sb, V, and Zn (Fi system), Mn and Na (Hi system); while for the Vi system there were no elements showing differences from RL already from the first one-month period onwards. Time-related progressive increases, with significance of the differences from RL, were observed for the elements As, Cr, Cu, Fe, Ti, and S (Fi system), Cl, Ni, S, Sb, and Cu (Hi system), and Cu and Zr (Vi system).

The results indicate that in Fi and Hi systems, most of the elements which remained similar to RL values may have a soil-dust origin. For most elements, the Fi system resulted in higher rates of element accumulation with the length of the exposure period (also shown by the highest number of elements different from RL values for Fi).

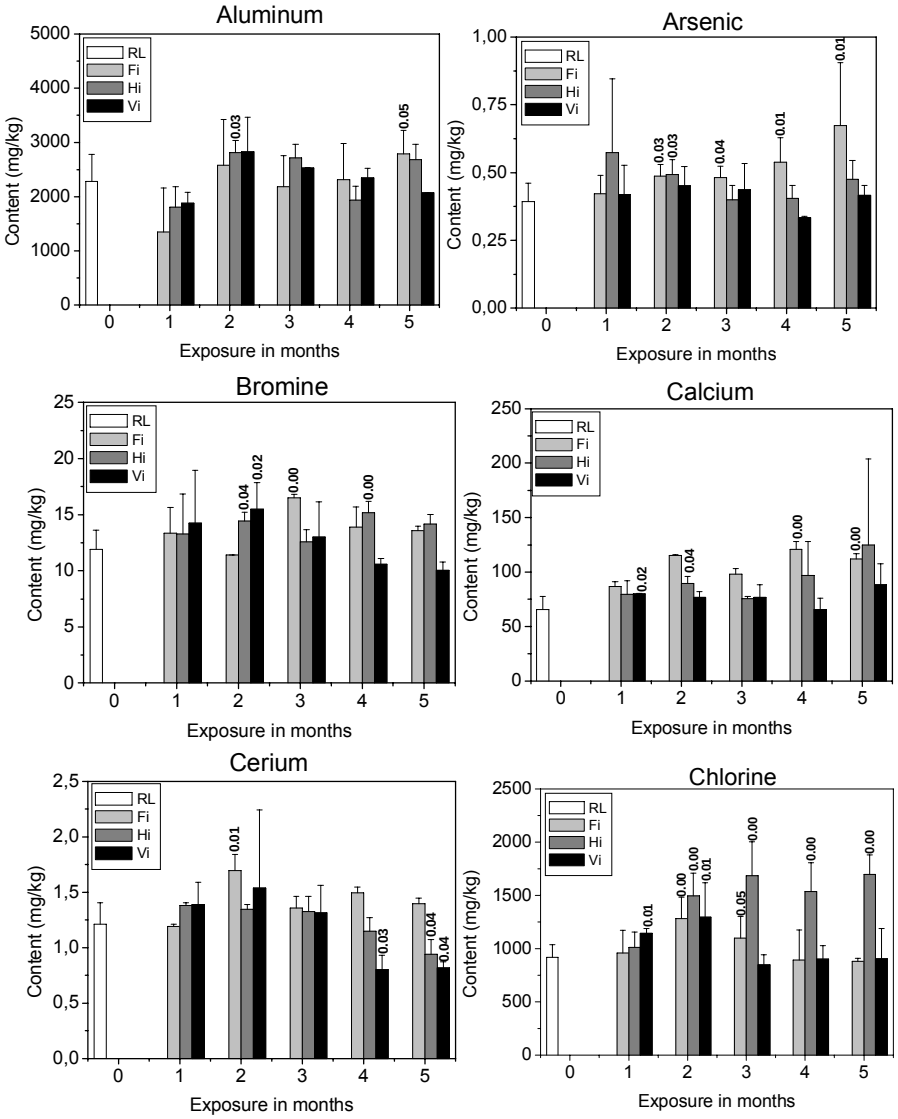

Fig. 4.4 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

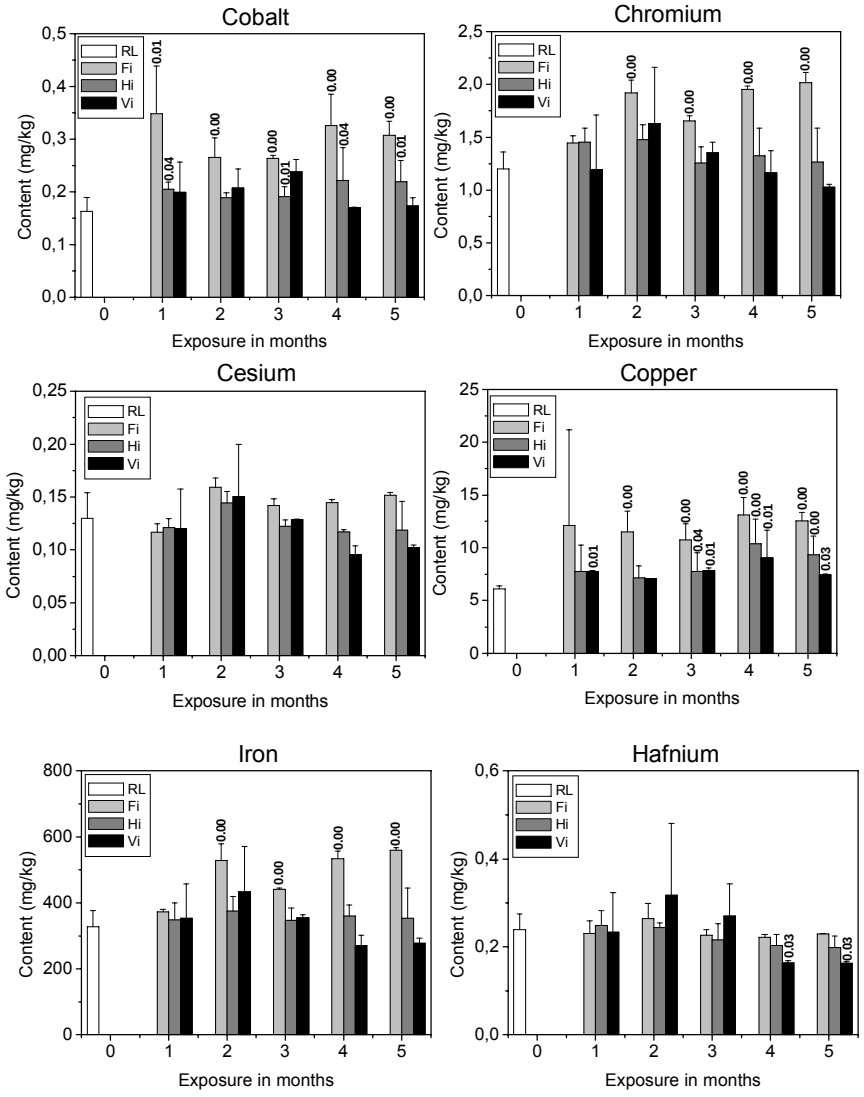

Fig. 4.5 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

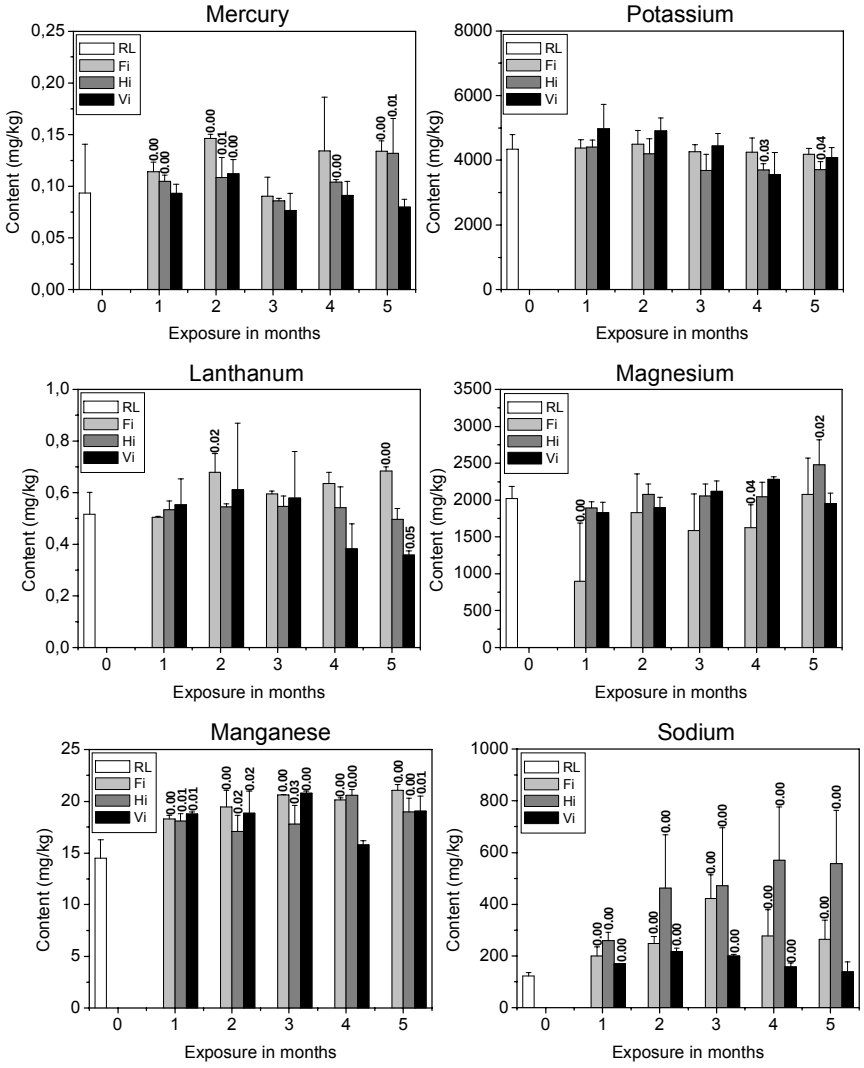

Fig. 4.6 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

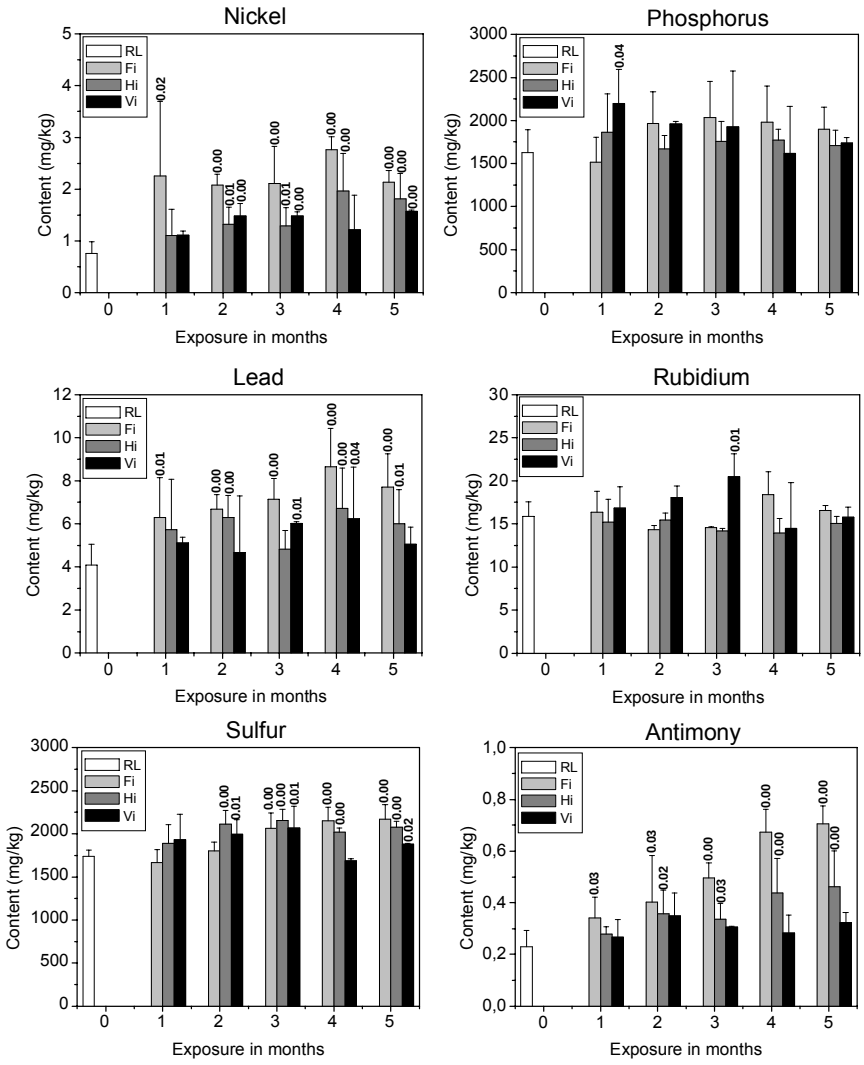

Fig. 4.7 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

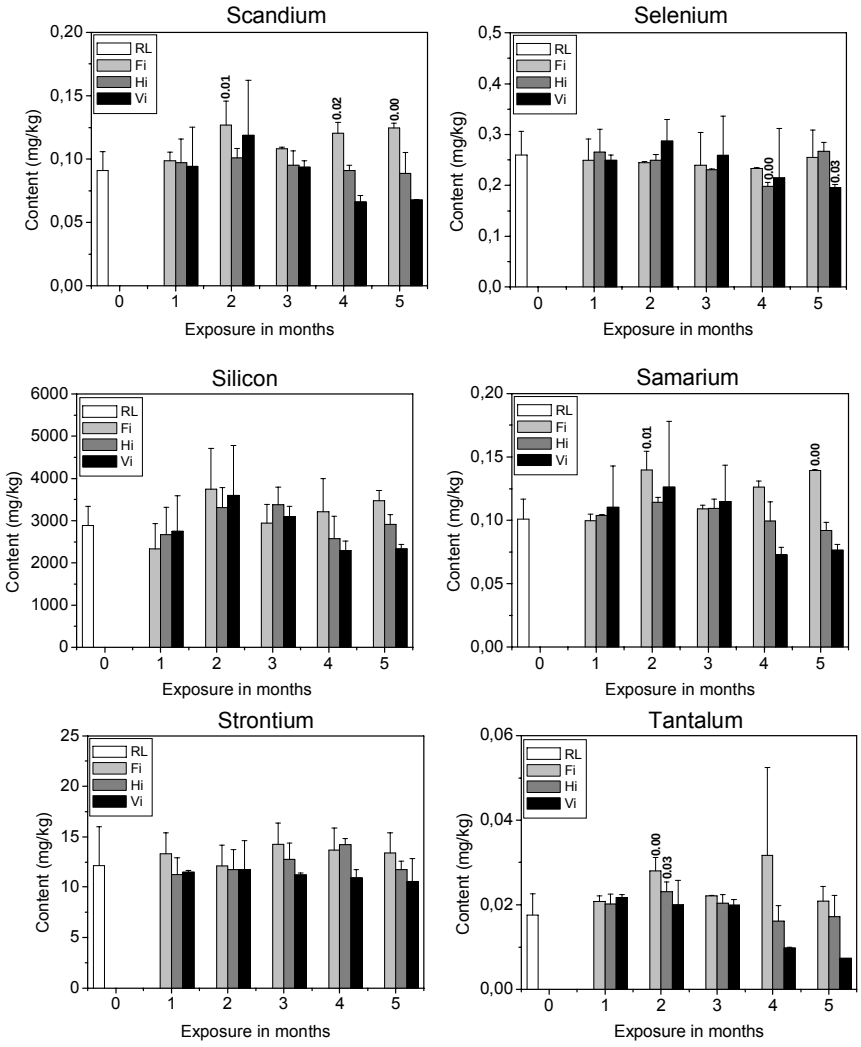

Fig. 4.8 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

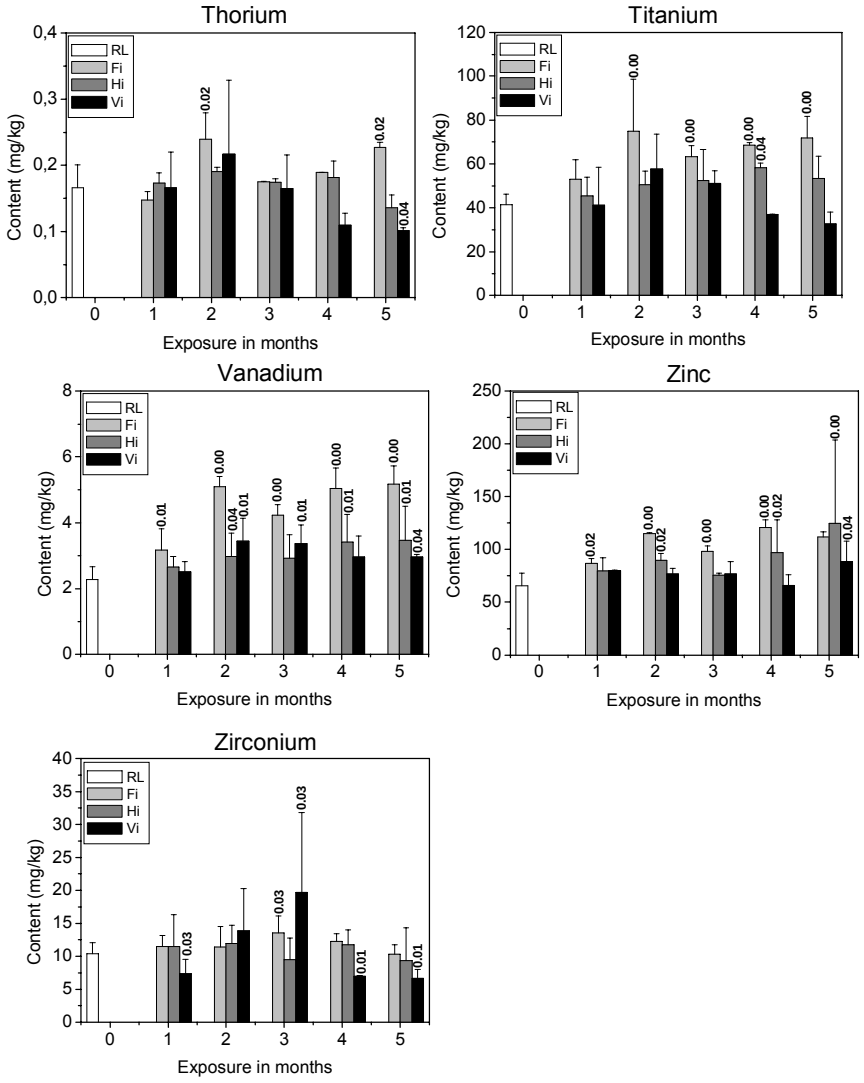

Fig. 4.9 - Average element contents with associated standard deviation obtained in each transplant system; Fi (free influx), Hi (horizontal influx) and Vi (vertical influx). Reference levels (RL) and associated standard deviation (based on nine replicates) are presented. Probability values P (student t-tests) indicate the significance of differences between the lichen element concentrations and initial levels (RL). P <0.05 indicate that differences are significant (95% level).

It should also be noted that variances between replicates (results not shown) were relatively high, and probably associated to intrinsic variable behaviour of biological systems, of the order of 20% (see data on *Parmelia sulcata* in Freitas and Nobre 1997). Finally, the progressive increase in differences from RL suggests that exposure time may be a very relevant issue in comparisons of the transplant systems: transplants within Fi systems may respond faster relative to those within Hi and especially Vi systems.

4.3.3 Lichen element content, precipitation volumes and total element deposition

4.3.3.1 Precipitation

The amount of rain was collected as 70, 90, 8, 31 and 14 $L.m^{-2}$ respectively for the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} month of exposure. In the rainwater samples the elements As, Ce, Co, Cr, Hg, Li, Rb, Sb, Sc, Se, Sm, Th, Ti, and Y were below the limits of detection of ICP-OES. Table 4.4 presents correlation values (R) for Al, Ba, Ca, Cs, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Si, Sr, V, and Zn as calculated for the correlation between precipitation (in $L.m^{-2}$) and total element deposition (in $mg.m^{-2}$).

The data indicate significant (positive) correlations for Fe and Ni only. For Co, Sc and Zn, Sloof (Sloof 1993) also reported the absence of any significant correlation between total element deposition and precipitation volumes; she reasoned that this may be attributed to additional effects from dry deposition and washout side effects from rainfall.

Table 4.4 - Correlation coefficient (R) between precipitation (in $L.m^{-2}$) and total element deposition (in $mg.m^{-2}$). Correlation coefficients R with $P < 0.05$ meet the 95% significance criterion (marked bold).

	Al	Ba	Ca	Cs
R	0.348	0.616	0.188	0.475
P	0.565	0.268	0.762	0.419
	Cu	Fe	K	Mg
R	0.006	**0.930**	0.193	0.871
P	0.993	**0.022**	0.755	0.054
	Mn	Na	Ni	Pb
R	0.834	0.867	**0.950**	0.721
P	0.079	0.057	**0.013**	0.169
	Si	Sr	V	Zn
R	-0.061	0.746	0.646	0.847
P	0.923	0.148	0.239	0.070

4.3.3.2 Total element deposition

Table 4.5 presents data on the correlations between the element concentrations in *Parmelia sulcata* transplants (Fi, Hi and Vi systems) and the total element deposition. Positive and significant correlations ($P < 0.05$) were obtained for Ca, Fe and Mn (Fi system), and for Na, Ni and V (Hi system); for the Vi system no significant correlations were found.

Table 4.5 - Correlation coefficient (R) between lichen element contents (in $mg.kg^{-1}$) exposed in systems Fi, Hi and Vi and cumulative total element deposition (in $mg.m^{-2}$). Correlations coefficients R with $P < 0.05$ meet the 95% significance criterion (marked bold).

Hanging system		Al	Ca	Cs	Cu	Fe
Fi	R	0.720	**0.935**	0.544	0.502	**0.879**
	P	0.171	**0.020**	0.489	0.388	**0.050**
Hi	R	0.146	0.408	-0.451	0.814	0.194
	P	0.815	0.496	0.588	0.094	0.754
Vi	R	-0.028	-0.842	-0.702	0.379	-0.576
	P	0.964	0.073	0.612	0.526	0.309
		K	Mg	Mn	Na	Ni
Fi	R	-0.820	0.840	**0.896**	0.393	0.275
	P	0.089	0.075	**0.039**	0.513	0.657
Hi	R	**-0.888**	0.735	0.592	**0.986**	**0.887**
	P	**0.044**	0.157	0.293	**0.002**	**0.045**
Vi	R	**-0.893**	0.653	-0.307	-0.382	0.571
	P	**0.041**	0.232	0.617	0.525	0.304
		Pb	Si	Sr	V	Zn
Fi	R	0.804	0.356	0.292	0.787	0.821
	P	0.101	0.557	0.634	0.115	0.088
Hi	R	0.239	-0.480	0.554	**0.981**	0.731
	P	0.694	0.413	0.333	**0.003**	0.203
Vi	R	0.364	-0.821	-0.864	0.190	-0.061
	P	0.548	0.089	0.059	0.763	0.922

A strong inverse correlation with cumulative total element deposition was observed for K in the Hi system and Vi system, possibly denoting some membrane damage in the transplants exposed within these systems. The release of K is related to cell membrane damage, which has notable consequences for the loss of electrolytes, particularly K and Mg (Nash III 1996). For the systems used, temperature may be ruled out as in causing losses in lichen vitality (increases in membrane permeability), since all systems experience similar temperatures. Future study may give additional attention to humidity: possibly the systems don't share the same humidity regimes and that can influence the biomonitor response (Figueira 2002). Another noteworthy observation is that the transplants within the Vi system, although none significant, show a number of negative correlations with element deposition (e.g. for Al, Ca, Cs, Fe, Mn, Na, Si, Sr and Zn, that is nine out of the 16 elements considered).

4.3.4 Comparison of transplant element contents

4.3.4.1 Element contents in lichen transplants, exposed in Fi, Hi and Vi systems

Since in the transplant observations throughout the 5-months experiment all samples were taken in duplicate, and sometimes processed in sets of four data (Fi, Hi in both wind-directional positions), a first exercise was performed on statistics. Out of the nine outcomes from RL, repeated and randomised (n=100) trials were taken of 2 or 4 samples each. Outcomes from these trials were compared to full RL data by t-testing, and screened for significant differences. The results indicate the risk of considering duplicate or four-fold observations as different from RL while in fact they belong to the same concentration population. The data (Table 4.6) suggest that this risk is less than 1% for every element considered.

Table 4.6 - Mean values and standard deviation of the time-zero reference lichen material (n=9 samples). The table also indicates the probability P (in %, based on n=100 trials) that a duplo sample (n=2) or a four-fold sample (n=4) is different from the element concentration population. The data indicate the risk that a sample from the experiment is calculated as different from time-zero while in fact it is belonging to the time-zero population. The data indicate that the risk is << 1% for every element considered.

Element	Mean values	Standard deviation	P sub sample	
			n=2	n=4
Al	2136	490	0	0
As	0.393	0.067	0	0
Br	11.90	1.72	0	0
Ca	3609	473	0	0
Ce	1.205	0.194	0	0
Cl	910.5	99.2	0	0
Co	0.162	0.027	0	0
Cr	1.488	0.864	0	0
Cs	0.129	0.024	0	0
Cu	6.115	0.687	0	0
Fe	328.2	48.3	0	0
Hf	0.239	0.036	0	0
Hg	0.094	0.047	0	0
K	4339	449	0	0
La	0.516	0.086	0	0
Mg	2001	231	0	0
Mn	14.43	1.66	0	0
Na	122.5	13.0	0	0
Ni	0.759	0.227	0	0
P	1627	265	0	0
Pb	3.965	0.879	0	0
Rb	15.88	1.72	0	0
S	1740	69	0	0
Sb	0.228	0.063	0	0
Sc	0.0908	0.0149	0	0
Se	0.264	0.047	0	0
Si	2852	539	0	0
Sm	0.101	0.016	0	0
Sr	10.95	3.57	0	0
Ta	0.0176	0.0050	0	0
Th	0.166	0.035	0	0
Ti	42.99	8.21	0	0
V	2.280	0.385	0	0
Zn	65.54	12.07	0	0
Zr	10.55	1.45	0	0

T-tests were also used throughout the total experiment period, to judge the comparability of the monthly results obtained with the three set-up systems Fi, Hi and Vi. To do this, monthly comparisons were made for Fi-Hi, Fi-Vi and Hi-Vi combinations. Here it should be noted that Fi and Hi were included in the analysis irrespective of wind-directional positioning: comparisons were on system level only. Results (Table 4.7) indicate that no significant differences (95% significance level) were observed for P and Ta, a single-case significant difference was indicated for Al, K, Hf, Hg, Pb, Rb, Se, and Sr and two-case significant differences were suggested for As, Ca, Cs, Mg, Mn, Sm, and Zn. It should also be noted, that the differences, if occurring, were mostly in later months (except for the Mg case). Furthermore, differences between systems progressively developed with exposure time: differences persisting for at least the last three months of exposure were observed between Fi and Hi for Cl, Cr, Fe, Sb, and V, between Fi and Vi for Cr, Fe, and Sb, and between Hi and Vi systems for Cl.

Table 4.7 - Probability values obtained by the application of Student t-tests for lichen transplants exposed on Fi (free influx), Hi (horizontal influx) and Vi (vertical influx) systems. Results lower than 0.05 are marked bold and should be considered non-equal.

Element	system	1 month	2 months	3 months	4 months	5 months
Al	Fi with Hi	0.346	0.611	0.139	0.335	0.710
	Fi with Vi	0.440	0.737	0.461	0.950	0.093
	Hi with Vi	0.827	0.966	0.383	0.116	**0.043**
As	Fi with Hi	0.324	0.872	0.052	**0.039**	0.152
	Fi with Vi	0.951	0.459	0.439	**0.038**	0.214
	Hi with Vi	0.501	0.462	0.554	0.123	0.347
Br	Fi with Hi	0.979	0.071	**0.017**	0.542	0.742
	Fi with Vi	0.788	**0.025**	0.108	0.299	0.062
	Hi with Vi	0.769	0.662	0.838	**0.016**	0.132
Ca	Fi with Hi	0.598	0.788	0.645	0.760	0.095
	Fi with Vi	0.162	0.671	0.667	**0.002**	**0.026**
	Hi with Vi	0.109	0.824	0.407	0.311	0.606
Ce	Fi with Hi	0.370	0.119	0.798	0.084	**0.001**
	Fi with Vi	0.455	0.710	0.778	**0.028**	**0.001**
	Hi with Vi	0.981	0.604	0.931	0.101	0.283
Cl	Fi with Hi	0.680	0.195	**0.021**	**0.016**	**0.000**
	Fi with Vi	0.318	0.951	0.185	0.971	0.865
	Hi with Vi	0.301	0.398	**0.027**	**0.038**	**0.012**
Co	Fi with Hi	0.136	**0.035**	**0.033**	0.069	**0.008**
	Fi with Vi	0.297	0.208	0.440	**0.033**	**0.002**
	Hi with Vi	0.891	0.548	0.185	0.371	0.195
Cr	Fi with Hi	0.960	0.113	**0.017**	**0.026**	**0.011**
	Fi with Vi	0.540	0.461	**0.017**	**0.027**	**0.004**
	Hi with Vi	0.410	0.664	0.620	0.554	0.408
Cs	Fi with Hi	0.799	0.288	**0.035**	0.208	0.078
	Fi with Vi	0.890	0.758	0.110	0.137	**0.005**
	Hi with Vi	0.977	0.808	0.509	0.198	0.495
Cu	Fi with Hi	0.392	**0.008**	**0.042**	0.107	**0.016**
	Fi with Vi	0.556	**0.037**	0.062	0.075	**0.001**
	Hi with Vi	0.991	0.919	0.961	0.557	0.219
Fe	Fi with Hi	0.689	**0.036**	**0.014**	**0.019**	**0.002**
	Fi with Vi	0.828	0.336	**0.002**	**0.010**	**0.000**
	Hi with Vi	0.947	0.515	0.859	0.219	0.253
Hf	Fi with Hi	0.729	0.586	0.696	0.361	0.213
	Fi with Vi	0.970	0.535	0.308	0.051	**0.033**
	Hi with Vi	0.812	0.421	0.286	0.078	0.243
Hg	Fi with Hi	0.525	0.099	0.769	0.503	0.951
	Fi with Vi	0.292	0.229	0.482	0.534	**0.006**
	Hi with Vi	0.394	0.850	0.567	0.251	0.257
K	Fi with Hi	0.859	0.371	0.081	0.064	**0.023**
	Fi with Vi	0.188	0.322	0.498	0.195	0.634
	Hi with Vi	0.192	0.146	0.143	0.687	0.179

Element	system	1 month	2 months	3 months	4 months	5 months
La	Fi with Hi	0.735	0.105	0.246	0.246	**0.000**
	Fi with Vi	0.674	0.650	0.866	0.068	**0.000**
	Hi with Vi	0.821	0.644	0.720	0.092	**0.014**
Mg	Fi with Hi	**0.046**	0.391	0.125	0.063	0.229
	Fi with Vi	0.194	0.870	0.233	**0.048**	0.749
	Hi with Vi	0.486	0.209	0.664	0.184	0.117
Mn	Fi with Hi	0.859	0.165	0.158	0.682	0.196
	Fi with Vi	0.696	0.811	0.909	**0.009**	0.399
	Hi with Vi	0.691	0.323	0.246	**0.023**	0.912
Na	Fi with Hi	0.052	0.084	0.687	**0.043**	**0.037**
	Fi with Vi	0.317	0.221	**0.033**	0.198	0.098
	Hi with Vi	**0.024**	0.187	0.177	0.056	0.055
Ni	Fi with Hi	0.181	**0.009**	0.086	0.082	0.284
	Fi with Vi	0.347	**0.034**	0.307	**0.011**	**0.030**
	Hi with Vi	0.991	0,582	0.516	0.288	0.547
P	Fi with Hi	0.236	0.192	0.293	0.371	0.273
	Fi with Vi	0.070	0.988	0.817	0.405	0.458
	Hi with Vi	0.426	0.066	0.628	0.581	0.841
Pb	Fi with Hi	0.718	0.565	**0.012**	0.183	0.175
	Fi with Vi	0.441	0.184	0.197	0.224	0.092
	Hi with Vi	0.739	0.297	0.142	0.793	0.487
Rb	Fi with Hi	0.630	0.475	0.846	0.052	0.405
	Fi with Vi	0.869	0.137	0.117	0.282	0.537
	Hi with Vi	0.601	0.100	**0.008**	0.855	0.770
S	Fi with Hi	0.141	**0.015**	0.435	0.166	0.340
	Fi with Vi	0.189	0.140	0.995	**0.018**	0.083
	Hi with Vi	0.846	0.429	0.567	**0.001**	**0.018**
Sb	Fi with Hi	0.196	0.667	**0.009**	**0.027**	**0.020**
	Fi with Vi	0.341	0.726	**0.011**	**0.006**	**0.002**
	Hi with Vi	0.791	0.934	0.547	0.218	0.257
Sc	Fi with Hi	0.926	0.146	0.160	0.083	**0.006**
	Fi with Vi	0.868	0.773	0.113	**0.027**	**0.001**
	Hi with Vi	0.905	0.494	0.893	0.159	0.139
Se	Fi with Hi	0.658	0.873	0.836	**0.012**	0.840
	Fi with Vi	0.999	0.103	0.764	0.699	0.405
	Hi with Vi	0.733	0.427	0.546	0.700	0.255
Si	Fi with Hi	0.470	0.449	0.208	0.223	**0.017**
	Fi with Vi	0.515	0.880	0.677	0.197	**0.004**
	Hi with Vi	0.909	0.664	0.454	0.539	**0.031**
Sm	Fi with Hi	0.796	0.142	0.960	0.147	**0.000**
	Fi with Vi	0.668	0.655	0.688	0.055	**0.000**
	Hi with Vi	0,765	0.679	0.713	0.125	0.126
Sr	Fi with Hi	0.179	0.812	0.304	0.666	0.181
	Fi with Vi	0.321	0.861	0.131	0.172	0.190
	Hi with Vi	0.846	0.993	0.288	**0.005**	0.370

Element	system	1 month	2 months	3 months	4 months	5 months
Ta	Fi with Hi	0.888	0.100	0.549	0.263	0.464
	Fi with Vi	0.819	0.113	0.464	0.222	–
	Hi with Vi	0.785	0.419	0.869	0.259	–
Th	Fi with Hi	0.539	0.175	0.947	0.793	**0.001**
	Fi with Vi	0.744	0.732	0.672	0.071	**0.001**
	Hi with Vi	0.873	0.666	0.727	**0.038**	0.118
Ti	Fi with Hi	0.276	0.095	0.201	0.332	**0.041**
	Fi with Vi	0.310	0.419	0.056	**0.046**	**0.008**
	Hi with Vi	0.686	0.438	0.905	0.119	0.058
V	Fi with Hi	0.201	**0.002**	**0.015**	**0.020**	**0.027**
	Fi with Vi	0.256	**0.013**	0.063	**0.019**	**0.006**
	Hi with Vi	0.606	0,487	0.497	0.538	0.558
Zn	Fi with Hi	0.581	0.060	0.142	0.163	0.736
	Fi with Vi	0.616	**0.013**	0.154	**0.002**	0.109
	Hi with Vi	0.993	0.413	0.957	0.232	0.541
Zr	Fi with Hi	0.995	0.809	0.102	0.678	0.716
	Fi with Vi	0.096	0.537	0.334	**0.003**	**0.036**
	Hi with Vi	0.330	0.611	0.154	**0.049**	0.513

4.3.4.2 Time- relations: comparisons between Fi, Hi and Vi systems

Apart from the results discussed in section 4.3.4.1., which were based on monthly comparisons between systems, the data were also processed in terms of full time relations. For this, the three systems were compared over the total exposure period, by comparing slopes in time of the changing element concentrations in the transplants (see Section 4.3.2 and Figs 4.4 to 4.9 also for direct time-curves).

First all elemental contents were normalized to the reference values. The systems were then compared by using y = a*x + b relationships with y and x as the two compared systems in time (for instance slope between Fi-Hi means that Fi=a*Hi+b). T-tests were applied to test both the significance of the regressions and the significance of the differences between slopes. Table 4.8 shows slopes (a values) obtained together with the standard errors, for all elements considered. The data indicate strong variability, and range from –2.15 (Sr in a Hi-Vi comparison) to 2.54 (Cr in a Fi-Hi comparison): they suggest both net accumulation and release.

Table 4.8 - Results for slopes *a* (and associated standard error = SE) in comparisons between the three different hanging systems Fi (free influx), Hi (horizontal influx) and Vi (vertical influx) during the 5 months of exposure for 35 elements. The notation is y = a*x + b, with e.g. Fi-Hi as y and x respectively.

	slope	SE	Slope	SE	slope	SE	Slope	SE	Slope	SE
	Al		As		Br		Ca		Ce	
Fi – Hi	1.14	0.45	0.48	0.54	0.33	1.10	0.90	0.25	0.36	0.82
Fi – Vi	0.75	0.73	-0.43	1.17	-0.28	0.42	-0.46	0.34	0.05	0.31
Hi - Vi	0.42	0.56	0.42	1.12	-0.24	0.18	-0.53	0.30	0.36	0.04
	Cl		Co		Cr		Cs		Cu	
Fi – Hi	0.36	0.43	-0.09	0.66	2.54	0.39	0.85	0.48	0.23	0.31
Fi – Vi	0.56	0.39	-0.84	0.61	-0.04	0.60	0.15	0.42	0.66	0.58
Hi - Vi	-0.37	0.58	-0.45	0.63	0.03	0.23	0.47	0.24	0.95	1.06
	Fe		Hg		Hf		K		La	
Fi – Hi	1.74	0.35	0.75	0.52	0.62	0.24	0.48	0.09	2.15	0.63
Fi – Vi	-0.34	0.64	1.05	0.67	0.19	0.10	0.16	0.08	-0.23	0.34
Hi - Vi	-0.04	0.36	0.48	0.72	0.29	0.10	0.37	0.12	-0.01	0.15
	Mg		Mn		Na		Ni		P	
Fi – Hi	0.77	0.89	0.48	0.23	0.51	0.26	0.29	0.33	0.74	0.86
Fi – Vi	0.60	1.34	0.08	0.34	0.79	1.46	-0.90	0.65	-0.66	0.39
Hi - Vi	-0.67	0.71	-0.12	0.55	-0.35	2.24	0.90	1.20	0.08	0.32
	Pb		Rb		S		Sb		Sc	
Fi – Hi	0.77	0.05	0.91	0.97	1.42	0.64	1.19	0.18	1.08	0.85
Fi – Vi	0.90	0.59	-0.64	0.19	-0.62	0.82	0.41	2.88	-0.05	0.31
Hi - Vi	1.02	0.83	-0.15	0.20	0.21	0.48	1.30	2.23	0.20	0.13
	Se		Si		Sm		Sr		Ta	
Fi – Hi	0.32	0.04	0.90	0.62	1.47	0.67	0.37	0.13	1.01	0.23
Fi – Vi	-0.04	0.13	0.23	0.55	-0.20	0.41	-0.85	0.80	-0.17	0.41
Hi - Vi	-0.02	0.41	0.50	0.28	0.15	0.21	-2.15	1.76	0.00	0.39
	Th		Ti		V		Zn		Zr	
Fi – Hi	1.76	0.71	0.77	0.49	1.21	0.14	1.01	0.25	0.57	0.43
Fi – Vi	0.07	0.46	0.13	0.48	1.36	1.05	-0.57	0.92	0.14	0.09
Hi - Vi	0.24	0.16	0.10	0.42	1.17	0.82	-0.22	0.88	0.10	0.11

Table 4.9 gives p-values for both regression and slopes: at $P < 0.05$, regression was significant and slopes were significantly different from unit value (95% probability level). Based on significant regression and slope data only, Tables 4.8 and 4.9 suggest that Fi shows higher accumulation of Cr, Fe, La, and V than Hi; Hi shows higher accumulation of K, Pb, and Se than Fi; no differences are present between Fi and Hi systems for Ca, Sb, Ta, and Z; Vi releases Rb, and shows higher accumulation of Ce than Hi.

Of the 35 elements and consequently the 105 possible combinations, only 13 elements show significant regressions (Table 4.9), indicating that the systems present comparable time-trends for that element. It should be noted also that, although the majority of the regressions are not significant, Table 4.8 shows negative slope values for a number of elements. Most of the negatives were related to Vi systems. For several elements, Vi may thus be interpreted as resulting in (increased) releases of element content rather than in accumulation.

Table 4.9 - P values for the system comparisons in element accumulation. Approaches are given on table 4.8, for Fi (free influx), Hi (horizontal influx) and Vi (vertical influx) for 35 elements. Pslope < 0.05 indicate that slopes ≠ unit value (95% level, marked bold). Pregression < 0.05 indicate significant regressions (95 % level, marked bold)

	P_{slope}	P_{regr}	P_{slope}	P_{regr}	P_{slope}	P_{regr}	P_{slope}	P_{regr}	P_{slope}	P_{regr}	P_{slope}	P_{regr}	P_{slope}	P_{regr}
	Al		As		Br		Ca		Ce		Cl		Co	
Fi - Hi	0.530	0.087	0.097	0.437	0.248	0.783	0.431	**0.035**	0.158	0.691	**0.028**	0.466	**0.020**	0.895
Fi - Vi	0.481	0.380	0.052	0.736	**0.002**	0.545	**0.001**	0.276	**0.002**	0.884	0.065	0.248	**0.002**	0.260
Hi - Vi	0.083	0.506	0.310	0.733	**0.000**	0.281	**0.000**	0.177	**0.000**	**0.003**	**0.006**	0.571	**0.007**	0.529
	Cr		Cs		Cu		Fe		Hf		Hg		K	
Fi - Hi	**0.001**	**0.007**	0.519	0.176	**0.005**	0.504	**0.009**	**0.015**	**0.023**	0.080	0.346	0.249	**0.000**	**0.012**
Fi - Vi	**0.018**	0.952	**0.010**	0.747	0.261	0.340	**0.010**	0.637	**0.000**	0.139	0.867	0.214	**0.000**	0.132
Hi - Vi	**0.001**	0.916	**0.007**	0.144	0.918	0.437	**0.003**	0.910	**0.000**	0.063	0.181	0.549	**0.000**	0.055
	La		Mg		Mn		Na		Ni		P		Pb	
Fi - Hi	**0.015**	**0.042**	0.588	0.451	**0.007**	0.123	**0.013**	0.145	**0.008**	0.442	0.545	0.452	**0.000**	**0.001**
Fi - Vi	**0.001**	0.547	0.539	0.687	**0.004**	0.835	0.764	0.625	**0.003**	0.263	**0.001**	0.186	0.734	0.225
Hi - Vi	**0.000**	0.969	**0.006**	0.414	**0.010**	0.841	0.248	0.885	0.862	0.508	**0.003**	0.816	0.961	0.307
	Rb		S		Sb		Sc		Se		Si		Sm	
Fi - Hi	0.838	0.418	0.216	0.113	0.072	**0.006**	0.843	0.293	**0.000**	**0.005**	0.740	0.245	0.193	0.117
Fi - Vi	**0.000**	**0.045**	**0.011**	0.501	0.672	0.895	**0.002**	0.892	**0.000**	0.764	**0.036**	0.704	**0.003**	0.655
Hi - Vi	**0.000**	0.516	**0.021**	0.691	0.776	0.600	**0.000**	0.216	**0.005**	0.962	**0.017**	0.178	**0.001**	0.542
	Sr		Ta		Th		Ti		V		Zn		Zr	
Fi - Hi	**0.000**	0.066	0.934	**0.023**	0.075	0.090	0.353	0.212	**0.030**	**0.030**	0.959	**0.029**	0.087	0.279
Fi - Vi	**0.007**	0.367	**0.003**	0.706	**0.010**	0.893	**0.015**	0.800	0.491	0.288	**0.019**	0.580	**0.000**	0.198
Hi - Vi	**0.016**	0.309	**0.005**	0.991	**0.000**	0.244	**0.009**	0.824	0.665	0.250	**0.036**	0.817	**0.000**	0.433

4.3.4.3 Elemental contents in lichen transplants positioned differently within Fi and Hi systems

The definition of the standardised difference (z scores) complies with the use of the z-values in proficiency testing (ISO 13528:2005) and has been applied in many studies (Bode 1996; Coquery, Carvalho et al. 1999; Mellado, Llauradó et al. 2002). The zeta-score is an estimate of the standard uncertainties of the results and shall be interpreted in the same way as the z-scores. When zeta is equal to 3 units of standard deviation, it means that 99.7% of all data should be within ± 3σ of the mean.

Within Hi and Fi systems, transplants were positioned either shielded from the incoming wind by their substrate (S), or such that they faced the wind continuously (F). The zeta-score (ζ) (ISO 13528:2005) comparisons were made in terms of elemental accumulation. Here, zeta-scores were calculated for every combination F and S as:

$$\zeta = \frac{\left| av_F - av_S \right|}{\sqrt{\sigma_F^2 + \sigma_S^2}}$$

with average av and variance σ. The combinations F and S were considered as different from each other when |z|>3. The results for Fi and Hi systems, in all possible F and S combinations, are presented in Table 4.10. The data are given on a monthly basis, and generally suggest that S and F positioning towards wind direction do not result in significant differences in response in terms of element accumulation. Only a few significant differences were observed.

Elements with zeta > 3 may here also be grouped on basis of the sign of the deviation. For $Fi_{\_F}$ - $Fi_{\_S}$ positive differences ($Fi_F > Fi_S$) were observed for K (1st month), Br (3rd month), Ce, Cs and Th (5th month) and negative differences for Co, P, Pb, Sr, and Zr (3rd month), Hf and Sm (5th month). For $Hi_{\_F}$ - $Hi_{\_S}$ positive differences were observed for Cl (3rd month), Na and Br (5th month) and negative differences for Pb (1st month), Zr (3rd month).

Table 4.10 - Zeta-scores for comparison in wind-directional positioning of transplants (Fi and Hi systems). Shielded from the wind is indicated by S, facing the wind is indicated by F; zeta-scores with absolute values >3 indicate significant differences (marked bold).

	$Fi_{\_F}$ - $Fi_{\_S}$					**$Hi_{\_F}$ - $Hi_{\_S}$**				
	1	2	3	4	5	1	2	3	4	5
Mg	-0.5	0.6	0.7	0.6	-0.6	1.5	0.0	**15.3**	-2.3	-0.3
Al	0.0	0.9	0.7	0.9	-0.5	-0.1	2.5	0.2	-0.3	-1.8
Si	0.2	0.7	0.2	1.2	-0.4	-0.4	2.5	0.9	-0.3	-0.8
P	1.9	-0.6	**-3.4**	-0.4	-0.2	-0.6	0.3	0.6	-0.9	-0.7
S	-0.5	-0.5	-0.7	0.9	-0.4	0.6	0.7	1.3	-0.3	0.5
Cl	-0.4	0.3	-0.7	-1.0	1.0	**6.2**	1.8	2.2	0.7	1.7
Ti	0.1	0.6	-1.2	1.7	0.1	-0.6	0.5	1.7	-0.2	0.1
Mn	0.5	0.7	-2.6	-0.7	-0.2	-0.6	0.0	2.4	1.1	1.3
Ni	1.5	0.4	-0.4	1.5	1.0	-2.0	1.1	1.7	0.2	0.2
Cu	0.8	0.3	-0.5	0.2	-1.8	-0.5	1.2	2.0	0.1	0.0
Sr	0.4	1.6	**-5.5**	-0.7	-0.2	-1.0	-0.3	0.9	-0.1	0.7
Zr	0.0	1.3	**-4.6**	1.3	-0.2	-1.0	2.1	**-3.8**	-1.5	-0.8
Pb	0.7	0.7	**-3.9**	-0.5	-0.6	**-3.9**	-0.1	0.8	1.4	1.1
V	1.9	0.6	1.3	1.1	-0.5	-1.0	0.8	2.5	0.2	0.0
Na	1.1	-1.0	-2.8	-1.1	-1.3	2.0	0.9	1.4	1.0	**5.3**
K	**3.0**	-0.4	-1.4	-0.1	-0.3	-1.9	0.2	0.0	-0.2	-0.3
La	0.5	1.5	0.3	2.7	-0.3	-1.5	1.6	0.6	0.6	0.5
Ca	-0.7	0.1	-0.5	0.4	-0.5	0.5	0.6	-1.7	1.2	0.7
Br	0.6	-1.0	**4.5**	0.9	-2.0	-0.2	1.2	0.7	0.9	**3.6**
As	0.4	2.7	1.2	1.6	-0.9	-0.8	1.3	-0.2	0.1	-0.2
Sm	0.1	1.4	1.1	2.0	**-7.8**	-1.1	1.8	0.1	0.3	-0.6
Sb	1.2	0.0	-0.5	0.6	0.2	-1.0	1.6	1.1	0.5	0.7
Co	0.8	0.6	**-6.2**	-0.4	-0.1	-2.1	0.4	1.7	0.0	0.0
Fe	0.8	1.1	-0.2	1.4	0.7	-1.1	1.1	0.9	0.1	0.1
Ta	1.1	-0.7	-1.3	-0.5	-0.2	-1.6	-0.5	1.2		-0.3
Sc	0.5	0.8	-0.2	1.2	1.4	-1.0	1.0	0.6	-0.1	0.0
Zn	0.8	-0.8	-1.3	0.8	-1.2	0.5	0.1	2.6	0.2	-0.4
Rb	1.2	-1.3	-1.0	-0.5	0.1	-0.1	0.5	0.6	0.8	-0.4
Cs	0.9	0.5	-0.7	3.0	-0.2	-0.7	0.7	-0.1	0.2	0.0
Hf	0.2	0.1	-0.5	0.3	**-13.7**	-1.5	0.6	0.1	-0.1	-0.7
Cr	1.7	1.4	-1.6	0.8	-0.6	-1.2	0.9	1.1	0.4	0.0
Th	0.1	0.6	-0.2	**6.2**	-1.3	-0.7	1.2	1.8	0.8	-0.1
Hg	0.0	0.5	0.3	1.2	0.3	-0.3	0.8	2.7	0.2	0.0
Se	-0.3	-0.9	0.0	-0.1	-0.6	-0.5	1.0	0.2	0.6	0.2
Ce	0.6	1.7	0.7	**3.0**	-1.1	-1.5	2.4	0.6	0.0	-0.4

Although generally being rare, differences between wind-facing and wind-shielded transplants seem to be more frequent with Fi systems. A further observation is that sea-related elements may accumulate to higher levels in wind-shielded rather than wind-facing Hi systems.

Other studies (Marques, Freitas et al. 2004b) have also used transplants of the epiphytic lichen *Parmelia sulcata* suspended in nylon bags using the Hi system device allowing a fixed orientation of the lichen towards the wind direction, facing the wind (F orientation) or shielded from the wind by its substrate (T orientation) exposed in a very polluted area of Portugal. The data were analysed via Monte Carlo Added Target Transformation Factor Analysis (MCATTFA) to get information on possible emission-source profiles and their contributions to total-element levels in transplants, in F- and T-orientations. Similar results were obtained, both orientations did not differ for Na, Mg, P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu and Ta, but showed some time-related differences for Cr and Zn. For the remaining elements the data presents a high variability. Under the conditions of the experiment, F- and T-oriented transplants generally did not result in differences in source profiles reflected, nor in differences in source contributions to element levels in the transplants.

In another study, a trace element deposition biomonitoring experiment with transplants of the fruticose lichen *Evernia prunastri* was developed (Ayrault, Clochiatti et al. 2007), aimed at monitoring the effects of different exposure parameters (exposure orientation - three positions used: horizontal, vertical and oblique - and direct rain) for the elements Ti, V, Cr, Co, Cu, Zn, Rb, Cd, Sb, and Pb. The accumulation trends were mainly affected by the exposure orientation and slightly less so by the protection from rain. The position of the thallus during exposure affected the accumulation levels. This position was strictly defined to ensure comparability. The use of a roof (non-metallic), allowing air to circulate, to protect the lichen from direct rain appeared desirable in this study.

The above results were obtained for specific lichens, thus, the results are not necessarily representative for other lichens.

4.3.5 Systems and grouped-elements

During the period of the present transplant study, aerosol collectors have also been positioned at the ITN campus, at the same sampling site, for the full year 2001 (Almeida 2004). Air particulate matter (both PM 2.5 and PM 10: smaller than 2.5 and 10 μm aerodynamic diameter particle size respectively) collected correlated well with air masses, which from February till July 2001 (the study period of the present work) were basically of maritime origin and for a few episodes maritime transformed, from continental south and continental north/centre origin (Almeida 2004). Principal components factor analysis (PCFA) performed on the aerosol data suggested three main groups of elements: the soil elements Al, Ca, Co, Fe, K, La, Mn, Sc, Si, Sm and Ti; the sea elements Na, Cl, K, Mg and S; and the industrial elements (e.g. due to traffic, fuel combustion, and metallurgic activities) As, Ca, Co, Cu, Hg, K, Mn, Ni, S, Sb, Se, V and Zn (Almeida 2004).

Based on the PCFA of the aerosol data, the present results may indicate, in terms of transplants to reflect total deposition, that Fi systems are preferable for elements associated with soil (Ca, Fe and Mn) and industry (Ca), and that Hi systems may be preferred for reflecting elements associated with seawater spray (Na) and industry (Ni and V). Na is reported as mostly associated with the superficial and intercellular lichen fractions, and is thus liable to be strongly affected by precipitation (Figueira 2002). This makes that the cover in Hi systems may have prevented excessive Na losses, and may also have had effects on net retention of other elements, like Ni and V. The data indicate that the Vi system does not show any correlation with element deposition. It should be noted here, however, that probability values shown in figures 4.4 to 4.9 indicate that rate differences in responses may exist between the systems. The Vi-system may be the slowest one in reflecting correlations between transplant element contents and total element deposition.

PCFA results, seen in the context of the groups presented at point 4.3.4.2, may be roughly discussed for all three systems used. No system-related differences were observed for most of the industry-associated elements (Ca, Zn, Sb) with the exceptions of V, K and Se. Fi is not different from Hi for Ca as a soil element,

shows highest responses for Fe, La and V, but the Hi system may be used for K and Se.

In a recent Portuguese Sado-estuary study, Hi systems were used to track emissions from the Electricity of Portugal (EDP) fuel-oil power station in the area, with emphasis on the emission of V and Ni (Reis, Freitas et al. 1999; Freitas, Reis et al. 2000b; Freitas, Reis et al. 2001; Marques, Freitas et al. 2004a; Marques, Freitas et al. 2004b). The present study suggests that the total deposition of V and Ni may be better reflected by the Hi system (Table 4.5), although in terms of V accumulation Fi may be preferred rather than Hi (Tables 4.8 and 4.9). The Hi systems used in the Sado estuary study gave V/Ni ratios which agreed well with literature (Marques, Freitas et al. 2004).

The overall data suggest that the Vi system should not be used in air-pollution studies with *Parmelia sulcata* transplants: the Vi system usually showed smallest element concentrations (Figs 4.4 to 4.9), and, for many elements, showed negative time trends and negative slopes in system comparisons (Tables 4.8 and 4.9). For 14 out of the 35 elements, Vi systems showed no differences with RL values at all. For Hi and Fi systems, no clear differences result from the present data: any choice should depend on the specific purposes of the study. It should be noted here that in terms of accumulation rates (slopes), Fi and Hi systems were considered not different for Ca, Sb, Ta and Zn, Fi may be favoured for Cr, Fe, La and V while Hi may be chosen for K, Se and Pb.

4.4 Conclusions

The present chapter recognises both system set-ups Vi, Hi and Fi, and wind-directional positioning S (shielded from the wind) and F (facing the wind). The results suggest the absence of any wind-directional effects on element accumulation within Fi (free influx) and Hi (horizontal influx) systems, but differences in response may be observed in relation with the transplant set-up systems. Here, the Vi (vertical influx) system generally shows poor results while Hi and Fi performance depends on the element involved.

As a general result, and on basis of accumulation data, Fi systems may be preferred for studies involving specific elements such as Cr, Fe, La and V and Hi systems may be preferred for K, Pb and Se. Comparing lichen data to element total deposition, the results suggest that the Fi system should be preferred for Ca, Fe and Mn, and the Hi system for Na, Ni and V; for the Vi system no significant correlations were found.

Overall, the present data suggest that the set-ups of transplant systems in exposure experiments are of significant relevance for the eventual results in (element-specific) transplant air pollution biomonitoring.

References

Almeida, S.M. (2004) *Composição elementar do aerossol atmosférico em zona urbano-industrial.* PhD Thesis, Universidade de Aveiro, Aveiro, Portugal, in Portuguese.

Ayrault, S., Clochiatti, R., Carrot, F., Daudin, L., and Bennett, J. (2007) Factors to consider for trace element deposition biomonitoring surveys with lichen transplants. *The Science of the Total Environment* **372**: 717–727.

Bargagli, R. (1998) Lichens as biomonitors of airborne trace elements. In *Trace elements in terrestrial plants: an ecophysiological approach to Biomonitoring and biorecovery*. Berlin, Germany: Springer Verlag, pp. 179-206.

Bode, P. (1996) *Instrumental and organisational aspects of a neutron activation analysis laboratory*. PhD Thesis, Delft University of Technology, Delft, The Netherlands pp. 1-251.

Boss C., Fredeen, K. (2004) *Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectrometry*, Perkin Elmer, USA.

Conti, M.E., and Cecchetti, G. (2001) Biological monitoring: lichens as bioindicators of air pollution assessment - a review. *Environ. Pollut.* **114**: 471-492.

Coquery, M., Carvalho, F.P., Azemard, S., and Horvat, M. (1999) The IAEA worldwide intercomparison exercises 1990-1997): determination of trace elements in marine sediments and biological samples. *The Science of the Total Environment* **237/238**: 501-508.

De Corte, F. (1987) *The k0 - Standardization Method, a move to the optimisation of neutron activation analysis.* PhD Thesis, Gent University, Gent, Belgium pp. 1- 464

Figueira, R. (2002) *Desenvolvimento de um sistema de biomonitorização ambiental.* PhD Thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal pp. 51-107.

Freitas, M.C., and Nobre, A.S. (1997) Bioaccumulation of heavy metals using *Parmelia sulcata* and *Parmelia caperata* for air pollution studies. *Journal of Radioanalytical and Nuclear Chemistry* **217**: 17-20.

Freitas, M.C., Catarino, F.M., Branquinho, C., and Máguas, C. (1993) Preparation of a lichen reference material. *Journal of Radioanalytical and Nuclear Chemistry* **169**: 47-55.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000a) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace Elements - their Distribution and Effects in the Environment*. Friese, M.a. (ed). Amsterdam, Netherlands: Elsevier Science, pp. 187-213.

Freitas, M.C., Reis, M.A., Marques, A.P., and Wolterbeek, H.T. (2000b) Dispersion of chemical elements in an industrial environment studied by

biomonitoring using *Parmelia sulcata*. *Journal of Radioanalytical and Nuclear Chemistry* **244**: 109-113.

Freitas, M.C., Reis, M.A., Marques, A.P., and Wolterbeek, H.T. (2001) Use of lichen transplants in heavy metal atmospheric deposition studies. *Journal of Radioanalytical and Nuclear Chemistry* **249**: 307-315.

GraphPad Software Inc. (1999) *Analysing Data with Graphpad Prism*. San Diego, USA.

ISO 13528:2005, Statistical methods for use in proficiency testing by interlaboratory comparisons by ISO/TC, 69/SC 6 N459.

Jacquiot, L., and Daillant, O. (2002) Bio-accumulation of heavy metals, trace elements and radioelements, review-part III. In. Mazille, France: Bulletin de l'Observatoire Mycologique, pp. 1-33.

Johansson, S.A.E., Campbell, J.L., and Malmqvist, K.G. (1995) *Particle Induced X-ray Emission Spectrometry (PIXE) in Chemical Analysis*. USA: John Wiley & Sons.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., and Verburg, T.G. (2004a) MCTTFA applied to differential biomonitoring in Sado estuary region. *Journal of Radioanalytical and Nuclear Chemistry* **259**: 35-40.

Marques, A.P., Freitas, M.C., Wolterbeek, H.T., De Goeij, J.J.M., and Steinebach, O. (2005) Cell membrane damage and element leaching in transplanted Parmelia sulcata lichen related to ambient SO2, temperature, and precipitation. *Environmental Science & Technology* **39**: 2624-2630.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., Verburg, T.G., and De Goeij, J.J.M. (2004b) Lichen-Transplant Biomonitoring in the Assessment of Dispersion of Atmospheric Trace-Element Pollutants: Effects of Orientation Towards the Wind

Direction. *Journal of Atmospheric Chemistry* **49**: 211–222.

Mellado, j., Llauradó, M., and Rauret, G. (2002) Determination of actinides and strontium in fish samples by extraction chromatography. *Analytica Chimica Acta* **458**: 367-374.

Montaser, A., Golighty, D. W. (1992) *Inductively Coupled Plasmas in Analytical Atomic Spectrometry.*VCH Publishers: New York, USA.

Mulgrew, A., and Williams, P. (2000) Biomonitoring of air quality using plants. In. Berlin, Germany: WHO Colaborating Centre for Air Quality Management and Air Pollution Control, Federal Environmental Agency, pp. 1-165.

Nash III, T.H. (1996) *Lichen biology*. England: Cambridge University Press.

Reis, M.A. (2001) *Biomonitoring and assessment of atmospheric trace elements in Portugal - methods, response modelling and nuclear analytical techniques.* PhD thesis, Delft University of Technology, Delft, Netherlands pp. 1-230.

Reis, M.A., Freitas, M.C., C., A.L., Marques, A.P., and Costa, C. (1999) Environmental assessment in an industrial area of Portugal. *Biol. Trace Elem. Res.* **71/72**: 471-479.

Sloof, J.E. (1993) *Environmental lichenology: biomonitoring trace element air pollution.* PhD Thesis Delft University of Technology, Delft, Netherlands pp. 1-191.

Weiss, N.A. (1999) Introductory Statistics. In.: Addison Wesley Longman, Inc., pp. 180-185.

Woolson, R.F., Clark, W.R. (2002) *Statistical Methods for the Analysis of Biomedical Data*: Wiley & Sons, Inc, USA.

Chapter 5[3]

Biomonitoring Study of Setúbal Peninsula Region

5.1 Introduction

In the period 1994–1996, an atmospheric exposure experiment with lichen transplants was held in Portugal using six sampling locations from north to south of Portugal. The results indicated that positioning of the transplants towards the wind direction was an important factor for the eventual interpretation (Reis, 2001). The experiment further showed that the amount of direct rain was an important factor to be accounted for the interpretation of the data. Moreover, a previous lichen–based national survey, held in Portugal in 1993, showed that the Setúbal peninsula region (50 km south of Lisbon, see Fig. 5.1) is a quite polluted area (Reis, Alves et al. 1996; Freitas and Nobre 1997; Freitas, Reis et al. 1999; Freitas, Reis et al. 2000; Reis 2001). This result was also obtained in moss surveys performed in 1990-1992 (Ruhling, 1994) and in 1996-1998 (Figueira, Sousa et al. 1999; Figueira 2002; Figueira, Pacheco et al. 2002).

Meteorological data of 30 years, as shown that the wind in this area is predominately from North mainly during the summer (about 45% of the

[3] This chapter is a modified version of:
A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ, "Biomonitoring-transplants: effects of positioning towards wind direction", Journal of Atmospheric Chemistry, Vol. 49, (2004), 211 – 222.
A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG, "MCTTFA applied to differential biomonitoring in Sado estuary region", Journal of Radioanalytical and Nuclear Chemistry, Vol. 259, Nº 1, (2004) 35-40.

occurrence), being South and Southwest the second predominant wind directions (about 25%) as reported by Garcia et al. (Garcia, Coelho et al. 2002). Garcia et al. (Garcia, Coelho et al. 2002) have related main pollution sources with meteorological data and the main conclusions are that under certain conditions the air quality in Setúbal city is very affected from the industries and traffic, especially with winds from south and low speed winds.

Therefore, the contribution to the composition of the atmosphere in this region, of the industries located South, at the North of the peninsula and those located at North of Tagus river, should also not be a priori underestimated (Fig. 5.2 and Fig. 5.3). There are factors that may contribute to the dispersion of pollutants such is the case of sea breeze and that are most of the times are not taken into account.

Fig.5.1 –Map of Portugal with the location of the study area (square in detail) on the right) location of Sado river estuary (circle) on the left.

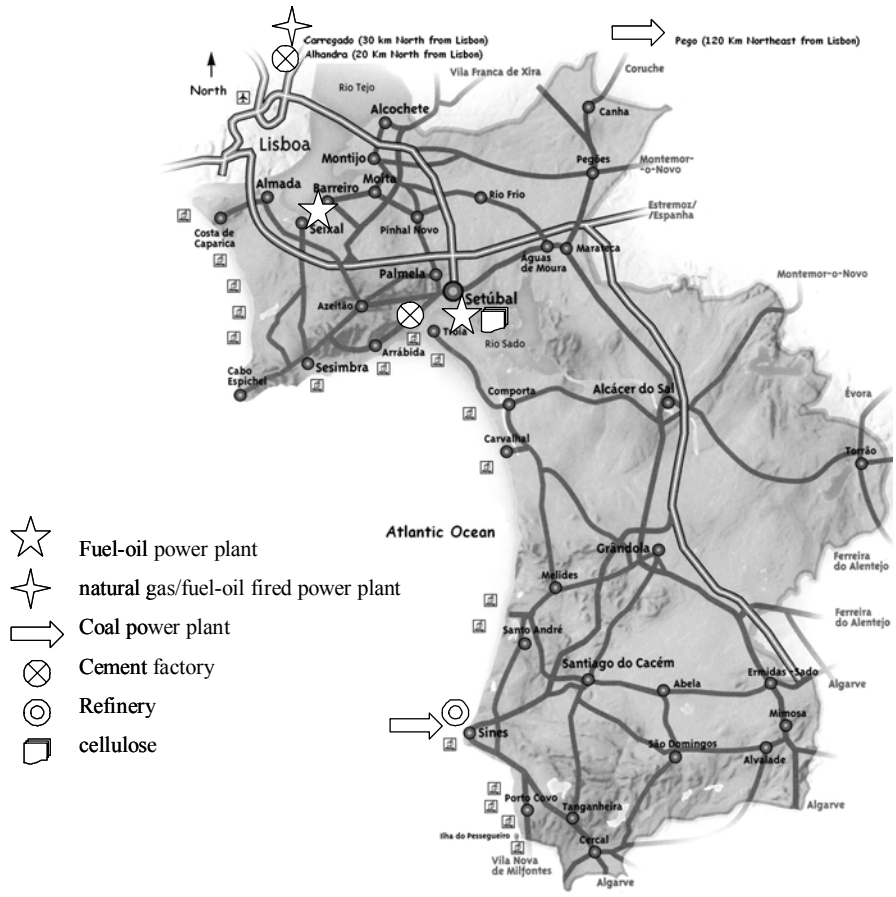

Fig.5.2 –Location of the study area and some of the main industries in the region.

The present chapter focuses on transplant positioning in biomonitoring of trace-element air pollution, in an exposure experiment performed in the Setúbal area. It addresses in particular the orientation of lichen transplants towards the wind direction, both in terms of total-element concentrations, time of exposure, and transplant expression of possible emission-source profiles. Use was made of Monte Carlo Target Transformation Factor Analysis (MCTTFA).

Fig. 5.3 – Principal industries located near Setúbal city (see further for sampling grid). From left to right top: cement factory (near S32 of the sampling grid), pyrites terminal with chimneys from the fuel-oil power plant on the back (near S33 of the sampling grid), fertiliser factory and terminal (near S39 of the sampling grid); from left to right bottom: cellulose and paper production (near S35 and S40 of the sampling grid), naval shipyard and deactivated iron-ore production (near S40 of the sampling grid).

5.2 Experimental Section

5.2.1 Sampling

Samples of the epiphytic lichen *Parmelia sulcata* were collected from olive trees at about 1.5 meters above the soil, in areas considered clean from the pollution point of view, between Fátima and Pombal (Reis, Alves et al. 1996; Freitas and Nobre 1997; Freitas, Reis et al. 1999; Freitas, Reis et al. 2000; Reis 2001). Before exposure 10 lichen transplants were separated randomly as reference base levels (RL). Samples of about 2 grams of the lichen were put into nylon net bags and suspended at about 1.5 meters above the soil, in a grid of 2.5 km × 2.5 km, within a rectangle of 15 per 25 km, having a fuel-fired power station in the centre. The lichen transplants support used contained up to 8 lichen transplants, and were equipped with a system, which rotates according the wind direction - 47 of these hanging systems were installed according to a grid (Fig. 5.4). Four transplants were hanged facing the wind direction (called F) and four transplants were facing the opposite direction – shielded by the wind or opposing the wind (called T).

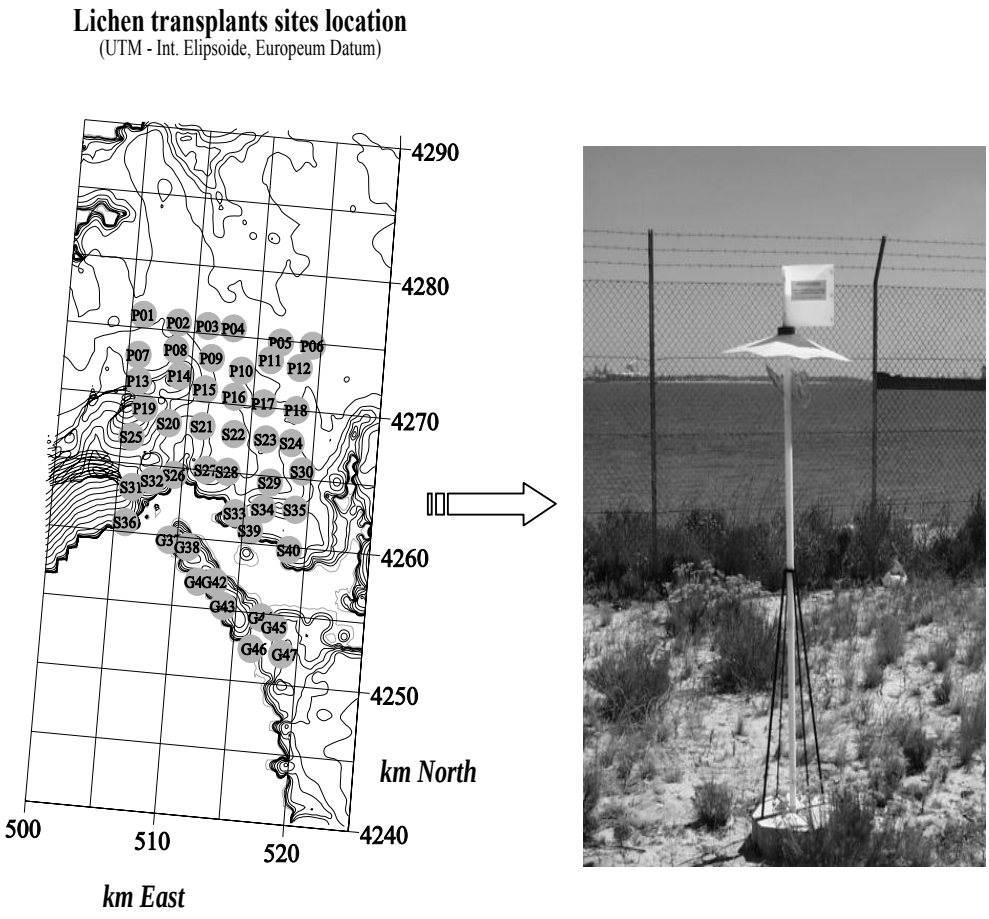

Fig. 5.4 – On the left, lichen transplants grid (Freitas, Marques et al. 2008) with a cement factory (site S32), a Power station (site S33), pyrites terminal (near site S33), fertiliser factory and terminal (near site S39), cellulose and paper production (near sites S35 and S40), naval shipyard and deactivated iron-ore production (near site S40), an automobile Plant (west site P13), and the city of Setúbal (sites S26 and S27). On the right, lichen transplant support system with the polyethylene cover used in each site.

Protection against leaching due to heavy rainfall was also provided by this system (Fig. 5.4). The lichen transplants were suspended in December 1997. Due to loss of

some systems, only 39 F-transplants (F3) and 39 T-transplants (T3) were removed after 3 months, 34 F-transplants (F6) and 35 T-transplants (T6) after 6 months and after 9 months exposure 25 F-transplants (F9) and 31 T-transplants (T9) were collected (Marques, Freitas et al. 2004b).

5.2.2 Sample preparation

In the laboratory transplants were cleaned from substrate and other lichens or mosses attached, rinsed for 30 s with de-ionised water and freeze-dried. Then they were transposed to a teflon capsule and freezed for about 2 minutes in liquid nitrogen and ground (for about 5 minutes at 1500 rpm) in a Teflon mill.

5.2.3 Analysis

Samples were analysed by the nuclear analytical techniques INAA (Erdtmann and Petri 1986) and PIXE (Johansson, Campbell et al. 1995). INAA analysis was carried out using pellets of 500 mg irradiated at the Portuguese Research Reactor (RPI) and a high-purity germanium detector for gamma spectra determination. Pellets of 500 mg were irradiated together with 0.1% Au-Al wires as comparators. Two irradiations were performed, short (18 s) and long irradiation (5 h). Samples were measured with a high-purity germanium detector for 5 minutes (short irradiation) after 5 - 10 minutes waiting time, and 2-4 h (long irradiation) after 4 and 30 days of waiting time. Concentrations were obtained by the k_0-factor method (De Corte 1987). PIXE analysis was made using a pellet of a thin layer of lichen powder in a boric acid support. Samples were irradiated in ITN Van de Graaff accelerator. The X-ray spectra were obtained with a Si(Li) detector, analysed by AXIL program and concentrations were obtained using DATTPIXE program and radiation damage correction procedures (Reis, Alves et al. 1996a). Quality control was pursued by both techniques analysing the IAEA-336 lichen and CTA-OTL-1 tobacco leaves reference materials (for accuracy of the techniques see Chapter 2 of the thesis). The analytical approach (INAA or PIXE) for elements determinable by both methods was selected following Freitas et al (Freitas, Reis et al. 2000) and the results from Chapter 2 of the present thesis.

5.2.4 Data handling

F-tests (Woolson and Clark 2002) were applied to the data (Table 5.1) to test the significance of the differences from reference level values (RL, t=0 lichen element content) of the exposed transplants element contents (Freitas, Marques et al. 2008). Student t-tests (GraphPad Software 1999, Weiss 1999, Woolson and Clark 2002) were used to test the significance of differences in values for the two applied positioning towards the wind direction (F for "facing" the wind, T for "shielded" from the wind by their substrates). Monte Carlo Added Target Transformation Factor Analysis (MCATTFA) (Kuik, Blaauw et al. 1993; Kuik, Sloof et al. 1993) was applied to the data at IRI (Delft University of Technology, The Netherlands) to study F- and T-related possible emission source profiles and their respective contributions to F- and T-associated total element contents. Contour plots (Surfer, Golden Software Inc) of contents variation of transplants facing and shielded by the wind direction (in units of standard variation of the reference values) and of transplants ratio (facing/shielded by the wind) were performed for 3, 6 and 9 months exposure. These plots are based in a $1/r^3$ extinction rule (see Fig. 5.6 for Cr and Zn). Data on the predominant wind direction during the exposure period were obtained from Electricity of Portugal – EDP measured within the perimeter of the fuel-oil power station (S33 of the grid).

5.3 Results and Discussion

5.3.1 Prevailing wind direction

EDP (Electricity of Portugal) measured wind direction in one of the sites of the grid (S33) and it is possible then to determine the prevailing wind direction affecting that region and the transplants during the exposure period (Fig. 5.5). Nevertheless it should be kept in mind that it is an estuary region with particular wind systems and a very specific topography.

Since the transplants were exposed for 3, 6 and 9 months the wind direction was also plotted that way. During the first 3 months of exposure (December 97 – March 98) the identification of a predominant wind direction on this region is not so clear since it was very variable, although it seems that it comes mainly from Northeast, Southeast (Continental wind) and in lesser extent Southwest. From December 1997

to June 1998 the transplants suffered wind coming mainly from the North/Northeast and in small percentage from West/Southwest. The transplants exposed till September 1998 had influence from North/Northeast and in a smaller percentage Southwest. The results reflect the typical behaviour of the wind in Setúbal city (Garcia, Coelho et al. 2002).

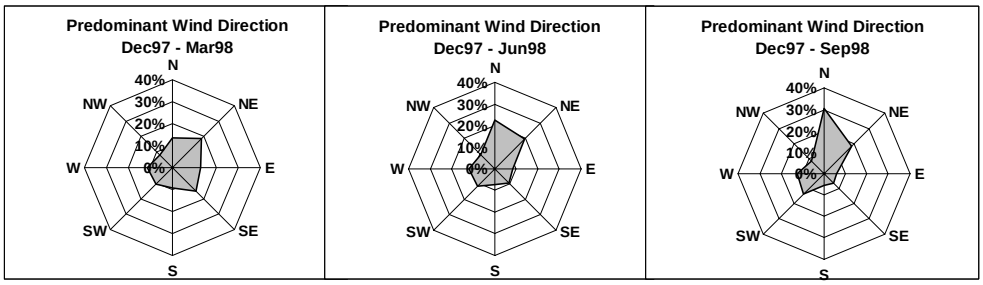

Fig. 5.5 - Predominant wind direction during the exposure period obtained from Electricity of Portugal – EDP measured within the fuel-oil power station perimeter (site S33 of the grid).

5.3.2 F and T differences from RL

Table 5.1 presents statistics on the element data obtained for 3, 6 and 9 months respectively for wind facing (F) and wind-shielded (T) transplants in Sado estuary region. The probability of F = T was determined (using a t-test) by calculating the ratio of F- and T-data variances. Whenever the ratio of F- and T-data variances leads to a probability of a different variance below 95%, the probability of an equal mean value was calculated assuming equal variances. The designation "different" means that the variances are different with a certainty larger than 95% and the t-test can not be applied, only approximate procedures can be used (Woolson and Clark, 2002) which was not the case. For each element the probability that F ≠ RL or T ≠ RL is presented (Table 5.1). Using a 95% probability threshold, results can be summarised as follows:

5.3.2.1 Equal behaviour of F and T towards RL

- Different from RL after 3, 6 and 9 months of exposure: Na, S, Cl, V, Mn, Ni, Cu, Zn, As, Se, Br, Sr, and Sb.

- Not different from RL after 3, 6 and 9 months of exposure: P, K, Sc, Fe, La, Ce, Nd, Sm, Eu, Tb, and Th.
- Different from RL for a certain exposure period: Mg (3 and 9 months), Co (9 months), Zr (3 and 9 months), Ba (9 months), and Pb (6 and 9 months).

5.3.2.2 Differences in behaviour of F and T towards RL

- In the F-set Ca and U differ from RL at 3, 6 and 9 months of exposure; for the T set this occurs at 9 months of exposure.
- In the F-set Si differs from RL at 6 and 9 months of exposure; for T set this occurs at 3, 6 and 9 months of exposure.
- In the F-set Cr differs from RL at 3, 6 and 9 months of exposure; for the T sets this occurs at 6 and 9 months of exposure.
- In the F-set Hg differs from RL at 9 months of exposure; T set does not differ from RL.

For elements behaving equally towards RL for F and T transplants, the first group elements include anthropogenic elements as well as sea-salt spray elements. The second group of elements include physiological and lithophilic elements. These observations indicate that with a few exceptions: (i) the wind direction does not significantly affect the bioaccumulation of elements into *Parmelia sulcata*; (ii) lithophilic elements did not accumulate during the exposure, for both F- and T-orientation; (iii) both F- and T-oriented transplants are sensitive enough to indicate trace-element pollutants; (iv) mostly physiological elements are not lost through the exposure for both F- and T-orientation. The latter observation serves to underline the absence of any visible toxicity action: losses of K and P are regarded as indicating cell-membrane damage (Nash III 1996). Based on Table 5.1 either F- or T-oriented transplants can be regarded as accumulating trace-element pollutants, in the present experiment without changes in physiological guide elements and without accumulating soil-related elements. Some time-related differences in behaviour between F- and T-orientation were observed for Al, P, Ti, Si, Ca, Cr, Hg and U, which might indicate F- and T-related differences in dynamics, but for the majority of elements F- and T-orientations behave similarly towards RL.

Table 5.1 – Statistics on the element data obtained for 3, 6 and 9 months respectively for wind facing (F) and wind-shielded (T) transplants in Sado estuary region. For each element the probability that F or T ≠ RL was calculated using a F-test (values ≥0.95 are marked bold). The probability that F = T was calculated using a T-test; whenever F and T present a probability higher than 0.95 of having different variances, F and T are considered "different" (marked bold).

Element	Probability of being different from RL						Probability of F=T		
	3 months		6 months		9 months		3 months	6 months	9 months
	F	T	F	T	F	T			
Al	0.392	0.905	**0.996**	0.775	0.891	0.720	0.896	**Different**	0.750
As	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**Different**	0.818	0.955
Ba	0.857	0.553	0.836	0.898	**0.999**	**0.995**	0.738	0.792	0.695
Br	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.864	0.782	0.712
Ca	**1.000**	0.575	**0.998**	0.688	**1.000**	**1.000**	**Different**	**Different**	0.874
Ce	0.469	0.731	0.079	0.488	0.903	0.691	**Different**	0.913	0.652
Cl	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.799	0.948	0.849
Co	0.403	0.344	0.817	0.918	**1.000**	**0.997**	0.880	0.831	0.713
Cr	**0.999**	0.798	**1.000**	**0.995**	**0.972**	**1.000**	**Different**	**Different**	**Different**
Cu	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**Different**	0.866	0.922
Eu	0.812	0.068	0.130	0.532	0.690	0.391	**Different**	0.846	**Different**
Fe	0.706	0.103	0.444	0.628	0.629	0.518	0.753	0.844	0.710
Ga							0.890	0.980	0.954
Hf	0.661	0.685	0.346	0.811	0.698	0.833	**Different**	**Different**	**0.710**
Hg	0.865	0.406	0.374	0.412	**1.000**	0.899	0.650	0.916	**Different**
K	0.815	0.892	0.394	0.243	0.919	0.556	0.788	0.556	0.882
La	0.857	0.553	0.329	0.281	0.497	0.075	0.758	0.899	0.715
Lu	0.558	0.104	0.170	0.530	0.868	0.118	0.597	0.775	0.704
Mg	**0.956**	**0.986**	0.791	0.922	**1.000**	**0.991**	0.957	0.674	0.817
Mn	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.852	**Different**	0.763
Na	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.882	0.828	0.873
Nd	0.071	0.684	0.769	0.902	0.877	0.925	0.774	0.942	0.986
Ni	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.987	0.700	0.828
P	0.698	0.933	0.487	0.049	0.109	0.101	0.811	0.926	0.876
Pb	0.848	0.495	**0.998**	**0.999**	**1.000**	**1.000**	0.964	0.781	**Different**
Rb	0.024	0.535	0.818	0.118	0.402	0.541	**Different**	0.950	0.790
S	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.874	0.671	**Different**
Sb	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.947	0.816	**Different**
Sc	0.830	0.013	0.504	0.403	0.229	0.274	Different	0.853	0.705
Se	**0.986**	**0.997**	**0.999**	**1.000**	**1.000**	**1.000**	0.518	0.755	0.739
Si	0.793	**0.997**	**1.000**	**0.991**	**0.998**	**0.963**	**Different**	**Different**	0.759
Sm	0.100	0.698	**0.956**	0.549	0.812	0.769	0.600	0.971	0.998
Sr	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.987	0.677	0.699
Ta	0.717	0.039	0.389	0.419	0.521	0.328	0.702	0.833	0.671
Tb	0.636	0.433	0.386	0.819	0.513	0.847	**Different**	0.984	0.767
Ti	0.692	**0.996**	**1.000**	0.914	**0.997**	**0.981**	**Different**	**Different**	0.757
Th	0.526	0.752	0.167	0.873	0.839	0.673	**Different**	0.922	0.729
U	**1.000**	0.310	**1.000**	0.873	**1.000**	**0.999**	**Different**	**Different**	0.789
V	**0.997**	0.949	**1.000**	**1.000**	**1.000**	**1.000**	0.895	**Different**	0.991
Zn	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	0.973	**Different**	**Different**
Zr	**1.000**	**1.000**	0.939	0.773	**1.000**	**1.000**	0.996	0.747	**Different**

5.3.3 Differences between F- and T-oriented transplants

Table 5.1 also presents statistics on the possible differences between F- and T-orientations of the transplants. This data may be summarised as follows: F- and T-orientations are significantly different for 3, 6 and 9 months exposure (Cr), for 3 and 6 months exposure (Si, Ca, Ti, Hf, and U), for 6 and 9 months exposure (Zn), for 3 and 9 months exposure (Eu), for 3 months exposure (Sc, Cu, As, Rb, Ce, Tb and Th), for 6 months exposure (Al, V and Mn) and for 9 months exposure (S, Sb, Zr, Hg and Pb). F and T are not significantly different (all exposure periods) for Na, Mg, P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu, and Ta.

General conclusions may be that the transplants behave similarly for Na, Mg, P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu and Ta and differently for Cr and Zn, while the elements Si, Ca, Ti, Hf and U show a behaviour that lies in between.

Fig. 5.6 presents contour plots of transplants ratio (facing/shielded by the wind) for Cr (3, 6 and 9 month exposure) and Zn (6 and 9 months exposure) the two elements for which F and T transplants were statistically considered to behave differently. For Cr, after 3 months of exposure, F over T ratio was higher than 3 in the western upper part of the grid. After 6 months, the patterns were shifted into another differentiation, which remained still identifiable at 9 months, in the eastern part of the grid (F/T between 2 and 3). For Zn, after 6 months of exposure, small F/T differentiation appeared in the North, Northwest and Southeast (F/T between 2 and 3). At 9 months the F/T differentiation persisted only at the Southeast part of the grid. The wind direction during the first 3 months of exposure was more diffuse and at 6 and 9 months of exposure was coming mostly from the north. Here it should be noted that concentrations may change with changing wind directions but no straightforward changes in F/T ratios are to be expected.

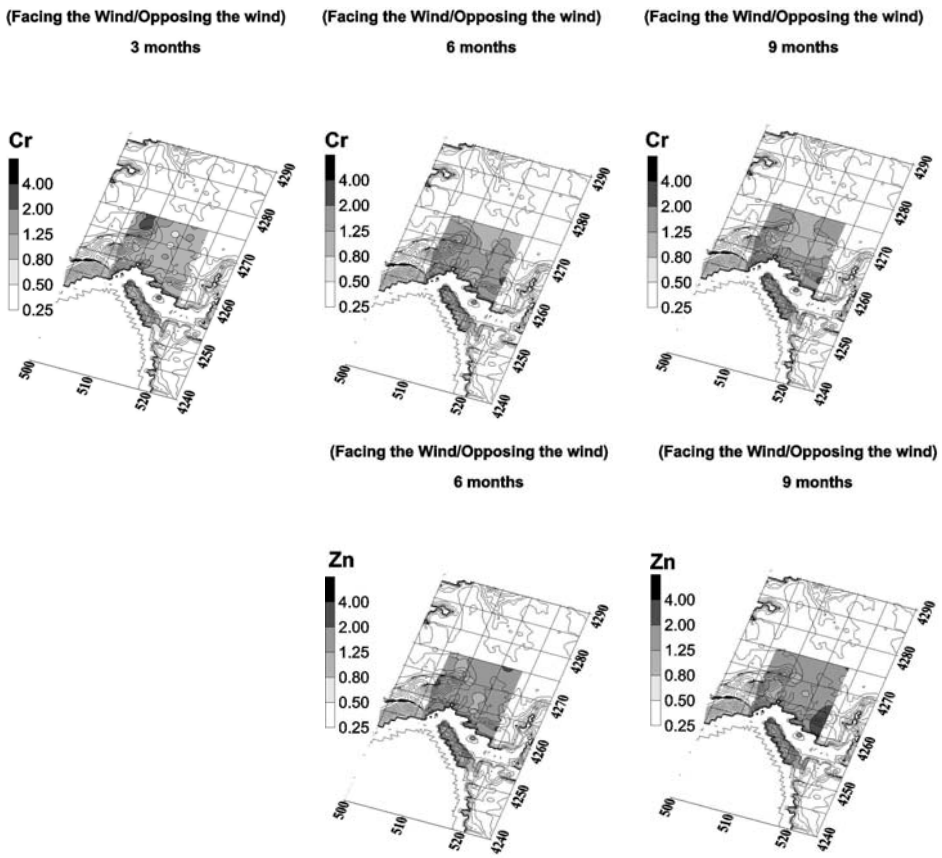

Fig. 5.6 – Contour plots (Surfer, Golden Software Inc) of transplants ratio (facing/shielded by the wind) for Cr (3, 6 and 9 month's exposure) and Zn (6 and 9 months exposure). These plots are based in a $1/r^3$ extinction rule.

5.3.4 F and T behaviour in reflecting emission source profiles

MCATTFA was used to compare F with T data. The comparisons were made for the common source profiles, both in time relations and in terms of the relative source contributions to total element levels.

MCATTFA was applied to the data at IRI (Delft, Netherlands) using 26 elements: Al, As, Ba, Br, Ca, Cl, Co, Cr, Cu, Fe, K, Mn, Na, Ni, P, Pb, Rb, S, Sb, Sc, Se, Si, Ti, U, V, Zn. Three data sets combinations were used: all data (F3, F6, F9, T3, T6, T9), facing the wind transplants (F3, F6, F9) and shielded by the wind transplants (T3, T6, T9). In order to choose the optimal number of factors in each case, use

was made of FIC (factor identification conflicts, see Fig. 5.7) (Kuik, Blaauw et al. 1993; Kuik, Sloof et al. 1993; Kuik and Wolterbeek 1995).

In the case of all data (201 samples used), FIC graph shows some sharp rises at 7, 9 and 10 factors. The total amount of explained variance is 0.74, 0.79 and 0.81 respectively. Also the rejected modified data sets due to assignment conflicts are 0%, 7% and 12% respectively (all centred in one factor) and so 9 was chosen as the optimal number of factors to be used. For the combination of F data (97 samples used), 7 and 10 factors are pointed out and the explained variance is 0.72 and 0.80 respectively. In the case of 10 factors, 12% of the generated modified data sets provided by Monte Carlo were rejected due to factor assignment conflicts (most of them centred in one single factor) and so 7 factors were chosen with only 0.2% of modified data sets rejected. For the T data sets combination (104 samples used), FIC points out to 7, 8 or 10 factors. Total explained variance is 0.82, 0.84 and 0.86 with 2, 4 and 13% of rejected modified data sets respectively. Since in the case of 8 factors, half of this sets were centred in one single factor, 7 was chosen as the optimal number of factors to be used.

Table 5.2 shows the normalized averaged factor loadings obtained for all Sado data. The element of a profile with the largest loading in the correlation domain is called the pilot element. Such a pilot element of a factor has the strongest correlation with that specific factor and consequently it is the most characteristic element in the profile.

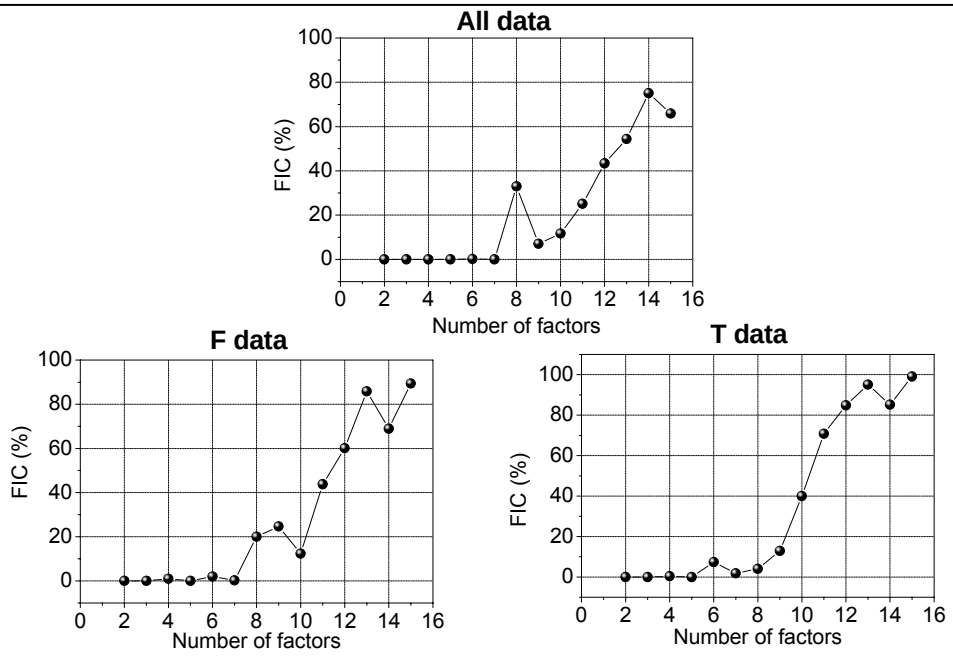

Fig. 5.7 – Percentage of factor identification conflicts (FIC) as a function of the retained number of factors for the three Sado estuary data combinations run by MCATTFA.

Factor 1, here named industrial/oil combustion factor, was associated with complex industry with an oil combustion component and Sb, Cu, V, Pb, and Ni as the most related elements. According to Nriagu (Nriagu 1989) V, Ni, Pb, and Cu are highly correlated with oil combustion but not Sb, usually related to smelting, coal combustion (www.atsdr.cdc.gov/toxprofiles/) and refuse incineration. Previous studies, some made in Portugal (Freitas and Nobre 1997) and other countries (Chueinta, Hopke et al. 2000) also found Sb associated with the oil combustion factor. The Ni/V ratio obtained of 0.54 is close to Nriagu value of 0.36 (Nriagu 1989) and agrees well with ratios for European aerosol (0.4 to 0.7) (Rahn and Huang 1999) combined with the presence of a fuel power station on the sampling grid.

Three soil factors are identified in factors 2, 4 and 8 with Mn, Sc and Co as pilot elements.

Table 5.2 – Normalized averaged factor loadings obtained after 500 Monte-Carlo variations for all Sado data. Indicated loadings were found to be significantly positive (P>95%), values marked with * are more than 99% significant and values marked with + are 95-99% significant. Pilot elements are marked with a P. Relative errors in percentage are given in parenthesis.

All Data Elements	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
Na	33361*		56.0*	224857*	9.66*	11811*			36748+
Al		2949*		103542+	7.58*	33461*	27352*	57505*	
Si		6312*		311166*		94661*	69614*	140858*	
P		273*			28.0*		2909*		
S	93847*	1387*	22.7*			17572*	10210*	31094*	
Cl		788*	100P		11.1*				
K				80205*(37)	100P	6958+			
Ca	173412*		61.3*	982766*(19)	24.1+	148967*			298947+
Sc	14.4*	0.14*		100P (7)		3.68*	2.98*	10.5*	
Ti		426*		26922* (16)			1245*		
V	1412*		0.05*	455* (39)	0.02*	35.4+	38.2*		140*
Cr	240*		0.01*	946* (17)			100P		
Mn		100P	0.24*	5021+ (21)	0.32*				954*
Fe	102881*	441*	2.55*	307688* (8)		12742*	14147*	37468*	
Co	93.4+		0.00+					100P (6)	
Ni	765* (6)	11.0*	0.05*			36.3*	44.4*	88.8*	
Cu	18776*	101*				1191*		1283+	1793+
Zn	25792*		3.31*				4095*		
As	106*	0.84+	0.00*			100P (5)	5.49+		89.9+
Se	16.5*	0.06+		59.1* (8)					6.07*
Br	1004*		0.37*	3197* (13)			155*		423*
Rb				1238* (8)	0.17*	70.8*		105+	
Sb	100P (6)	0.22*	0.00*	34.7* (28)			1.33+	7.43*	12.9*
Ba	1329*	4.62*	0.06*	3019* (8)		267*	67.8*	362*	283*
Pb	3550*	17.9*	0.10*	1040* (34)					
U			0.00+				2.33+		100P

Factor 3 was identified as the sea salt spray factor with Cl and Na the elements presenting the highest correlation with the factor. The Na/Cl ratio obtained of 0.56

for factor 3 equals to the ratio for seawater presented by Bowen (Bowen 1979), which was already expected due to the proximity of the sea.

The physiological factor is related with the biomonitors itself, in this case the lichen, and can be observed in factor 5 with K and P as the pilot elements and also with Rb associated. Potassium and phosphorous are plants macronutrients like C, N, S, Mg and Mn, essential for plants (Franzle and Markert 2002). Potassium is also indicator of cell membrane damage when loss of this element is observed (Nash III 1996). According to Bowen (Bowen 1979), the P/K ratio for lichen lies between 0.087 and 1.23, which is compatible with the obtained value of 0.28.

Factor 6 has As as the pilot element and Ca, Si, Ba, Al, Rb and Sc as the most correlated elements. This factor was associated with agricultural activities. The northern part of the grid has still intense agriculture mainly wine production with the associated fumigations of pesticides and herbicides in the vineyards. These products are one of the possible sources of arsenic (NORD 1987; NORD 1994; Gonzales, Soto et al. 1997), in combination with the use of arsenious anhydride and sodium arsenite in cuts-protection when vineyards are trimmed (www.dgpc.min-agricultura.pt/), and the use of mixed Ca2OH and CuSO4 in control of vineyard diseases.

Factor 7 has Cr as pilot element and some other correlated elements are Si, Zn, Al, Fe, Sc, S, Ni, Ti and Br. According to Nriagu (Nriagu 1989) Cr and Zn are the most important elements associated with iron and steel manufacture although the ratio for these two elements obtained in this work is not in agreement with literature (Nriagu 1989). Other works have pointed out Zn and Cr as coming from metal industry (NORD 1987; NORD 1994; Jeran, Jacimovic et al. 2002) such as steel (Jeran, Jacimovic et al. 1996). Other associated elements are Si, Al, Sc, S and Fe.

Factor 9 has uranium as pilot element and Ca, As, Se, Sb, Mn, Ba, V, Cu and Na as associated elements. This element association points out to cement production since it has Ca associated with coal combustion elements (coal is the main combustion source of cement plant), but still the correlation of uranium with this factor is too high. This industry is situated Southwest part of the sampling grid (site S32) but this factor might be a mixture of different sources.

The use of all data to run MCATFA was base on the assumption that factor loadings were not depending on time, facing or shielded by the wind direction. MCATTFA also run with F3, F6, F9 and T3, T6, T9 separately assuming that the factor loadings were only not depending on time. A comparison between the results provided by the three different combinations was performed by calculating the angles between the factors obtained. The factors can be written as vectors in an n-dimensional space where the co-ordinates are just the elements content. The cosines of the angles (marked bold italic) correspond to a correlation between the factors higher than 0.75 (Table 5.3).

Comparing the all data factors with the F data factors, 6 to 7 factors are very similar for both combinations since the cosine values range from 0.80 to 0.99. Factor 4 (all data) presents a higher cosine value with factor 7 (F data) than with factor 4 (F data) and so the first correlation was the one considered. Factor 7 (all data) and factor 4 (F data) present a correlation of 0.80, lower than the others (of 0.90 or higher). In the case of the comparison of all data factors with T data factors, 5 to 7 factors can be considered similar in both combinations (cosines between 0.74 and 0.98). Factor 6 (all data) resembles factor 6 (T data) but the correlation is only 0.74. There is also a similarity between factor 7 in both combinations although correlation is only 0.77. Factors 8 and 9 (all data) do not present any similarity with any of the factors of the two other combinations.

This is in agreement with the results obtained for F and T comparison where it was found that 5 factors are similar for both combinations (cosines range from 0.87 to 0.96) and 2 factors seem F or T specific with cosine values lower than 0.75. Although factor 4 (F data) is similar to factor 2 (T data) the correlation with all data is not the same. Factor 4 (all data) resembles more factor 7 in the case of F data and factor 2 in the case of T data. Nevertheless the correlation of factor 4 (all data) with factor 4 (F data) is 0.79, which is a considerable value.

Table 5.3 – Cos (N-dimensional vector angles) between the MCATTFA factors for the three different combinations performed: all data (A), facing the wind transplants data (F) and shielded by the wind transplants data (T). Values larger than 0.75 are in bold.

A / F	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
Factor 1	**0.97**	0.36	0.37	0.37	0.03	0.29	0.39	0.51	0.16
Factor 2	0.33	**0.99**	0.26	0.35	0.14	0.40	0.59	0.40	0.09
Factor 3	0.44	0.25	**0.98**	0.33	0.05	0.24	0.43	0.18	0.22
Factor 4	0.41	0.37	0.23	**0.79**	0.15	0.56	**0.80**	0.74	0.09
Factor 5	0.06	0.17	0.18	0.32	**0.97**	0.22	0.11	0.19	0.18
Factor 6	0.40	0.41	0.32	0.37	0.17	**0.90**	0.50	0.39	0.59
Factor 7	0.52	0.38	0.41	**0.89**	0.19	0.39	0.30	0.31	0.31
A / T	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Factor 5**	**Factor 6**	**Factor 7**	**Factor 8**	**Factor 9**
Factor 1	**0.93**	0.39	0.36	0.43	0.02	0.43	0.38	0.45	0.40
Factor 2	0.39	0.48	0.16	**0.84**	0.13	0.49	0.70	0.71	0.27
Factor 3	0.40	0.25	**0.98**	0.26	0.05	0.21	0.30	0.19	0.16
Factor 4	0.38	**0.95**	0.26	0.32	0.16	0.52	0.61	0.46	0.12
Factor 5	0.04	0.20	0.00	0.35	**0.97**	0.22	0.20	0.19	0.01
Factor 6	0.42	0.26	0.36	0.51	0.20	0.74	0.17	0.43	0.50
Factor 7	0.63	0.20	0.46	0.43	0.06	0.34	**0.77**	0.42	0.44
F / T	**Factor 1**	**Factor 2**	**Factor 3**	**Factor 4**	**Factor 5**	**Factor 6**	**Factor 7**		
Factor 1	**0.87**	0.32	0.36	0.39	0.09	0.50	0.49		
Factor 2	0.38	0.50	0.22	**0.88**	0.20	0.48	0.63		
Factor 3	0.37	0.25	**0.96**	0.21	0.17	0.25	0.38		
Factor 4	0.35	**0.95**	0.24	0.46	0.19	0.53	0.28		
Factor 5	0.05	0.21	0.01	0.27	**0.93**	0.21	0.26		
Factor 6	0.37	0.23	0.30	0.43	0.36	0.70	0.58		
Factor 7	0.59	0.26	0.54	0.62	0.13	0.49	0.36		

Table 5.4 presents average contributions (%) to total element occurrence for MCATTFA performed with F data and T data respectively. Observing the fractional variances explained by the factors, for the factors identified as the same in both F and T combinations, it is possible to observe that factors 1, 2 and 5 (F data) have similar strength when compared with the corresponding factors 1, 4 and 5 (T data). Despite the fact that the average contributions of each element might be somewhat different the mean is similar. For instance, for factor 5 the mean of the averaged contributions to total element occurrence is 14% and 12% for F data and T data respectively but K contribution is not the same (77% and 90% for F and T respectively). On the other hand, factors 2 and 3 (T data) are somewhat stronger than the corresponding factors 4 and 3 (F data) although average contribution values don't differentiate that much.

Based on the results above, nine factors were identified using all Sado data sets and seven factors were identified using F and T data separately. The number of samples used (201 against 97 and 104, respectively) might be the reason for this result since the double of samples were available in the first case. Two factors are only identified in all data combination. Five factors were identified for the three combinations performed, oil combustion, 2 soils, marine and physiological factors. The factors identified as agricultural activities and ferrous metal processing and handling, seem to be transplant specific. For these 2 factors, F data presents better correlation with all data than T data. Overall the relative strength of the factors is similar for F and T in common sources. All in all there are no significant differences in F and T average contributions to total element occurrence for the 5 common factors.

Table 5.4 – Averaged contributions (%) to total element occurrence obtained after 500 Monte-Carlo variations for facing the wind transplants (F) and shielded by the wind transplants (T). Indicated loadings were found to be significantly positive (P>95%), values marked with a * are more than 99% significant and values marked with + are 95-99% significant. Pilot elements are marked with a P. Total fraction is given in percentage.

Element	T data								F data							
	Fact 1	Fact 2	Fact 3	Fact 4	Fact 5	Fact 6	Fact 7	Total	Fact 1	Fact 2	Fact 3	Fact 4	Fact 5	Fact 6	Fact 7	Total
Na	1.16*	15.08+	69.41*			17.70*		108			28.50*		25.39*	1.44*	37.22*	93
Al		49.51*		31.52P	13.31*		0.68+	96		32.86*		36.99*		2.46*		73
Si		48.32*	0,03	29.67*	7.30*		0.95*	89		29.06*		41.72*		2.69*		74
P				13.02*	73.82*		0.42+	91		9.23*			43.93*			54
S	1.96*		17.67*	18.02*		2.57+	0.77*	43	1.70*	17.69*	12.44*	16.49*		1.43*		54
Cl			65.31P	4.99*				78		5.49+	34.90P		19.74*			71
K	0.23+				90.05P	2.64+		93					77.18P	1.09*	7.36+	87
Ca	1.28+		11.64*			37.61P	1.91*	60			7.30*	18.98*	15.10*	3.25*	38.61*	85
Sc	1.39*	85.09P			15.34*	6.52*	1.17*	113	1.66*	9.62*	2.69*	44.70P	4.50+	0.92*	23.60*	88
Ti		48.51*		28.60*	14.89*			95		60.35P		13.04*			29.35*	103
V	12.18*		10.77*		4.40+	22.68*	9.70*	60	9.98*		13.35*	27.56*	5.39*	1.29*	34.34*	93
Cr	4.71*	71.40*	5.09*	12.46*			6.59*	102			5.06*	76.61*				92
Mn			4.79*	30.26*		14.65*		60		49.23*	4.13*		24.74*		31.31*	110
Fe	2.66*	79.50*	1.97+	5.98*	4.03+	5.62*	3.20*	103	2.88*	9.60*	3.18*	45.41*		1.09*	22.17*	86
Co	4.22*	53.15*	6.40*	7.73*		16.85*	2.73*	91	7.08*			71.67*	22.70*			103
Ni	5.47*	26.93*	18.90*	38.90*			1.12*	92	5.53*	50.74*	10.46*			1.60*		81
Cu	19.46P			67.28*		26.44*		123	21.66P	50.85*				6.53*		110
Zn			60.42*	10,29			29.51P	116	8.50*	18.84*	32.67*	50.60*		2.79*		124
As	7.57*	24.88*		29.74*		47.02*	2.37+	112		16.68*				15.96P		77
Se	3.42*	49.42*			9.80*			68	0.44+			10.29+			42.47P	56
Br	1.69*	32.72*	26.85*				3.45*	69	0.84*		15.23*	7.63+			30.09*	54
Rb		43.26*			55.67*	13.41*		113	0.31+			29.90*	34.10*	0.84+	17.20*	84
Sb	14.33*	20.51*	13.01*	6.52+		10.21*	6.77*	72	12.23*	12.08+	4.65*				47.85*	83
Ba	3.69*	49.17*	4.11+	9.57*		23.52*	1.93*	93	3.31*	4.10+	4.13*	43.38*	8.30*	1.99*	33.53*	99
Pb	6.60*	7.98+	16.99*	23.66*		4.51+		61	8.88*	16.30*	4.51*			1.46*	25.53*	62
U	2.79*	47.28*				13.09*	5.27*	75			11.48*	0	63.12+	13.32*		115
Mean	4	30	13	15	12	11	3	88	3	15	8	22	14	2	21	85

5.3.5 F and T orientation in reflecting emission-source profiles for different time relations

MCATTFA was used again to compare data from F- and T-orientations. The comparison was made for the common source profiles, both in time relations, and in terms of the relative source contributions to total-element levels. Due to loss of some systems, 39 F-transplants (F3) and 39 T-transplants (T3) were removed after 3 months, 34 F-transplants (F6) and 35 T-transplants (T6) after 6 months and after 9 months exposure 25 F-transplants (F9) and 31 T-transplants (T9) were collected (Marques, Freitas et al. 2004). Since a few sampling sites were available per set, MCATTFA analyses ran with a selection of only 10 elements (Na, Cl, Ca, Sc, V, Fe, Ni, Cu, As, and Pb) following Henry (Henry 1991) in his reasoning towards a rule-of-thumb relationship between number of sampling sites and number of elements to be taken into the factor analysis. The approach to determine the number of factors was based on the selection of common factors in F- and T-orientations, and further essentially followed Kuik et al. (Kuik, Blaauw et al. 1993; Kuik, Sloof et al. 1993). Source profiles (factor loadings, Table 5.5) could be associated to industrial emissions (Cu, Pb, Ni and V associated elements), sea-salt spray (Na and Cl associated elements), soil (Sc and Fe associated elements) and agricultural activities (As and Ca associated elements). Once more, these factor interpretations largely follow Nriagu (Nriagu 1989), who showed V, Ni, Pb and Cu as highly correlated with industrial emissions especially oil combustion but presently Ni/V ratios are not in agreement with ratios for European aerosol (0.4 to 0.7) (Rahn and Huang 1999). Na and Cl are the elements more associated to sea salt spray although the Na/Cl ratio is also not in agreement with literature (Bowen 1979). The As, Ca factor could be related to a pesticide- or herbicide-associated use of As in vineyards (Ruhling 1987; Ruhling 1994).

Average contributions of common factors (source profiles) to total-element occurrences in F- and T-oriented transplants were calculated and compared. Table 5.6 gives data on these comparisons: factor contributions were calculated for both F- and T-oriented transplants, for each exposure period.

Table 5.5 - Averaged contributions (%) to total element occurrence obtained after 500 Monte-Carlo variations for facing the wind transplants (F) and shielded by the wind transplants (T) for 3, 6 and 9 months exposure separately. Indicated loadings were found to be significantly positive (P>95%), values marked with a * are more than 99% significant and values marked with + are 95-99% significant. Pilot elements are marked with a P. Total fraction is given in percentage.

	F3					T3				
	1	2	3	4	Total	1	2	3		Total
Na	0.07	68.70P	19.73	5.24+	94	5.37+	93.74*	0.96		100
Cl	0.54	76.66*	22.60	5.18+	105	2.36	97.94P	0.37		101
Ca	0.01	2.74	25.17+	15.02+	43	2.99	8.64*	44.70*		56
Sc	5.00	3.44	82.37P	1.70+	92	6.87	0.01	98.72P		106
V	16.43*	10.37	23.83	0.46	51	14.99*	9.92	6.54		31
Fe	7.37*	8.31*	85.42*	1.56+	103	8.24+	2.66+	94.73*		106
Ni	45.64*	21.37*	4.71	0.94	73	32.73*	20.69*	36.45*		90
Cu	65.64*	15.38*	39.79*	1.37	122	65.16P	17.92*	48.08*		131
As	13.98	14.31	13.62	63.55P	105	33.45*	3.15	20.99+		58
Pb	55.66P	0.05	17.66	1.34	75	28.08*	9.32*	15.25+		53
Mean	21	22	33	10	86	20	26	37		83
	F6					T6				
	1	2	3	4	Total	1	2	3	4	Total
Na	0.03	100.82*	0.28	0.17	101	0.46	82.76*	36.01*	2.56	122
Cl	0.32	93.96P	0.13	0.88	95	0.00	63.78P	0.00	0.03	64
Ca	13.82	13.12*	0.00	41.40*	68	0.78	1.65	22.87*	18.42*	44
Sc	84.13P	0.05	1.93	2.52	89	3.48+	0.00	86.00P	0.78	90
V	17.61	3.76*	15.13*	0.00	36	24.10P	0.00	1.50	0.02	26
Fe	89.52*	0.01	3.51+	1.53	94	7.28*	2.36*	85.55*	2.70	98
Ni	87.87*	3.62*	10.18*	1.22	103	25.19*	25.92*	33.72*	13.12*	98
Cu	60.85	0.05	31.96P	15.96+	109	56.40*	1.12	6.59	48.99*	113
As	2.80	0.00	10.82*	83.79P	97	11.37*	0.26	21.95*	64.83P	98
Pb	73.17*	0.00	11.96*	8.13*	93	28.19*	17.27*	22.57*	14.56*	82
Mean	43	22	8	16	89	16	19	32	17	84
	F9					T9				
	1	2	3	4	Total	1	2	3	4	Total
Na	0.00	0.40	51.16+	24.65P	76	1.95*	98.72P	9.41	0.15	110
Cl	0.37	1.35	115.82P	0.80	118	0.00	102.88*	0.21	0.34	103
Ca	0.15	20.22*	26.51*	7.39*	54	0.35	32.76*	5.77	7.63P	46
Sc	0.41	22.92P	22.78+	0.48	46	2.80*	1.11+	74.30P	0.25	78
V	9.18*	27.29*	36.58*	0.12	73	20.23*	9.09*	45.69*	5.37*	80
Fe	2.23*	23.67*	18.80+	1.16	46	6.40*	0.59	77.78*	1.24*	86
Ni	10.91*	25.94*	60.90*	5.48*	103	17.29*	40.06*	21.24*	2.44*	81
Cu	33.57*	65.70*	0.33	24.48*	124	49.97P	0.00	49.54+	9.85*	109
As	2.80+	49.03*	0.00	27.06*	79	11.69*	0.66	81.26*	13.49*	107
Pb	12.90P	1.81	12.31+	0.98	28	14.81*	23.44*	0.63	0.04	39
Mean	7	24	35	9	75	12	31	37	4	84

Table 5.6 - Comparison in relative factor contributions in F- and T-oriented lichen transplants*. All factor contributions in F - T comparisons with slopes with P values < 0.05 should be considered as non-identical (in blank).

P – values				
	T3 – 1	T3 – 2	T3 - 3	
F3 – 1	0.053			
F3 – 2		0.98		
F3 – 3			0.088	
F3 – 4				
	T6 – 1	T6 – 2	T6 - 3	T6 - 4
F6 – 1				
F6 – 2		0.31		
F6 – 3				
F6 – 4				0.33
	T9 – 1	T9 – 2	T9 - 3	T9 - 4
F9 – 1	0.17			
F9 – 2			0.55	0.73
F9 – 3		0.11		
F9 – 4				0.089

*Relative contributions obtained for F- and T orientations after initial normalisation of factor-explained total variances per element to 100%. In the Table, the Fa-b or Ta-b notation indicates F- or T-oriented transplants, the character "a" denotes the months of exposure and the character "b" the bth source factor; thus, F3-1 means the F transplant after 3 months of exposure and the 1st source factor. In MCTTFA, identical factors are recognised by the calculation of so-called "factor-conflicts" (Kuik et al. 1993a, 1993b), but it should be noted here that common factors do not need to carry the same factor number. Industrial emissions: F3-1, T3-1, F6-3, T6-1, F9-1 and T9-1, sea-salt spray: F3-2, T3-2, F6-2, T6-2, F9-3 and T9-2, soil: F3-3, T3-3, F6-1, T6-3, F9-2 and T9-3 and agricultural activities: F3-4, F6-4, T6-4, F9-4 and T9-4. In the calculations the contribution vectors were compared by calculating the slopes between two outcomes: unit slope values indicate identical contributions.

The data in Table 5.6 indicates that, apart from difficulties in interpreting two factor contributions in the 6 months of exposure, and a double significance occurrence for the F-T comparison in the 9-month exposure, in most cases F- and T-oriented transplants show comparable source contributions over all exposure periods considered. Nevertheless, this is not the case of the industrial and soil factors in the 6 months data.

Although it cannot be considered that F and T are different, is it possible that the factor values are higher for facing or shielded by the wind lichen transplants? To answer this question the fractional calculated concentration was calculated:

$$FCC = \frac{\text{calculated concentration for each sample and element of factor i}}{\text{measured concentration}}$$

Due to the fact that the sum of the calculated concentrations over all the factors is not always 100%, the FCC had to be "corrected" for each element with the total averaged contribution:

$$FCC = \frac{\text{calculated concentration for each sample and element of factor i}}{\text{measured concentration}} \times \frac{100\%}{\text{explained \%}}$$

This procedure was only applied to the cases where the slopes (calculated using the average contribution to total element occurrence in Table 5.6) were statistically equal to 1 or with the P values close to 0.05, which performed a total of 11 possible situations. Tables 5.7 and 5.8 show the results for the 10 selected elements for which regression is significant and the slope is not equal to 1 (where $F = y$ and $T = x$ in $y = mx$). It is possible to observe that for the 10 selected elements, F is sometimes higher than T and vice-versa depending on the obtained slope and considered element and so none of the sets can be considered to present higher factor values than the other.

Apart from the data above, which predict that F- and T-oriented transplants do not differ in factor contributions, the initial data were interpreted also in terms of time relations. Here, factor contributions were compared between exposure periods, to judge shifts in relative source importance with time.

Table 5.9 gives results for 3,6- 6,9- and 3,9-month exposure periods comparisons for both F- and T-oriented transplants, and generally indicates that relative source contributions do not change in time, although there are some exceptions.

Table 5.7 – Regression, intercept and slopes calculated using fractional calculated concentration (FCC) for Na, Cl, Ca and Sc. The regression is statistically significant if $P_{regression}<0.05$. The slope is not equal to 1 if $P_{slope}<0.05$.

		3T1-3F1	3T2-3F2	3T3-3F3	6T1-6F3	6T2-6F2	6T3-6F1	6T4-6F4	9T1-9F1	9T2-9F3	9T3-9F2	9T4-9F4
Na	intercept			9.53E-02	-2.95E-03		1.15E-04	1.07E-03	5.57E-07			2.44E-01
	error int.			4.02E-02	1.54E-03		1.22E-04	6.85E-04	2.08E-06			3.96E-02
	N			39	32		32	32	24			24
	slope			1.32E+01	1.39E+00		7.07E-04	5.62E-02	2.91E-04			4.01E+01
	error slope			2.03E+00	1.20E-01		2.70E-04	1.43E-02	7.00E-05			1.50E+01
	P slope			1.28E-31	2.53E-18		2.09E-112	3.26E-58	2.78E-97			6.17E-12
	P regr.			1.35E-07	1.24E-12		1.47E-02	4.56E-04	4.25E-04			1.38E-02
Cl	intercept	3.10E-03	-5.68E-02	1.49E-01	-1.60E-03	8.11E-01			-9.00E-04			6.40E-03
	error int.	1.40E-03	3.30E-01	5.05E-02	6.00E-04	8.55E-02			4.00E-04			1.00E-03
	N	35	35	35	29	29			23			23
	slope	1.15E-01	7.66E-01	2.76E+01	9.30E+01	1.94E-01			1.61E+03			3.75E-01
	error slope	3.28E-02	3.42E-01	7.88E+00	5.76E+00	9.12E-02			3.96E+01			7.46E-02
	P slope	1.82E-50	2.85E-04	2.35E-20	1.77E-35	2.45E-28			4.30E-37			4.36E-22
	P regr.	1.28E-03	3.21E-02	1.35E-03	2.15E-15	4.28E-02			1.95E-21			5.63E-05
Ca	intercept	1.05E-04	3.63E-02			1.30E-01			-9.26E-04	2.73E-01		
	error int.	3.50E-05	1.46E-02			3.69E-02			3.41E-04	8.80E-02		
	N	35	35			31			25	25		
	slope	2.57E-03	2.30E-01			2.30E+00			5.26E-01	3.15E-01		
	error slope	5.30E-04	7.43E-02			9.07E-01			2.04E-02	1.08E-01		
	P slope	3.82E-113	2.12E-36			6.21E-09			1.49E-34	4.08E-21		
	P regr.	3.04E-05	4.00E-03			1.67E-02			1.69E-18	7.74E-03		
Sc	intercept	3.41E-02			-3.19E-03	2.68E-04			-4.14E-03			
	error int.	8.63E-03			7.57E-03	1.06E-04			1.76E-03			
	N	35			32	32			25			
	slope	2.99E-01			6.96E-01	4.47E+00			3.75E-01			
	error slope	1.08E-01			1.33E-01	1.13E+00			2.41E-02			
	P slope	1.59E-29			5.00E-14	1.42E-17			1.11E-35			
	P regr.	9.32E-03			1.21E-05	4.15E-04			1.06E-13			

Table 5.8– Regression, intercept and slopes calculated using fractional calculated concentration (FCC) for V, Fe, Ni, Cu, As and Pb. The regression is statistically significant if $P_{regression}<0.05$. The slope is not equal to 1 if $P_{slope}<0.05$.

		3T1-3F1	3T2-3F2	3T3-3F3	6T1-6F3	6T2-6F2	6T3-6F1	6T4-6F4	9T1-9F1	9T2-9F3	9T3-9F2	9T4-9F4
V	intercept					7.80E-02			-2.19E-02			
	error int.					2.27E-02			1.93E-02			
	N					32			25			
	slope					5.20E+02			4.78E-01			
	error slope					2.38E+02			5.61E-02			
	P slope					1.75E-13			4.92E-25			
	P regr.					3.70E-02			1.40E-08			
Fe	intercept	4.82E-02		5.41E-01	-3.38E-04	5.49E-05			-1.93E-02			
	error int.	1.14E-02		1.27E-01	1.31E-02	2.20E-05			9.16E-03			
	N	35		35	32	32			25			
	slope	2.78E-01		3.27E-01	5.14E-01	3.13E-03			8.86E-01			
	error slope	1.25E-01		1.40E-01	1.27E-01	7.70E-04			7.15E-02			
	P slope	6.77E-28		2.61E-25	2.68E-20	2.90E-98			3.14E-08			
	P regr.	3.30E-02		2.56E-02	3.26E-04	3.25E-04			1.17E-11			
Ni	intercept	4.07E-01		2.79E-02	-2.66E-02		7.30E-01		-2.82E-02	3.20E-01		
	error int.	8.69E-02		1.35E-02	3.43E-02		1.00E-01		1.55E-02	1.33E-01		
	N	35		35	30		30		23	23		
	slope	4.86E-01		1.08E-01	4.82E-01		4.99E-01		5.74E-01	6.55E-01		
	error slope	2.32E-01		2.92E-02	1.24E-01		2.29E-01		6.74E-02	2.15E-01		
	P slope	7.85E-15		2.50E-52	4.26E-20		9.76E-13		1.93E-19	1.18E-07		
	P regr.	4.41E-02		7.92E-04	5.78E-04		3.80E-02		2.97E-08	6.19E-03		
Cu	intercept					3.88E-04	5.61E-01		-1.04E-02			
	error int.					1.16E-04	6.82E-02		3.40E-02			
	N					30	30		23			
	slope					1.95E-02	1.92E+00		3.37E-01			
	error slope					8.09E-03	7.11E-01		9.25E-02			
	P slope					3.40E-62	8.62E-08		1.27E-20			
	P regr.					2.30E-02	1.17E-02		1.54E-03			

		3T1-3F1	3T2-3F2	3T3-3F3	6T1-6F3	6T2-6F2	6T3-6F1	6T4-6F4	9T1-9F1	9T2-9F3	9T3-9F2	9T4-9F4
As	intercept								-8.24E-03			
	error int.								1.22E-02			
	N								22			
	slope								4.10E-01			
	error slope								8.56E-02			
	P slope								2.13E-19			
	P regr.								1.11E-04			
Pb	intercept			1.34E-01	1.92E-02	2.99E-06			-1.15E-01			3.92E-02
	error int.			4.64E-02	4.23E-02	1.28E-06			5.90E-02			8.40E-03
	N			36	30	30			23			23
	slope			4.46E-01	2.65E-01	1.89E-05			1.50E+00			6.16E+00
	error slope			1.46E-01	1.23E-01	0.00E+00			1.21E-01			2.37E+00
	P slope			1.38E-22	1.82E-24				1.99E-15			5.47E-10
	P regr.			4.29E-03	3.95E-02	5.51E-04			6.50E-11			1.43E-02

Table 5.9. Comparison in shifts with exposure periods relative to factor-contributions in F- and T-transplants*. P-values > 0.05 (shown) indicate unit slope values. All factor contributions in F - T comparisons with slopes with P < 0.05 values should be considered as non-identical. The table only shows P > 0.05 values. For explanation of notations and source profiles, see Table 5.6.

P – values				
	F3 – 1	F3 - 2	F3 - 3	F3 - 4
F6 - 1				
F6 - 2		0.74		
F6 - 3	0.14			
F6 - 4				0.17
	F6 – 1	F6 - 2	F6 - 3	F6 - 4
F9 - 1	0.17			
F9 - 2				
F9 - 3		0.069		
F9 - 4		0.082		0.38
	F3 – 1	F3 - 2	F3 - 3	F3 – 4
F9 - 1	0.59			
F9 - 2				
F9 - 3				
F9 - 4				0.052
	T3 – 1	T3 - 2	T3 - 3	
T6 - 1				
T6 - 2		0.29		
T6 - 3			0.14	
T6 - 4				
	T6 – 1	T6 - 2	T6 - 3	T6 – 4
T9 - 1	0.33			
T9 - 2		0.10		
T9 - 3				
T9 - 4	0.27			
	T3 – 1	T3 - 2	T3 - 3	
T9 - 1	0.20			
T9 - 2		0.068		
T9 - 3				
T9 - 4	0.37		0.56	

*In the calculations the contribution-vectors were compared by calculating the slopes between two outcomes; a unit slope values indicate identical contributions.

It is stressed that, although in general no shifts in time relations are observed, some elements do present a higher accumulation sufficient enough to produce a change in the factor contribution of related factors. For instance, F3-2 and F9-3 in Table 5.9 both related to sea-salt spray were considered non-identical. The sampling area is next to the sea and it is well known that sodium and chlorine are elements easily

accumulated and also easily leached from lichens (Figueira 2002). A high accumulation rate and no leaching effects (by the use of a proper exposure device equipped with a "hat") are expected for the sea-related elements. For facing-the-wind transplants (F) this has led to a shift in element contents observed between 3 and 9 months of exposure for the sea factor.

5.4 Conclusions

Generally reviewing the present results, the data indicate that F- and T positioned transplants do not differ regarding the accumulation of Na, Mg, P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu and Ta, regardless of the examined periods of exposure. Differences between F and T transplants were obtained for Cr and Zn. For the remaining elements, high variability is observed. This observation answer a hypothesis raised to explain the differences between replicates data found by Reis (Reis 2001), who reasoned that both positioning and precipitation were influencing accumulation results. Although the present results show consistent data for a large variety of elements, one may reason that differences between study hanging systems may have caused some differentiation in transplant behaviour. The present study implied transplant protection against direct rain, and it was of different length of the exposure periods, and the location of the sampling sites.

All in all, for the majority of elements determined, the data indicate the absence of any significant differences in the behaviour of F and T positioned transplants. F and T positioned transplants accumulate elements to similar levels, and they both do not show any appreciable accumulation of litophylic elements. Moreover, F and T positioned transplants both do not show appreciable losses of K and P, and in general they show similar source profiles in MCATTFA approaches and similar outcomes on calculated source-contributions to total element concentrations, although some exceptions are observed. Uptake and release processes of elements in lichens are mechanisms still not very well known, especially in field conditions (Wolterbeek, Garty et al., 2002) and more work should be done in this area in order to be able to fully understand and explain the differences found.

References

Bowen, H.J.M. (1979) *Environmental Chemistry of the Elements*. London, Great Britain: Academic Press.

Caeiro, S., Costa, M.H., Goovaerts, P., and Martins, F. (2005a) Benthic biotope index for classifying habitats in the sado estuary: Portugal. *Marine Environmental Research* **60**: 570-593.

Chueinta, W., Hopke, P.K., and Paatero, P. (2000) Investigation of sources of atmospheric aerosol at urban and suburban residential areas in Thailand by positive matrix factorization. *Atmospheric Environment* **34**: 3319-3329.

De Corte, F. (1987) *The k0 - Standardization Method, a Move to the Optimisation of Neutron Activation Analysis*. Gent, Belgium: Gent University, pp. 1- 464.

Erdtmann, G., and Petri, H. (1986) Nuclear activation analysis: fundamentals and techniques, Part 1. In *in Treatise on Analytical Chemistry*. Elving, P.J. (ed). USA: John Wiley & Sons Inc., pp. 422-626.

Figueira, R. (2002) *Desenvolvimento de um Sistema de Biomonitorização Ambiental.* PhD Thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal pp. 51-107, in Portuguese.

Figueira, R., Sousa, A.J., Pacheco, A.M.G., and Catarino, F.M. (1999) Saline variability at ground level after kriging data from *Ramalina* spp. biomonitors. *The Science of the Total Environment* **232**: 3-11.

Figueira, R., Pacheco, A.M.G., Sousa, A.J., and Catarino, F.M. (2002) Development and calibration of epiphytic lichens as saltfall biomonitors - dry-deposition modelling. *Environmental pollution* **120**: 69-78.

Franzle, S., and Markert, B. (2002) The biological system of the elements (BSE) - a brief introduction into historical and applied aspects with special reference on "ecotoxicological identity cards" for different element species (e.g. As and Sn). *Environmental pollution* **120**: 24-45.

Freitas, M.C., and Nobre, A.S. (1997) Bioaccumulation of heavy metals using *Parmelia sulcata* and *Parmelia caperata* for air pollution studies. *Journal of Radioanalytical and Nuclear Chemistry* **217**: 17-20.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (1999) Distribution in Portugal of some pollutants in the lichen *Parmelia sulcata*. *Environmental pollution* **106**: 229-235.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace Elements - their Distribution and Effects in the Environment*. Friese, M.a. (ed). Amsterdam, Netherlands: Elsevier Science, pp. 187-213.

Freitas, M.C., Marques, A.P., Reis, M.A., Farinha, M.M. (2008) Atmospheric dispersion of pollutants in Sado estuary (Portugal) using biomonitors. *International Journal of Environment and Pollution* **32** (4) 434-455.

Garcia, J.M., Coelho, L.M.R., Ribeirete, A., Nogueira, M., Machado, P., and Magalhães, R. (2002) Air quality analysis in the city of Setúbal - scenarios generation to support municipality decisions. In *8Th International Conference*

on Hamonisation within Atmospheric Dispersion Modelling Regulatory Purposes, pp. 468-473.

Gonzales, E.B., Soto, E.G., Mahía, P.L., and Rodrígues, D.P. (1997) Total arsenic and selenium levels in atmospheric particulate matter of La Coruña (Spain). *The Science of the Total Environment* **208**: 207 - 211.

GraphPad Software Inc. (1999) *Analysing Data with Graphpad Prism*. San Diego, USA

Henry, R.C. (1991) Receptor modelling for air quality management. In *Multivariate receptor models*. Hopke, P.K. (ed). Amsterdam, Netherlands: Elsevier Science BV, pp. 117-147.

Jeran, Z., Jacimovic, R., Batic, F., and Mavsar, R. (2002) Lichens as integrating air pollution monitors. *Environmental Pollution*: 107-113.

Jeran, Z., Jacimovic, R., Batic, F., Smodis, B., and Wolterbeek, H.T. (1996) Atmospheric heavy metal pollution in Slovenia derived from results for epiphytic lichens. *Fresenius Journal of Analytical Chemistry* **354**: 681-687.

Johansson, S.A.E., Campbell, J.L., and Malmqvist, K.G. (1995) *Particle Induced X-ray Emission Spectrometry (PIXE) in Chemical Analysis*. USA: John Wiley & Sons.

Kuik, P., and Wolterbeek, H.T. (1995) Factor analysis of atmospheric trace-element deposition data in the Netherlands obtained by moss monitoring. *Water, Air and Soil Pollution* **83**: 323-346.

Kuik, P., Sloof, J.E., and Wolterbeek, H.T. (1993) Application of Monte Carlo-assisted Factor Analysis to large sets of environmental pollution data. *Atmospheric Environment* **13**: 1975-1983.

Kuik, P., Blaauw, M., Sloof, J.E., and Wolterbeek, H.T. (1993) The use of Monte Carlo methods in factor analysis. *Atmospheric Environment* **13**: 1967-1974.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., and Verburg, T.G. (2004a) MCTTFA applied to differential biomonitoring in Sado estuary region. *Journal of Radioanalytical and Nuclear Chemistry* **259**: 35-40.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., Verburg, T.G., and De Goeij, J.J.M. (2004b) Lichen-transplant biomonitoring in the assessment of dispersion of atmospheric trace-element pollutants: effects of orientation towards the wind direction. *Journal of Atmospheric Chemistry* **49**: 211–222.

Nash III, T.H. (1996) *Lichen Biology*. England: Cambridge University Press.

Nriagu, J.O. (1989) Natural versus anthropogenic emissions of trace metals to the atmosphere. In *Control and Fate of Atmospheric Trace Metals*. J. M. Pacyna and B. Ottar (ed). Amsterdam, Netherlands: Kluwer Academic Publishers, pp. 3-13.

Rahn, K.A. (1999) A graphical technique for determining major components in a mixed aerosol. I. Descriptive aspects. *Atmospheric Environment* **33**: 1141-1455.

Rahn, K.A., and Huang, S. (1999) A graphical technique for distinguishing soil and atmospheric deposition in biomonitors from the plant material. *The Science of the Total Environment* **232**: 79-104.

Reis, M.A. (2001) *Biomonitoring and Assessment of Atmospheric Trace Elements in Portugal - Methods, Response Modelling and Nuclear Analytical Techniques.* PhD Thesis, Delft University of Technology, Delft, The Netherlands pp. 1-230.

Reis, M.A., Alves, L.C., and Jesus, A.P. (1996a) Matrix effects correction for quantitative TTPIXE analysis. *Nuclear Instruments and Methods in Physiscs Research B* **109/110**: 134-138.

Reis, M.A., Alves, L.C., Wolterbeek, H.T., Verburg, T.G., Freitas, M.C., and Gouveia, A. (1996b) Main atmospheric heavy metal sources in Portugal by biomonitor analysis. *Nuclear Instruments and Methods in Physiscs Research B* **109/110**: 493-497.

Ruhling, A. (1987) *NORD 1987 Survey of Atmospheric Heavy Metal Deposition - Monitored by Moss Analysis*: The Nordic Council of Ministers.

Ruhling, A. (1994) *NORD 1994 Atmospheric Heavy Metal Deposition in Europe - Estimation Based on Moss Analysis*. Copenhagen: The Nordic Council of Ministers.

Wolterbeek, H. Th., Garty, J., Reis, M. A., Freitas, M. C., 2002: Biomonitors in use: Lichens and metal air pollution, in Markert, Breure and Zechmeister (eds.), *Bioindicators and Biomonitors*, Elsevier Science BV, Amsterdam, The Netherlands, pp. 377–419.

Weiss, N.A. (1999) Introductory Statistics. In.: Addison Wesley Longman, Inc., pp. 180-185.

Woolson, R.F., Clark, W.R. (2002) *Statistical Methods for the Analysis of Biomedical Data*: Wiley & Sons, Inc., USA.

Chapter 6

Discussion, Conclusions and Outlook

6.1 Biomonitoring

The emphasis that is put on lichens as bioindicators and biomonitors, has much to do with their abundance both in remote areas and in areas near pollution sources, where the variety of responses of different species towards pollution permits detailed patterns to be obtained even at low levels of pollution (Batzias and Siontorou 2007). Lichens are one of the most valuable long-term biomonitors of atmospheric pollution (Bargagli 1989; Henderson 1996; Beeby 2001; Conti and Cecchetti 2001; Wolterbeek 2002; Batzias and Siontorou 2007): 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 trace element content. Because the concentrations of trace elements in lichen thalli are directly correlated with environmental levels of these elements (Wolterbeek 2002), lichens are very useful for monitoring not only spatial patterns but also temporal trends of trace element deposition (Zschau, Getty et al. 2003).

The present thesis therefore focused on lichens as biomonitor organisms; its various chapters deal with aspects such as exposure characteristics (Chapter 4), vitality (Chapter 3) and elemental analysis (Chapter 2). The emphasis was on lichen transplants: the use of transplants permits sampling at any selected site, standardisation of the initial material, and the study of the dynamics of the responses.

6.2 Exposure

The exposure protocols of lichen transplants used in monitoring surveys have raised growing attention, thereby focusing on both aspects of transplant vitality during the course of the survey, and the transplant orientation towards wind direction and fluxes. These set-ups in the field are shown as of significant relevance for the eventual results in (element-specific) transplant air pollution biomonitoring (Chapter 4 and Chapter 5, and see (Ayrault, Clochiatti et al. 2007)). The use of a (non-metallic) roof system, which allows air to circulate, but protects the transplant lichen from direct rain influxes appeared positive, but the improvement was element-specific: for certain elements hardly any gain was established (see Chapter 4 and Chapter 5). It should be noted here that applying roof systems changes the fully natural appearance of a biomonitor network: the network starts being more vulnerable to vandalism and theft. Therefore, roof systems may be applied predominantly in well-kept and guarded sites.

On basis of the elemental accumulation data, transplant set-ups which allow free elemental influx may be preferred for studies focused on specific elements such as Cr, Fe, La and V and roofed transplant set-ups may be preferred for elements such as K, Pb and Se. In relation to elemental total deposition, free influx systems should be preferred for e.g. Ca, Fe and Mn, and the roofed transplant systems for Na, Ni and V. The transplant orientation towards wind direction appeared to have no significant influence on the element levels in the transplants for a large range of elements except for Zn and Cr (Chapter 5 and see (Marques, Freitas et al. 2004b)).

6.3 Case study

Transplant positioning was not only studied in dedicated small scales, but was also investigated on a bigger scale: a grid was designed to monitor the Sado industrial region, a region indicated by various other studies as strongly polluted (Ruhling 1994; Reis, Alves et al. 1996; Freitas and Nobre 1997; Figueira, Sousa et al. 1999; Freitas, Reis et al. 1999; Freitas, Reis et al. 2000; Reis 2001; Figueira 2002; Figueira, Pacheco et al. 2002). In this study (Chapter 5), the transplants were exposed in “horizontal influx” mode (exposure system with a cover) and orientated

according to the wind direction (facing or shielded from the wind), by a free-rotating device. The study comprised more exposure sites (use of a grid), a prolonged period of exposure (9 months exposure instead of 5). The outcomes indicated that for the majority of the elements determined, differences in wind-directional positioning of the transplants did not result in significant differences in element concentrations and geographical patterns (Chapter 5 and (Marques, Freitas et al. 2004a; Marques, Freitas et al. 2004b)).

The geographical patterns and concentrations were used and processed by Monte Carlo Added Target Transformation Factor Analysis (MCATTFA), to assess possible pollution source profiles (Kuik, Blaauw et al. 1993; Kuik, Sloof et al. 1993; Sloof 1993; Kuik and Wolterbeek 1995; Wolterbeek, Bode et al. 1996; Reis 2001; Wolterbeek and Verburg 2004; Sarmento, Wolterbeek et al. 2008). The main objective here was to test the possibility that wind-directional positioning of the transplants could be used to enhance the assessment potential for either local or remote (and possibly diffuse) sources. The underlying reasoning was that wind-directed (facing) transplants may be "highlighting" local sources rather than remote ones. For both set-ups, the MCATTFA results show similar source profiles and similar calculated source-contributions to total element concentrations in transplants (Chapter 5): these data again support the idea that transplant positioning does not affect results neither in terms of concentrations, nor in factor compositions (source profiles).

6.4 Vitality of lichens

The results presented in this thesis strongly suggest that the comparability of lichen vitality in large geographical areas may be limited: lichen performance may be governed by the area's geographical or time-related variability in temperature and precipitation. Wet deposition may severely affect lichen elemental levels (Chapter 3, (Marques, Freitas et al. 2005)), possibly related to flush-out effects (Sloof 1993; Reis 2001; Figueira 2002; Bergamaschi, Rizzio et al. 2007; Branquinho, Gaio-Oliveira et al. 2007; Pacheco, Freitas et al. 2007). On the other hand, desiccation of lichens may cause disruption of the plasma membrane (Bargagli 1998; Marques,

Freitas et al. 2005; Godinho, Wolterbeek et al. 2008) and so this point should be kept in attention when exposing lichens using a covering device.

These observations make that any comparison of outcomes in terms of element concentrations for time- or spatial series of lichen samples should be accompanied by a comparably careful monitoring of (preceding) ambient conditions, and assessment of the lichen's vitality. The lichens response in terms of element accumulation/release depends on vitality: only if vitality is comparable for the full sampling area/time series, the direct processing of the lichen's elemental contents in terms of geographical/time patterns of (element) atmospheric concentrations/deposition may yield meaningful results (Marques, Freitas et al. 2005). The point to be raised here is which measure of lichen vitality could be a meaningful indication of lichen's comparability: in the literature many assessments are proposed (Garty, Cohen et al. 1998; Branquinho, Catarino et al. 1999; Garty, Weissman et al. 2000; Mulgrew and Williams 2000; Carreras and Pignata 2001; Garty 2001; Raa, Geiserb et al. 2005; Tretiach, Adamo et al. 2007). In practise, however, and especially in studies where large numbers of lichen transplants (or *in situ* samples lichens) have to be processed, high through-put vitality tests may be preferred. Electric conductivity has been pointed out as the most sensitive parameter for physiological response to environmental stress, when compared to NDVI (normalized difference vegetation index) and chlorophyll degradation, being also related to the whole lichen and not to just the photobiont as are many other parameters (Mulgrew and Williams 2000). Also it is a very simple and easy to handle test and time consuming negligible. So in the present thesis, vitality testing was by conductivity measurements and potassium-efflux determinations: the reasoning was that this kind of testing was as close as possible to any effect on element intake/release phenomena.

On basis of the above, the design of a biomonitor experiment should involve transplants of similar and well-defined initial condition: 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 (Chapter 3, (Marques, Freitas et al. 2005; Godinho, Wolterbeek et al. 2008)). Understanding all these aspects is

fundamental to the development of appropriate protocols to biomonitor chemical element deposition with lichen transplants.

6.5 Sample analysis: multielemental techniques?

In many environmental (bio)monitoring studies on elements, nuclear techniques are regarded as especially useful (Freitas, Reis et al. 2000; IAEA 2001; Smodis 2007): the solid samples need not be digested, and the general sensitivity, precision and accuracy is compatible with what is needed, based on available sample sizes and number of samples. There is, however, no single technique by which all (multi)elemental information can be obtained. In many larger-scaled studies, contributors make use of a variety of analytical techniques, and much effort should be devoted to the testing of the comparability of outcomes (Ruhling 1987; Ruhling 1994; Ruhling and Tyler 2004; Harmens, Norrisa et al. 2007).

Also for INAA (Instrumental Neutron Activation Analysis) should be remarked that it cannot yield information on all elements (INAA is insensitive or has low sensitivity to elements such as B, Be, Cd, Cu, Ni, Pb, Si and Tl, etc.) some of which are of potential interest from an atmospheric (bio)monitoring's point of view. Therefore, the use of a complementary technique is asked for. Maintaining the advantages of nuclear approaches, the thesis focused on the PIXE (Proton Induced X-ray Emission) technique, by which, in addition to INAA, information on elements such as Cu, Ni, S, Si, P, Pb, etc. can be generated. The question raised was whether PIXE could be seen as fully complimentary to INAA, that is, does PIXE yield results similar to INAA for elements both techniques can be used for? In Chapter 2, INAA and PIXE are discussed, their principal differences (large sample mass *versus* small sample mass, bulk- versus surface-related information), the relevant sample characteristics (homogeneity), and the comparability of outcomes for selected elements (Cl, Fe, K, Mn, and Zn). In general terms, and notwithstanding both the limited amount of material "seen" in the PIXE analysis and the grain-size distributions in milled and sieved samples, PIXE and INAA yielded comparable results, the latter judged by a variety of statistical test approaches (Chapter 2 and (Marques, Freitas et al. 2007)). The study also indicates, however, that much effort should be devoted to harmonize analytical techniques, in

the sense that protocols should be developed and tested which ensure the techniques' comparability under all experimental conditions. Here, the point should be that expressions of the laboratory's general level of performance are not enough: they should be supplemented with inter-laboratory verification of the outcomes in comparative analytical testing of the sample materials of interest.
A particular and additional point of interest emerged from Chapter 2: in the set of samples of the used reference material IAEA-336 and various grain-size fractions prepared from it, the elemental content was shown to change with grain size. These changes are discussed as possibly related to the size-related contribution of lichen components (algae, fungi) in the eventual sample, which has not been anticipated before. The decreasing partial abundance of fungus material in samples of decreasing grain size (probably due to the predominant discarding of fungal material in size-fraction preparation protocols), and the large fungal accumulation capacity for specific elements (Asta and Garrec 1980; Goyal and Seaward 1981; Richardson, Kiang et al. 1985; Garty and Delarea 1991; Asta 1992; Clark, Mangelson et al. 1999; Garty 2001; Budka, Przybylowicz et al. 2002; Paul, Hauck et al. 2003; Marques, Freitas et al. 2007) might explain the observed phenomenon.
The conclusion to be drawn here, is that, apparently, during the careful preparation of the different grain size lichen materials from the original reference material, the milling and sieving processes may have lead to changes of the bulk initial material, and the question to be raised is whether the eventual IAEA-336 can be still regarded as a full analytical reference for all possible techniques (including matrix-sensitive techniques such as AAS, ICP and/or MS), to be used for unprocessed lichen materials.

6.6 Basic thoughts on lichen-based air pollution studies

On basis of presented results, the thesis generates several thoughts on approaches in future lichen-based air pollution studies:

- Lichen material should be collected with attention for the positioning of native lichens, (e.g. horizontal, vertical, covered, not covered, shaded, not-shaded etc). This applies both for studies in which *in situ* sampling is

performed (direct data comparability), as for studies in which the sampled material is set out again as a transplant (comparability of start-up material).

- In collecting native lichens, or in transplanting, the material's orientation towards (predominant) wind direction has no significant effect on the element levels in the lichen transplants for a large range of elements except for Zn and Cr (Chapter 5 and (Marques, Freitas et al. 2004b)): lichen's orientation may be therefore be regarded as of limited significance. In a more general sense, the question remains, however, what effect the orientation may have on the lichen's vitality.
- The lichen's vitality should be assessed by selected analytical approaches: The comparability (usability) of any further study-data depends on the homogeneity of the vitality data in the lichen material. This applies both to *in situ* sampled lichen material as for lichen transplants, and for both spatial – as time-related data.
- Dry and hot periods affect lichen's vitality. For some lichen transplants experiments it might be preferable to avoid this period (Chapter 3 and (Freitas and Pacheco 2004; Marques, Freitas et al. 2005));
- In principle, current analytical techniques are mostly sensitive enough for the studies considered, and hardly ask for higher levels of element accumulation (except for e.g. Cd, Ni, Pb). On basis of the accumulation data, however, "free influx" positioning highlights specific elements such as Cr, Fe, La and V and "horizontal influx" positioning may be preferred if studies focus on elements such as K, Pb and Se (Chapter 4). On basis of correlations of lichen data to data on element total deposition, "free influx" positioning highlights Ca, Fe and Mn, and "horizontal influx" positioning highlights Na, Ni and V (Chapter 4). The multi-element characteristics generally asked-for in lichen air pollution monitoring (see above) may make it difficult to decide on specific positioning. Any positioning, however, should be maintained and ensured in sampling throughout the whole study.
- PIXE can be regarded as fully complimentary to INAA in lichen air pollution (bio)monitoring studies. For PIXE, samples should be reduced to powder and all the sample mass, as a first approximation, should pass

through a 125 μm nylon net sieve. To ensure full comparability and rule out any milling-effects, the powdered material should be used in both PIXE and INAA protocols.

6.7 Future work

6.7.1 Lichen basics under ambient conditions

The quantitative use of biological monitors, including the presently used lichens, requires further investigation of aspects such as element uptake/release mechanisms, interspecies calibration, non-atmospheric uptake sources, vitality/accumulation-affecting atmospheric conditions (pH, temperature, etc), and the quantitative relations between concentrations in the monitor and element levels in specified atmospheric components (atmospheric dust, wet and dry precipitation) (Sloof, Bruin et al. 1988), including lichen dynamics, associated to simultaneously occurring uptake and release (Reis 2001; Godinho, Wolterbeek et al. 2008).

6.7.2 Analysis

Generally speaking, INAA can be considered as one of the most powerful techniques for the determination of the element content in biomonitors. However, in air-pollution studies, the application of "standard" INAA (fully instrumental, thermal neutrons used) alone does not yield information on all elements which are considered as main elemental pollutants under European Union legislation like Pb, Ni, As, Cd, and Hg, due to their demonstrated carcinogenic properties. Lead cannot be determined by standard INAA (no gamma radiation emitted) and for elements such as cadmium and nickel high limits of detection (limited sensitivity) have been reported (Freitas, Révay et al. 2008). Approaches, such as short irradiations with epithermal neutrons, replicate-sample analysis (RSINAA) (Ventura, Freitas et al. 2007; Dung, Freitas et al. 2008), cyclic analysis (CINAA), prompt gamma activation analysis (PGAA), Compton suppression, measurements in well-type detectors, chemical NAA (isolation of the elements of interest before measurement) all meant to enhance sensitivity (Dung, Freitas et al. 2008), are ways

of obtaining the necessary results without destroying the sample (except for chemical NAA) which can then be re-analyzed by normal INAA, for the rest of the elements of interest. Recent literature shows PGAA in determinations in lettuce of H, C, Si, P, and S, and the trace elements B and (Freitas, Révay et al. 2008), and Compton suppression applications to improve the INAA sensitivity for Cr, Fe, Hg, Ni, Rb, Sr and Zn (Dung, Freitas et al. 2008).

6.7.3 Biomonitors versus filters (air particulate matter)

In studying quantitative relationships, biomonitor data should be compared not only to (total) deposition data, but also to air filter data. Here, in filtering, aerosol sizes should be taken into consideration as are the possible chemical abundances and associations of the elements of interest. First set-ups should comprise joined studies, where both lichen data and filter data are obtained from the same sites. Here, the size relations are of particular interest, since epidemiological and laboratory studies have indicated that the fraction of atmospheric particulates which can be inhaled (PM10: smaller than 10 μm aerodynamic diameter particle size) and associated transition metals are among the factors responsible for the development of cardio-pulmonary diseases (Chapman, Watkinson et al. 1997)

Simple linear modelling suggested correlations between the element concentrations in lichens and in air particulate matter, but only for a few elements (Costa, Marques et al. 2002). The difficulty in finding good correlations between the lichens and the air particulate matter in the air has also been reported in other studies (Sloof 1993; Rossbach, Jayasekera et al. 1999). Rizzio *et al.* (Rizzio, Bergamaschi et al. 2001) evaluated air pollution data obtained with lichens and with filtering and argued that the lichen-based results mostly pinpointed the predominant direction of pollutants transportation, while filter-results were highlighting the degree of the local trace element atmospheric pollution. This line of reasoning, however, is not supported by the present thesis' results. More plausible could be the lichen's dynamics (Reis 2001; Godinho, Wolterbeek et al. 2008), which complicates a straight-forward and direct comparison of lichen-data with short-term filtering data. Other studies (Bari, Rosso et al. 2001) found a significant correlation between several heavy metals

(Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) accumulated in thalli of the lichen *Pseudevernia furfuracea* transplanted for one year in a rural site and the concentration of air particulate of the same chemical elements. This subject requires however further investigations in order for biomonitoring be consider has and fundamental and indispensable complementary tool for trace element air pollution studies.

6.7.4 Organic Compounds

Furthermore, apart from the elemental content, effort should be devoted to also include organics in the analysis of the biomonitor (Wolterbeek 2002) which are emitted in association with human activities such as the production of coke from coal, the combustion of fossil fuels, or the aluminium and carbon electrode industries.

Mosses were applied as monitors of the deposition of organic micropollutants from the 1980s onwards (Thomas and Herrmann 1980). Most of the early work concentrated on the deposition of organochlorine compounds such as pesticides and polychlorobiphenyls (PCBs). In later work, both lichens and mosses have been applied also in monitoring of atmospheric polycyclic aromatic hydrocarbons (PAHs) (Carlberg, Baumann Ofstad et al. 1983; Thomas 1984; Thomas and Schunke 1984; Wegener, Van Schaik et al. 1992; Jacob, Grimmer et al. 1993). Some recent works (Augusto, Pereira et al. 2007) showed the potential of lichens for monitoring organic polychlorinated dibenzofurans (PCDD/F) atmospheric deposition trying to assess human exposure to dioxins.

6.7.5 Epidemiology

Several studies with epidemiological data consistently demonstrate the adverse effects of air pollution on human health (Schwartz, Dockery et al. 1996; Cislaghi and Nimis 1997; Beeson, Abbey et al. 1998 275; Laden, Neas et al. 1999; Harrison and Yin 2000; Wappelhorst, Kuhn et al. 2000; Cassee, Muijser et al. 2002; Wolterbeek and Verburg 2004; Fuga, Saiki et al. 2008). Therefore, one of the most relevant issues in air pollution biomonitoring is the relationship to human health monitoring. Much work has been performed so far, with variable success. Possibly,

the biomonitor dynamics should be known better, to judge deposition periods which are reflected by the biomonitor. Notwithstanding the above remarks, several studies which correlated data from lichen and moss biomonitoring with health epidemiological data have shown remarkable results: Cislaghi and Nimis (Cislaghi and Nimis 1997) found a strong correlation between lichen biodiversity and lung cancer. Other authors (Wappelhorst, Kuhn et al. 2000) found that a connection exists between the thallium content of mosses and the occurrence of cardiovascular disease and between Ce, Fe, Ga and Ge levels in the mosses and the incidence of diseases of the respiratory system. Wolterbeek and Verburg (Wolterbeek and Verburg 2004) found better than 95% probability correlations both for total moss elements and mortality due to specific diseases and for fractionated moss elements and mortality rates summed for group diseases. In the latter study, total selenium concentrations in moss could be correlated to mortality rates due to prostate cancer and fractionated moss elements could be correlated to mortality rates due to grouped diseases of the genitor-urinary system. Exploratory analyses revealed that the accumulation of toxic elements on the lichen *Canoparmelia texana* may be of use in determining the human risk of cardiopulmonary mortality due to prolonged exposure to ambient levels of air pollution (Fuga, Saiki et al. 2007). Correlation studies between biomonitoring data on chemical element air pollution and (epidemiological) health data may prove valuable in turning attention to specific chemical element-health issues and in directing further study into possible dose–response mechanisms in air-associated chemical element epidemiology (Wolterbeek and Verburg 2004).

For a more complete and informative data set the biomonitor content should be compared with precipitation, particulate matter fractionation, and speciation data. On basis of this extensive information the biomonitor data may be compared to medical statistics, to evaluate the possible relationships between atmospheric pollutants and the human responses (Szczepaniak and Biziuk 2003). Markert (Markert 2008) states that the focus and future goals of biogeochemical research must consider the direct effects on human health, by including modelling of active biogeochemical processes than they have done so far. Newly developed strategies as the multi-markered bioindication concept (MMBC) with its functional and

integrated windows on prophylactic healthcare are essential tools for successfully observing the environment with respect to trace chemical elements. More work needs to be performed, however, to further standardize the biomonitoring approach. Standardization may make that this low cost method gains importance in assessments of the impact of environmental pollutants on human health.

References

Asta, J. (1992) Constitution minérale de quelques espèces de lichens. *Bulletin de la Société Botanique de France*. **139**: 81-97.

Asta, J., and Garrec, J.P. (1980) Étude de la répartition du calcium, potassium, magnésium et phosphore dans les différentes couches anatomiques de dix lichens par analyse directe à la microsonde électronique. *Cryptog. Bryol. Lichénol.* **1**: 3-20.

Augusto, S., Pereira, M.J., Soares, A., and Branquinho, C. (2007) The contribution of environmental biomonitoring with lichens to assess human exposure to dioxins. *International Journal of Hygiene and Environmental Health* **210**: 433-438.

Ayrault, S., Clochiatti, R., Carrot, F., Daudin, L., and Bennett, J. (2007) Factors to consider for trace element deposition biomonitoring surveys with lichen transplants. *The Science of the Total Environment* **372**: 717–727.

Bargagli, R. (1989) Determination of metal deposition patterns by epiphytic lichens. *Toxicological and Environmental Chemistry* **18**: 249–256.

Bargagli, R. (1998) Lichens as biomonitors of airborne trace elements. In *Trace Elements in Terrestrial Plants: an Ecophysiological Approach to Biomonitoring and Biorecovery*. Berlin, Germany: Springer Verlag, pp. 179-206.

Bari, A., Rosso, A., Minciardi, M.R., Troiani, F., and Piervittori, R. (2001) Analysis of heavy metals in atmospheric particulates in relations to their accumulation in explanted *Pseudevernia furfuracea* thalli. *Environmental Monitoring and Assessment* **69**: 205-220.

Batzias, F., and Siontorou, C. (2007) A novel system for environmental monitoring through a cooperative/synergistic scheme between bioindicators and biosensors. *Journal of Environmental Management* **82**: 221–239.

Beeby, A. (2001) What do sentinels stand for? *Environmental Pollution* **112**: 285–298.

Beeson, W.L., Abbey, D.E., and Knutsen, S.F. (1998) Long-term concentrations of ambient air pollutants and incident lung cancer in California adults: results from the AHSMOG study. *Environmental Health Perspectives* **106**: 813-822.

Bergamaschi, L., Rizzio, E., Giaveri, G., Loppi, S., and Gallorini, M. (2007) Comparison between the accumulation capacity of four lichen species transplanted to a urban site. *Environmental Pollution* **148**: 468-476.

Branquinho, C., Catarino, F.M., Brown, D.H., Pereira, M.J., and Soares, A. (1999) Improving the use of lichens as biomonitors of atmospheric metal pollution. *The Science of the Total Environment* **232**: 67-77.

Branquinho, C., Gaio-Oliveira, G., Augusto, S., Pedro Pinho a, Máguas, C., and Correia, O. (2007) Biomonitoring spatial and temporal impact of atmospheric dust from a cement industry. *Environmental Pollution* **151**, (2), 292-299.

Budka, D., Przybylowicz, J., Mesjasz-Przybylowicz, J., and Sawicka-Kapusta (2002) Elemental distribution in lichens transplanted to polluted forest sites

near Krakow (Poland). *Nuclear Instruments and Methods in Physics Research B* **189**: 499-505.

Carlberg, G.E., Baumann Ofstad, E., Drangsholt, E., and Steinnes, E. (1983) Atmospheric deposition of organic micropollutants in Norway studied by moss and lichen analysis. *Chemosphere* **12**: 341-356.

Carreras, H.A., and Pignata, M.L. (2001) Comparison among air pollutants, meteorological conditions and some chemical parameters in the transplanted lichen *Usnea amblyoclada*. *Environmental Pollution* **111**: 45 - 52.

Cassee, F.R., Muijser, H., Duistermaat, E., Freijer, J.J., Geerse, K.B., and Marijnissen, J.C.M. (2002) Particle size-dependent total mass deposition in lungs determines inhalation toxicity of cadmium chloride aerosols in rats. Application of a multiple path dosimetry model. *Archives of Toxicology* **76**: 277-286.

Chapman, R.S., Watkinson, W.P., Dreher, K.L., and Costa, D.L. (1997) Ambient particulate matter and respiratory and cardiovascular illness in adults: particle-borne transition metals and the heart–lung axis. *Environmental Toxicology and Pharmacology* **4**: 331-338.

Cislaghi, C., and Nimis, P. (1997) Lichens, air pollution and lung cancer. *Nature* **387**: 463-464.

Clark, B.M., Mangelson, N.F., St Clair, L.L., Gardner, J.S., Cooper, L.S., Rees, L.B. et al. (1999) Analysis of lichen thin sections by PIXE and STIM using a proton microprobe. *Nuclear Instruments and Methods in Physics Research B* **150**: 248-253.

Conti, M.E., and Cecchetti, G. (2001) Biological monitoring: lichens as bioindicators of air pollution assessment - a review. *Environmental Pollution* **114**: 471-492.

Costa, C., Marques, A.P., Freitas, M.C., Reis, M.A., and Oliveira, O.R. (2002) A comparative study for results obtained using biomonitors and PM10 collectors in Sado Estuary. *Environmental Pollution* **120**: 97 - 106.

Dung, H., Freitas, M., Sarmento, S., and Blaauw, M. (2008) The calibration of gamma-ray spectrometers coupled to Compton suppression and fast pneumatic systems for the k_0-NAA method. *Journal of Radianalytical and Nuclear Chemistry (in press)*.

Figueira, R. (2002) *Desenvolvimento de um Sistema de Biomonitorização Ambiental.* PhD thesis, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal pp. 51-107, in Portuguese.

Figueira, R., Sousa, A.J., Pacheco, A.M.G., and Catarino, F.M. (1999) Saline variability at ground level after kriging data from *Ramalina* spp. biomonitors. *The Science of the Total Environment* **232**: 3-11.

Figueira, R., Pacheco, A.M.G., Sousa, A.J., and Catarino, F.M. (2002) Development and calibration of epiphytic lichens as saltfall biomonitors - dry-deposition modelling. *Environmental pollution* **120**: 69-78.

Freitas, M., and Pacheco, A. (2004) Bioaccumulation of cobalt in *Parmelia sulcata*. *Journal of Atmospheric Chemistry* **49**: 67-82.

Freitas, M., Révay, Z., Szentmiklósi, L., Dionísio, I., Dung, H., and Pacheco, A. (2008) Different methodologies in neutron activation to approach analysis of environmental and nutritional samples. *Journal of Radianalytical and Nuclear Chemistry (in press)*.

Freitas, M.C., and Nobre, A.S. (1997) Bioaccumulation of heavy metals using *Parmelia sulcata* and *Parmelia caperata* for air pollution studies. *Journal Radioanalytical and Nuclear Chemistry* **217**: 17-20.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (1999) Distribution in Portugal of some pollutants in the lichen *Parmelia sulcata*. *Environmental Pollution* **106**: 229-235.

Freitas, M.C., Reis, M.A., Alves, L.C., and Wolterbeek, H.T. (2000) Nuclear analytical techniques in atmospheric trace element studies in Portugal. In *Trace Elements - their Distribution and Effects in the Environment*. Markert and Friese. (ed). Amsterdam, Netherlands: Elsevier Science, pp. 187-213.

Fuga, A., Saiki, M., Marcelli, M.P., and Saldiva, P.H.N. (2008) Atmospheric pollutants monitoring by analysis of epiphytic lichens. *Environmental Pollution* **151**, (2), 334-340.

Garty, J. (2001) Biomonitoring atmospheric heavy metals with lichens: theory and application. *Critical Reviews in Plant Sciences* **20**: 309-371.

Garty, J., and Delarea, J. (1991) Localization of iron and other elements in the lichen *Nephoroma arcticum* (L) Torss. *Environmental and Experimental Botany* **3**: 367-375.

Garty, J., Cohen, Y., and Kloog, N. (1998) Airborne elements, cell membranes, and chlorophyll in transplanted lichens. *Journal of Environmental Quality* **27**: 973-979.

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A., and Orlovsky, L. (2000) Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiology Plantarum.* **109**: 410-418.

Godinho, R.M., Wolterbeek , H.T., Verburg, T.G., and Freitas, M.C. (2008) Bioaccumulation behaviour of transplants of the lichen Flavoparmelia caperata in relation to total deposition at a polluted location in Portugal. *Environmental Pollution* **151, (**2), 318-325.

Goyal, R., and Seaward, M.R.D. (1981) Metal uptake in terricolous lichens; I. Metal localization within the thallus. *New Phytologist* **89**: 631-645.

Harmens, H., Norrisa, D.A., Koerbera, G.R., Busea, A., Steinnes, E., and Ruhling, A. (2007) Temporal trends in the concentration of arsenic, chromium, copper, iron, nickel, vanadium and zinc in mosses across Europe between 1990 and 2000. *Atmospheric Environment* **41**: 6673-6687.

Harrison, R.M., and Yin, J. (2000) Particulate matter in the atmosphere: which particle properties are important for its effects on health? *The Science of the Total Environment* **249**: 85-101.

Henderson, A. (1996) Literature on air pollution and lichens *Lichenologist* **28**: 279–285.

IAEA (2001) NAHRES-63 - Validation and Application of Plants as Biomonitors for Trace Element Atmospheric Pollution Analysed by Nuclear and Related Techniques. Vienna, Austria: IAEA.

Jacob, J., Grimmer, G., and Hildebrandt, A. (1993) The use of passive samplers for monitoring polycyclic aromatic hydrocarbons in ambient air. *The Science of the Total Environment* **139**: 307-321.

Kuik, P., and Wolterbeek, H.T. (1995) Factor analysis of atmospheric trace-element deposition data in The Netherlands obtained by moss monitoring. *Water, Air and Soil Pollution* **83**: 323-346.

Kuik, P., Sloof, J.E., and Wolterbeek, H.T. (1993) Application of Monte Carlo-Assisted Factor Analysis to large sets of environmental pollution data. *Atmospheric Environment* **13**: 1975-1983.

Kuik, P., Blaauw, M., Sloof, J.E., and Wolterbeek, H.T. (1993b) The use of Monte Carlo methods in factor analysis. *Atmospheric Environment* **13**: 1967-1974.

Laden, F., Neas, L.M., Dockery, D.W., and Schwartz, J. (1999) The association of elemental characteristics of fine particles with mortality in six cities. *Epidemiology* **10**: S613.

Markert, B. (2008) Definitions and principles for bioindication and biomonitoring of trace metals in the environment. *Journal of Trace Elements in Medicine and Biology* **(in press)**.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., and Verburg, T.G. (2004a) MCTTFA applied to differential biomonitoring in Sado estuary region. *Journal of Radioanalytical and Nuclear Chemistry* **259**: 35-40.

Marques, A.P., Freitas, M.C., Wolterbeek, H.T., De Goeij, J.J.M., and Steinebach, O. (2005) Cell membrane damage and element leaching in transplanted Parmelia sulcata lichen related to ambient SO_2, temperature, and precipitation. *Environmental Science & Technology* **39**: 2624-2630.

Marques, A.P., Freitas, M., Wolterbeek, H.T., Verburg, T.G., and De Goeij, J.J.M. (2007) Grain-size effects on PIXE and INAA analysis of IAEA-336 lichen reference material. *Nuclear Instruments and Methods in Physics Research B* **255**: 380–394.

Marques, A.P., Freitas, M.C., Reis, M.A., Wolterbeek, H.T., Verburg, T.G., and De Goeij, J.J.M. (2004b) Lichen-transplant biomonitoring in the assessment of dispersion of atmospheric trace-element pollutants: effects of orientation towards the wind direction. *Journal of Atmospheric Chemistry* **49**: 211–222.

Mulgrew, A., and Williams, P. (2000) Biomonitoring of air quality using plants. In. Berlin, Germany: WHO Colaborating Centre for Air Quality Management and Air Pollution Control, Federal Environmental Agency, pp. 1-165.

Pacheco, A., Freitas, M.C., and Sarmento, S. (2007) Nuclear and non-nuclear techniques for assessing the differential uptake of anthropogenic elements by atmospheric biomonitors. *Nuclear Instruments and Methods in Physics Research A* **579**: 499-502.

Paul, A., Hauck, M., and Fritz, E. (2003) Effects of manganese on element distribution and structure in thalli of the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*. *Environmental and Experimental Botany*. **50**: 113-124.

Raa, H.S.Y., Geiserb, L.H., and Crang, R.F.E. (2005) Effects of season and low-level air pollution on physiology and element content of lichens from the U.S. Pacific Northwest. The *Science of the Total Environment* **343**: 155-167.

Reis, M.A. (2001) *Biomonitoring and Assessment of Atmospheric Trace Elements in Portugal - Methods, Response Modelling and Nuclear Analytical Techniques*. PhD thesis, Delft University of Technology, Delft, The Netherlands pp. 1-230.

Reis, M.A., Alves, L.C., Wolterbeek, H.T., Verburg, T.G., Freitas, M.C., and Gouveia, A. (1996) Main atmospheric heavy metal sources in Portugal by biomonitor analysis. *Nuclear Instruments and Methods in Physiscs Research B* **109/110**: 493-497.

Richardson, D.H.S., Kiang, S., Ahmadjian, V., and Nieboer, E. (1985) Lead and uranium uptake by lichens. In *Lichen Physiology and Cell Biology*. Brown, D.H. (ed). New York, USA: Plenum Publishing Corporation, pp. 227-246.

Rizzio, E., Bergamaschi, L., Valcuvia, M.G., Profumo, A., and Gallorini, M. (2001) Trace elements in lichens and in airborne particulate matter for the evaluation of the atmospheric pollution in a region of northern Italy. *Environment International* **26**: 543-549.

Rossbach, M., Jayasekera, R., Kniewald, G., and Thang, N.H. (1999) Large scale air monitoring: lichens *versus* air particulate matter. *The Science of the Total Environment* **232**: 59-66.

Ruhling, A. (1987) *Survey of Atmospheric Heavy Metal Deposition - Monitored by Moss Analysis*: Copenhagen, Denmark: The Nordic Council of Ministers.

Ruhling, A. (ed) (1994) *Atmospheric heavy metal deposition in Europe - estimation based on moss analysis*. Copenhagen, Denmark: The Nordic Council of Ministers.

Ruhling, A., and Tyler, G. (2004) Changes in the atmospheric deposition of minor and rare elements between 1975 and 2000 in south Sweden, as measured by moss analysis. *Environmental Pollution* **131**: 417-423.

Sarmento, S., Wolterbeek , H.T., Verburg, T.G., and Freitas, M.C. (2008) Correlating element atmospheric deposition and cancer mortality in Portugal: Data handling and preliminary results. *Environmental Pollution* **151, (**2), 341-351.

Schwartz, J., Dockery, D.W., and Neas, L.M. (1996) Is daily mortality associated specifically with fine particles? *Air Waste Management Association* **46**: 927-939.

Sloof, J.E. (1993) *Environmental Lichenology: Biomonitoring Trace Element Air Pollution.* PhD Thesis, Delft University of Technology, Delft, The Netherlands pp. 1-191.

Sloof, J.E., Bruin, M., and Wolterbeek, H.T. (1988) Critical evaluation of some commonly used biological monitors for heavy metal air pollution. *3rd International Conference of Environmental Contaminants*. Venice, Italy, pp. 296-298.

Smodis, B. (2007) Investigation of trace element atmospheric pollution by nuclear analytical techniques at a global scale: Harmonised approaches supported by the IAEA. *Journal of Environmental Management* **85**: 121-127.

Szczepaniak, K., and Biziuk, M. (2003) Aspects of the biomonitoring studies using mosses and lichens as indicators of metal pollution. *Environmental Research* **93**: 221-230.

Thomas, W. (1984) Statistical models for the accumulation of PAH, chlorinated hydrocarbons and trace metals in epiphytic Hypnum cupressiforme. *Water, Air and Soil Pollution* **22**: 351-371.

Thomas, W., and Herrmann, R. (1980) Nachweis von Chlorpestiziden, PCB, PCA und Schwermetallen mittels epiphytischer Mooseals Biofilter entlang eines Profils durch Mitteleuropa. *StaubReinhaltung der Luft* **40**: 440, in german.

Thomas, W., and Schunke, E. (1984) Polyaromatic hydrocarbons, chlorinated hydrocarbons, and trace metals in moss samples from Iceland. *Lindbergia* **10**: 7-32.

Tretiach, M., Adamo, P., Bargagli, R., Baruffo, L., Carletti, L., Crisafulli, P., Giordano, S., Modenesi, P., Orlando, S., Pittao, E. (2007) Lichen and moss bags as monitoring devices in urban areas. Part I: Influence of exposure on sample vitality. *Environmental Pollution* **146**: 380-391.

Ventura, M., Freitas, M., Pacheco, A., Meerten, T., and Wolterbeek, H.T. (2007) Selenium content in selected Portuguese foodstuffs. *European Food Research and Technology* **224**: 395-401.

Wappelhorst, O., Kuhn, I., Oehlmann, J., and Markert, B. (2000) Deposition and disease: a moss monitoring project as an approach to ascertaining potential connections. *The Science of the Total Environment* **249**: 243-256.

Wegener, J.W.M., Van Schaik, M.J.M., and Aiking, H. (1992) Active biomonitoring of polycyclic aromatic hydrocarbons by means of mosses. *Environmental Pollution* **76**: 15-18.

Wolterbeek, H.T. (2002) Biomonitoring of trace element air pollution: principles, possibilities and perspectives. *Environmental Pollution* **120**: 11-21.

Wolterbeek, H.T., and Verburg, T.G. (2004) Atmospheric metal deposition in a moss data correlation study with mortality and disease in the Netherlands. *The Science of the Total Environment* **319**: 53-64.

Wolterbeek, H.T., Bode, P., and Verburg, T.G. (1996) Assessing the quality of biomonitoring via signal-to-noise ratio analysis. *The Science of the Total Environment* **180**: 107-116.

Zschau, T., Getty, S., Gries, C., Ameron, Y., Zambrano, A., and Nash III., T.H. (2003) Historical and current atmospheric deposition to the epilithic lichen *Xanthoparmelia* in Maricopa County Arizona. *Environmental Pollution* **125**: 21–30.

Summary

The topic of the present thesis is the (bio) monitoring of (trace) element air pollution, with the attention focused on used techniques and selected approaches. Lichens are used in all experiments, and elemental analyses are by nuclear multi-elements techniques. The thesis is focused on lichen transplants aspects, aiming predominantly at a) transplant vitality, b) the effects of the positioning of the transplants used, and c) on analytical aspects, thereby concentrating on aspects of comparative PIXE and INAA. The thesis consists of four parts (divided in six chapters): the first part is dedicated to elemental analysis, the second is about viability and vitality of lichens, third is focused on possible set-ups in transplant monitoring, and the fourth is a transplant case study in an industrialised region, aimed at the recognition of emission source profiles of industrial sources and possible differentiation between selected positioning, but variable in wind-directional exposure.

The main issues of the thesis are presented in Chapter 1 (Introduction).

Grain-size effects on PIXE and INAA analysis of IAEA-336 Lichen Reference Material is presented in Chapter 2. It focuses on the complementary use of the nuclear analytical techniques INAA and PIXE, discusses accuracy and precision, and specifically addresses grain sizes of the initial bulk samples used for PIXE. IAEA-336 lichen certified reference material (grain size < 125 μm) was milled and sieved through nylon sieves with 64 μm, 41 μm and 20 μm pores. Particle sizes were determined by Laser Light Scattering technique: the data indicate that, after sieving, the IAEA-336 lichen reference material's particle size distribution follows a bimodal distribution, which is turning more and more monomodal after further fine sieving. PIXE and INAA were compared for the elements Cl, K, Mn, Fe and Zn, where by the used z-tests no differences could be shown between both techniques. However, fractionation into smaller grain sizes showed to be associated with lower element content, for Na, Cl, K, Mn and Sr. The observed increases of the proportion of algae in the smaller grain-size fractions and the possible accumulation capacity for certain elements in the fungal part of the lichen may

explain the observed phenomenon. The sieving process and consequently the discarding of part of the material may have lead to a change of the properties of the original sample, and thus to differences in element concentrations.

Viability and vitality of lichen transplants was discussed on Chapter 3 Lichens (Parmelia sulcata) were transplanted from a clean background site to an industrial area, there exposed for in total 12 months, and collected on a regular basis to verify membrane damage. The main objective was to relate variability in lichen vitality to variabilities in ambient conditions (temperature, precipitation, SO2) as well as to time-related changes in accumulated trace elements, to gain insight into the possibilities to use and compare lichens throughout larger geographical areas.

The electrical conductivity of solutions used to rinse the lichens was used to assess cell-membrane damage, whereas the element contents of the solutions and lichens were determined by ICP-OES and INAA respectively. Factor Analysis (MCATTFA) was used to determine grouping of elements of similar origin and/or behaviour. All in all, the data indicate that, apart from lichen Na and Cl levels, and for temperature and precipitation, no clear relationships with conductivity could be observed. Conductivity was mostly related to released Na, Cl, K, Mg and Cs. On basis of concentrations, Na, Cl and K could be considered as largely determining the conductivity. The data suggest a different origin of K than that of Na and Cl: the latter two are most probably due to effects from sea salt sprays.

Parmelia sulcata was sensitive enough to reflect appreciable ambient rises in air SO2 and resistant enough to recover afterwards with latyer decrease in SO2. Factor Analysis on selected elements (K, Sc, Cu, V, As and Sb) indicated the absence of any comparability between K and V, As and Sb suggesting differences in origin and/or chemical-physical occurrence. Generally speaking, the present data suggest that the comparability of lichen vitality in large geographical areas may be limited and governed by the area's variability in temperature and precipitation rather than by variability in metal deposition rates. The leaching data on all elements and element groups, however, strongly suggest that wet deposition (rainfall) may also severely affect lichen elemental levels. This latter observation makes that comparing outcomes for time- or spatial series of lichen samples should be

accompanied by a comparably careful monitoring of (preceding) ambient conditions.

Chapter 4 addresses transplant positioning, comprising physical positioning, the orientation towards the dominant wind direction and rain-shielding. In experiments, three options for transplant positioning were studied; totally free positioning ("Fi" = free influx), positioning with vertical shielding ("Hi" = horizontal influx), and positioning with horizontal shielding ("Vi" = vertical influx), the first two combined with a wind-directional adaptation: (continuously "in" the wind direction ("F" = facing), or continuously shielded by their substrates ("S" = shielded).

For all systems, transplant element contents progressively differed from the initial reference values ("time zero" values) , the changes going faster for Fi systems (e.g. Co, Mn, Na, Ni, Pb, Sb, V and Zn) and Hi systems (e.g. Mn and Na) than for Vi systems. The element contents in lichen transplants and in total element deposition showed significant correlations for Ca, Fe and Mn in the Fi system and for Na, Ni and V in the Hi system. No significant positive correlations were found for the Vi system, possibly due to the slow response rate within the Vi system. The results also suggested the absence of any wind-directional effects on element accumulation within Fi and Hi systems. On basis of the results (response rates) a Fi set-up . may be preferred for studies involving specific elements like Cr, Fe, La and V while Hi should be preferred for K, Pb and Se.

Biomonitoring study of Setúbal peninsula region (Chapter 5) focuses on a case study on transplant positioning in a survey carried out in the Setúbal area that includes the Sado river estuary and a very industrial city. It addresses the wind-directional positioning of lichen "F" and "S" transplants, exposed for 3,6 and 9 months. Element determinations were performed by both PIXE and INAA, and data processing (e.g. Factor Analysis for the recognition of emission source profiles) was focused on processing of both "F" and "S" samples and the total data set. The main objective was to test the possibility of using the wind-directional positioning as a tool for the enhancement of the detection strength of local and remote sources. In all cases 5 factors were found, with for 2 factors a "F' and "S" differentiation. The "F" and "S" orientations did not show differences for Na, Mg,

P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu and Ta, but showed some time-related differences for Cr and Zn. Under the conditions of the experiment, F- and S-oriented transplants generally did not result in differences in source profiles reflected, nor in differences in source contributions to element levels in the transplants.

In Chapter 6 all findings are discussed, summarized, and an outline is given for possible future biomonitoring, including the data-coupling to epidemiological data.

Samenvatting

Het onderwerp van dit proefschrift is de (bio)monitoring van luchtverontreiniging met (spore)elementen, met de aandacht met name gericht op te gebruiken technieken en aanpak. In alle experimenten werden korstmossen gebruikt, en bepalingen waren alle multi-element en via nucleaire technieken. Het proefschrift is gericht op het gebruik van korstmos-transplanten, waarbij aandacht werd besteed aan a) de transplantvitaliteit, b) de effecten van de transplantpositionering, en c) de analytische aspecten. Bij deze laatste werd met name geconcentreerd op vergelijkingen tussen PIXE en INAA.
Het proefschrift bestaat uit drie onderdelen en zes hoofdstukken: het eerste deel is gericht op elementanalyse, het tweede op mogelijke set-ups in monitoring met transplanten, en het derde deel is een transplant case-study in een geïndustrialiseerd gebied, gericht op de herkenning van emmissie-bronprofielen van industriële bronnen, met aandacht voor differentiatie in positionering bij variabele windrichting-georienteerdheid.

Hoofdstuk 1 introduceert de voornaamste aandachtspunten van het proefschrift.

Hoofdstuk 2 (Effecten van deeltjesgrootte op PIXE en INAA analyse van IAEA-336 korstmos referentiemateriaal) is gericht op het complementaire gebruik van de nucleaire analytische technieken PIXE en INAA, geeft aandacht aan accuratesse en precisie, en gaat in op de korrelgrootte van het oorspronkelijke sample voor gebruik via PIXE. Het gebruikte IAEA-336 referentiemateriaal (korrelgrootte < 125 μm) werd gemalen en gezeefd door nylon zeven met 64, 41 en 20 μm poriëngrootte. De deeltjesgrootten werden bepaald met laser verstrooiingstechnieken: na zeving werd een bimodale grootteverdeling vastgesteld, die meer en meer modal werd na fijnere zeving. PIXE en INAA werden vergeleken voor de elementen Cl, K, Mn, Fe en Zn, waarbij via de gebruikte z-tests geen significante verschillen bleken tussen beide technieken. Fractionering in kleinere korrelgrootten leverde een concentratiedaling op voor Na, Cl, K, Mn en Sr. De gemeten vergroting van de alg-bijdrage in de kleinere korrelgrootte-massa en de accumulatiecapaciteit van de schimmelcomponent voor

bepaalde elementen zou hier een verklaring voor kunnen zijn. De zeving en het daarmee gepaard gaande uiteindelijke gebruik van slechts een fractie van het oorspronkelijke materiaal kan dus aanleiding zijn tot veranderingen in de eigenschappen van het originele sample, en dus tot verschillen in elementconcentraties.

In hoofdstuk 3 wordt aandacht besteed aan de transplant-vitaliteit. Korstmossen (*Parmelia sulcata*) werden getransplanteerd van de achtergrond-gebied naar een industriegebied, daar blootgesteld voor in totaal 12 maanden en geregeld gemonsterd en bemeten op membraanschade. Het doel was de variabiliteit in transplantvitaliteit te onderzoeken in relatie met variabiliteit in omgevingscondities (temperatuur, neerslag, SO_2), en daarbij ook de elementaccumulatie te betrekken: de te beantwoorden vraag was of korstmossen gesampled uit grotere geografische gebieden wel zonder voorbehoud in accumulatievergelijkingen betrokken mogen worden.
Het electrisch geleidingsvermogen (conductiviteit) van vloeistoffen waarmee de korstmossen gespoeld werden werd gebruikt als maat voor membraanschade, terwijl vloeistoffen en korstmossen werden doorgemeten op elementinhoud via ICP-OES en INAA. Factoranalyse (MCTTFA) werd toegepast om tot groepen elementen te komen van vergelijkbare origine of vergelijkbaar gedrag. De resultaten gaven aan dat behalve voor Na en Cl, en voor temperatuur en precipitatie, geen duidelijke relaties met conductiviteit te vinden zijn. Conductiviteit was vooral gerelateerd aan uitgespoeld Na, Cl, K, Mg en Cs, waarbij, op basis van hun concentraties, met name Na, Cl en K van belang bleken te zijn. De gegevens duidden op een verschil in oorspong voor K enerzijds en Na en Cl anderzijds: deze laatste twee zijn waarschijnlijk afkomstig uit zee-aerosol.
Paermelia sulcata was gevoelig genoeg om omgevingsstijging in SO_2 te reflecteren, en resistent genoeg om te herstellen bij latere SO_2-verlaging. Factoranalyse op geselecteerde elementen K, Sc, Cu, V, As en Sb gaf de afwezigheid aan van enige vergelijkbaarheid tussen K en V, As en Sb, wat verschil aangeeft in origine en/of chemisch/fysische verschijningsvorm. In algemene zin gaf het resultaat aan dat de vergelijkbaarheid van korstmossen in grotere gebieden gelimiteerd is, en eerder bepaald wordt door variabiliteit in temperatuur en precipitatie dan door verschillen in depositiesnelheden van metalen en overige

elementen. De spoelexperimenten gaven aan dat precipitatie (regen) van grote betekenis kan zijn voor de uiteindelijk te meten elementconcentraties: dit maakt dat tijdseries of geografische series in korstmosbemonsteringen altijd moeten worden vergezeld van een even zorgvuldig uitgevoerde monitoring van (voorgaande) omgevingsomstandigheden.

Hoofdstuk 4 geeft aandacht aan (transplant)positionering, waaronder de fysische positionering, de oriëntatie ten aanzien van de dominante windrichting, en de afscherming tegen directe regen. In experimenten werden drie transplant-positioneringsmogelijkheden betrokken: volledig vrije positionering ("Fi" = free influx), positionering met vertical beschutting ("Hi" = horizontal influx) en positionering met horizontal beschutting ("Vi" = vertical influx), waarbij de eerste twee gecombineerd werden met winddirectionele aanpassing (continue "in" de windrichting ("F" = facing), of juist continue afgeschermd door hun substraat ("S" = shielded).
Voor alle set-ups verschilden de elementconcentraties in de transplanten van de "time-zero" waarden, waarbij de verschillen gedurende de expositieperiode sneller opliepen voor Fi-systemen (Co, Mn, Na, Ni, Pb, Sb, V, Zn) en Hi-systemen (Mn, Na) dan voor Vi-systemen. De elementconcentraties in de transplanten waren significant gecorreleerd met de elementdepositie voor Fi-systemen (Ca, Fe, Mn) en Hi-systemen (Na, Ni, V) maar niet voor de Vi-systemen, dit laatste waarschijnlijk als gevolg van de daarbij geldende trage response. De resultaten geven de afwezigheid aan van significante wind-directionele effecten in Hi- en Fi-systemen. Op basis van de resultaten (response-rates) zou een Fi set-up de voorkeur kunnen hebben voor elementen zoals Cr, Fe, La of V, terwijl een Hi set-up te prefereren zou zijn voor elementen als K, Pb en Se.

Hoofdstuk 5 richt zich op een case-studie, in de Portugese zwaar-geïndustrialiseerde Setúbal regio, met inbegrip van het estuarium van de Sado rivier. In deze case-studie , waar de aandacht gericht was op de wind-directionele positionering van transplantmateriaal, werd gebruik gemaakt van zowel "F"- als "S" transplanten, beide beschermd tegen directe regen, en blootgesteld voor 3, 6 en 9 maanden. Elementbepalingen werden uitgevoerd via zowel PIXE als INAA, en dataprocessing (o.a. factoranalyse i.v.m. mogelijke herkenning van

emmissiebronprofielen) werd gericht op bewerking van zowel de “F”-, de “S”- als de totale sampleset. De vraag was of winddirectionele positionering tot verbeterde detectie zou leiden van locale en/of diffuse bronnen. In alle gevallen waren 5 factoren onderscheidbaar, waarbij voor 2 factoren een “F”- en “S”- differentiatie werd gevonden. “F”- en “S”- orientaties gaven geen verschillen te zien voor Na, Mg, P, Cl, K, Fe, Co, Ni, Ga, Se, Br, Sr, Ba, La, Nd, Sm, Lu en Ta, en leidden tot tijds-gerelateerde verschillen voor Cr en Zn. In algemene zin leken: “F”- en “S”- oriëntaties van de transplantmaterialen weinig verschillen op te leveren in gevonden bronprofielen, en ook geen verschil te geven in de fractionele bronbijdragen aan de diverse elementconcentraties.

In Hoofdstuk 6 worden de bevindingen kort bediscussieerd, samengevat, en wordt een outline gegeven van mogelijke toekomstige biomonitoring, waaronder de data-koppeling aan epidemiologische gegevens.

Acknowledgements

Since secondary school I have felt the need to be connected to the environment and feel that some how I have given a contribution to understand our world and its unfortunate pollution problems. There are some people that I need to thank for given me the opportunity to do that.

My special thanks for the Institutions that have accepted me as a PhD student and gave me finantial 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 one of the persons who had the privilege to meet and work under the supervision of Professor De Goeij. He was my first promoter. In our meetings his critical refereeing of my work and approaches of data interpretations improved the scientific quality of the experimental results shown. Moreover he kept reminding me of the important things for a PhD student.

I am specially grateful to my supervisor Maria do Carmo Freitas who encouraged me and helped me on my first steps of Science, introduced me to the Neutron Activation Analysis technique, to the wonderful world of lichens and gave me a chance to work in her group. One day she proposed me to start a PhD at ITN by the Delft University of Technology which I gladly accepted. She has been a good friend and an excellent daily supervisor, given me orientation and pushing me to the end of it.

My very special thanks to my promoter, Bert Wolterbeek. I have learnt so much with him. I have specially appreciated our discussions and debates. He tought me to be more consistent in my research and due to his ideas and suggestions, the research presented in this thesis has enriched and gained form. Also he gave me the opportunity to enrich my English and make this thesis and propositions nicely

readable. He has a wonderful perspective of life as it is, more than a promoter, a friend, truly a unique person.

A very big kiss to my special friend Tona Verburg. I don't have words to express my gratitude to her. She has been wonderful introducing me and explaining me MCATTFA and performing all statistical analysis of the results on this thesis. She was also a close presence on the time I have spent in Delft. I will always remember our dinners and our conversations. Millions of kisses for her.

Without Dr. Miguel Reis my understanding of PIXE would not be complete and many questions would remain unanswered. It has been a great pleasure to have his help and cooperation doing the PIXE analysis and just be able to ask him anything at any time. Also he has developed the rotating exposure systems for lichen transplants used in this thesis. Also most of the field work, had his contribution. A GPS in person, without him the location of the transplants on the grid would be impossible. He is really someone that likes his work and likes to discuss science.

I also would like to thank Orlando R. Oliveira and Paula Cristina Chaves from ITN Physics Department (Portugal) for assistance in PIXE analysis, for the long nights at the accelerator machine and for their friendship.

Many thanks for Dr. Ales Fajgelj from International Atomic Energy Agency, Agency's Laboratories Seibersdorf (Austria) for the particle size measurements performed (Low Angle Laser Light Scattering).

My special thanks to all the Reactor Department at ITN, to the head of the Department Dr. José Marques and specially the reactor operators. Thanks for all the conversations during all the time spent at the Hot Chemistry. Also many thanks to all the people that have made part of my daily life at ITN, Isabel Dionisio, Rita Godinho, Susana Sarmento (from Carmo's group), Teresa Fernandes and Mr. Silva. Many, many, millions of kisses for my group colleagues Mané, Marta, Márcia and Carlos. We were a wonderful group!

Many thanks for all the people of Radiation and Isotopes for Health group of the department of Radiation, Radionuclides and Reactors and all the persons that I have contacted at IRI where everyone is always ready to help and the atmosphere is professional, encouraging and friendly. I was lucky to have the technical and analytical support of Dr. Olav M. Steinebach who performed my ICP-OES analysis and help through some experiments performed at IRI Laboratories. I would also like to thank Astrid van der Meer for the conversations, laughs and analytical support performing some experiments at the Biology University. Also I would like to thank Ramon for the help to work with some equipment and Delft laboratory rules. Outside work I wish to thank all the people I had the privilege to meet at IRI, Wim, Astrid Fischer, Folkert, Ula, Heleen Brasser, Yvonne, etc.

But my most special thanks goes to my family!

To the boys of my life. My husband, my love Sérgio that has always encouraged me to finish the PhD and my little popcorn André who gives me joy everyday!

To my dear sister and her help and support.

To my parents for everything they have done for me. For pushing me to keep on studding through my life and for all the sacrifices they have done through their own life. For hearing me, listening to my problems and giving their advices. For taking care of André whenever I need.
Thank You Mum and Dad.

Curriculum Vitae

Ana Paula Valério Marques was born on the 23th June 1972 in Vila Franca de Xira, Portugal. She attended the primary and secondary chemistry school in Alverca do Ribatejo where she presently lives and in 1991 she obtained the school level certificate. In September 1991 she continued her education in the Faculty of Sciences - University of Lisbon and in September 1996 got her degree in Technological Chemistry. It included a one year period of training that was performed at the Reactor Department of the Nuclear and Technological Institute (ITN) at Sacavém under the supervision of Dr. Carmo Freitas, where she developed a study using APM filters to compare trace element air pollution from an urban area with a rural area ("Monitorização da poluição atmosférica em elementos-traço numa zona rural do país: comparação com resultados obtidos numa zona urbana"), an IAEA Coordinated Research Program. In March 1997 she started research work at ITN integrated on Dr. Carmo Freitas research group where she analysed several materials by k_0 INAA (lichens, mosses, olive leaves, APM, human blood, marbles). She also participated in several intercomparison trials for possible CRM namely NAT-3 Urban Dust Artificially Loaded on Air Filters, NAT-5 Lichen, NAT-6 Moss, Tea Leaves – INCT-TL-1, Mixed Polish Herbs – INCT-MPH-2, Almejas – CCHEN. In April 2003 she started her PhD promotion study at the Reactor Institute Delft in a joint project between the Atmospheric Dispersion Group from ITN headed by Dr. Carmo Freitas and the Department of Radiation, Radionuclides and Reactors (R3) Section at that time under the supervision of Prof. J.J.M. De Goeij, and today by Dr. H. Th. Wolterbeek. On March 2004 she started her education on Quality Management and also Safety, Higiene and Health at Work. From February 2005 till August 2005 she worked as a Safety, Higiene and Health at Work Superior Technician on the basis of consultory and on April 2006 she was invited to be responsible for the Quality Management and Safety Department of a small electricity services Portuguese company where she is till this date.

List of Publications

1) **M.C. FREITAS, A.P. MARQUES, M. A. REIS, M.M. FARINHA**, "Atmospheric dispersion of pollutants in Sado estuary (Portugal) using biomonitors", *International Journal of Environment and Pollution,* Vol. 32, N.º 4 (2008), 434–455.
2) **A.P. MARQUES, M.C. FREITAS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ** Grain-size effects on PIXE and INAA analysis of IAEA-336 lichen reference material, *Nuclear Instruments and Methods in Physics Research B,* Vol. 255 (2007), 380–394.
3) **A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ**, "Cell-membrane damage in transplanted Parmelia sulcata lichen related to ambient SO2, temperature and precipitation", *Environmental Science & Technology*, Vol. 39, (2005), 2624–2630.
4) **A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG, J.J.M. De GOEIJ**, "Biomonitoring-transplants: effects of positioning towards wind direction", *Journal of Atmospheric Chemistry*, Vol. 49, (2004), 211–222.
5) **A.P. MARQUES, M.C. FREITAS, M.A. REIS, H.TH.WOLTERBEEK, T. VERBURG**, "MCTTFA applied to differential biomonitoring in Sado estuary region", *Journal of Radioanalytical Nuclear Chemistry,* Vol. 259, Nº 1, (2004) 35-40.
6) **M.C. FREITAS, M.A. REIS, A.P. MARQUES, S.M. ALMEIDA, M.M. FARINHA, O. DE OLIVEIRA, M.G. VENTURA, A.M.G. PACHECO, L.I.C. BARROS**, "Monitoring of environmental contaminants, 10 years of use of k_0 INAA", *Journal of Radioanalytical and Nuclear Chemistry*, Vol. 257, Nº 3 (2003) 621-625.
7) **C. J. COSTA, A. P. MARQUES, M. C. FREITAS, M. A. REIS, O. R. OLIVEIRA**, "A comparative study for results obtained using biomonitors and PM10 collectors in Sado estuary", *Environmental Pollution*, Vol. 120, (2002), 97-106.

8) **M. C. FREITAS, M. A. REIS, A. P. MARQUES, H. Th. WOLTERBEEK**, "Use of lichen transplants in heavy metal atmospheric deposition studies", *Journal of Radioanalytical and Nuclear Chemistry,* Vol. 249, Nº 2 (2001), 307-315.

9) **M.C. FREITAS, M. A. REIS, A. P. MARQUES, H. Th. WOLTERBEEK**, "Dispersion of chemical elements in an industrial environment studied by biomonitoring using *Parmelia sulcata*", *Journal of Radioanalytical Nuclear Chemistry,* Vol. 244, Nº 1 (2000), 109-113.

10) **M. A. REIS, M. C. FREITAS, L.C. ALVES, A. P. MARQUES, C. COSTA**, "Environmental assessment in an industrial area of Portugal", *Biological Trace Element Research*, 71/72 (1999), 273-280.