Universidade de Aveiro Departamento de Biologia
2012

SANDRINA AZEVEDO RODRIGUES

BIODIVERSIDADE LIQUÉNICA E BIOMONITORIZAÇÃO DE POLUIÇÃO ATMOSFÉRICA

LICHEN BIODIVERSITY AND BIOMONITORING OF ATMOSPHERIC POLLUTION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Doutor Arsenio Terrón Alfonso, Professor Titular da Faculdade de Ciências Biológicas e Ambientais da Universidade de Léon (Espanha).

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Aos meus pais e ao Filipe

o júri

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palavras-chave

biodiversidade, biomonitorização, *Chrysothrix flavovirens,* fluorescência da clorofila *a*, *Hypotrachyna lividescens, H. pseudosinuosa,* indústria de pasta de papel, líquenes epífitos, *Lecanora sorediomarginata, Lepraria elobata, Ochrolechia arborea,* pH do ritidoma, pinhais dunares, poluição atmosférica.

resumo

Esta tese debruça-se sobre a biodiversidade de líquenes epífitos de pinhais dunares portugueses e sobre uso de líquenes como biomonitores de poluição atmosférica nesse habitat. A Mata Nacional das Dunas de Quiaios (Figueira da Foz) foi o ponto de partida dos estudos de biodiversidade efetuados nesta tese, mas alguns deles estenderam-se à maior parte da costa portuguesa. Como resultado, encontrou-se uma espécie nova para a ciência, *Lecanora sorediomarginata* Rodrigues, Terrón & Elix, epifítica sobre *Pinus pinaster* Aiton e *P. pinea* L, que se encontra distribuída na maior parte da costa. Esta espécie caracteriza-se morfologicamente por um talo crustáceo, de cor esbranquiçada a acinzentada ou esverdeada e que desenvolve sorálios a partir de pequenas verrugas marginais. Quimicamente caracteriza-se pela presença dos ácidos 3,5-dicloro-2'-*O*-metilnorestenospórico [maior], 3,5-dicloro-2'-*O*-metilanziaico [menor], 3,5-dicloro-2'-*O*-metilnordivaricático [menor], 5-cloro-2'-*O*-metilanziaico [traço] e úsnico [traço]; atranorina [menor] e cloroatranorina [menor]. É quimicamente semelhante a *L. lividocinerea* Bagl., com a qual apresenta afinidades filogenéticas com base na análise da sequência ITS do rDNA, e a *L. sulphurella* Hepp. Adicionalmente, espécies *Chrysothrix flavovirens* Tønsberg e *Ochrolechia arborea* (Kreyer) Almb, também se encontraram epifíticas sobre *P. pinaster* e *P. pinea* em vários pinhais ao longo da costa, representando novos registos para a flora liquénica portuguesa, bem como a espécie *Lepraria elobata* Tønsberg encontrada epifítica sobre *P. pinaster* apenas nas Dunas de Quiaios. Além disso, as espécies *Hypotrachyna lividescens* (Kurok.) Hale e *H. pseudosinuosa* (Asahina) Hale encontraram-se epifíticas sobre *P. pinaster* e outros forófitos nas Dunas de Quiaios, constituindo novos registos para a flora liquénica da Península Ibérica. Estes resultados põe em evidência a importância dos pinhais dunares como habitat para líquenes epífitos. Num estudo conduzido entre janeiro e julho de 2008 num pinhal dunar (Mata do Urso, Figueira da Foz), em cuja bordadura existe uma fábrica de celulose de papel, usaram-se transplantes de líquenes da espécie *Flavoparmelia caperata* (L.) Hale para avaliar a acumulação de trinta e três elementos putativamente emitidos por fábricas de papel e pasta de papel. A cinética da fluorescência da clorofila *a* foi estudada nos líquenes transplantados, através da análise dos parâmetros F_v/F_m, F_0, F_m, q_P, NPQ, Φ_{PSII}, e Φ_{Exc}, de forma a avaliar os efeitos decorrentes da acumulação de elementos na vitalidade dos líquenes. Pretendeu-se avaliar se a acumulação de elementos e a cinética da fluorescência da clorofila *a* variavam significativamente com o local e o tempo de exposição, tendo em consideração os resultados obtidos de transplantes colocados num local de referência (Dunas de Quiaios) durante o mesmo período de tempo. (*Continua no verso*)

resumo

A maior parte dos elementos — Al, B, Ba, Ca, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, P, S, Sb, Sc, Sr, Ti e V — ocorreu em concentrações significativamente mais elevadas nos transplantes expostos a 500 m da fábrica. Cerca de metade dos elementos estudados — B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb e V — encontraram-se em concentrações significativamente mais elevadas nos transplantes expostos durante 180 dias. O solo foi identificado como uma fonte parcial da maior parte dos elementos. Os parâmetros F_v/F_m, F_m, Φ_{PSII} e Φ_{Exc} variaram significativamente com o local e/ou com o tempo de exposição. Observou-se um decréscimo significativo nos parâmetros F_v/F_m e F_m nos transplantes expostos a 500 e 1000 da fábrica, e também naqueles expostos durante 135 e 180 dias. Observou-se também um decréscimo significativo nos parâmetros Φ_{PSII} e Φ_{Exc} expostos durante 180 dias. Estes parâmetros correlacionaram-se de forma negativa e significativa com a acumulação de elementos: F_v/F_m: B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb e Zn; F_m: Ba, Co, Hg, Mn, Mo, N, P, S, Sb e Zn; Φ_{PSII}: N e P; Φ_{Exc}: Mn, N, P e S. Estudos acerca da diversidade liquénica efetuados nos mesmos locais onde os transplantes foram colocados no local impactado, revelaram um menor valor de diversidade liquénica a 500 m da fábrica, que foi também o único local onde se encontraram espécies nitrófilas, o que se poderá dever à deposição de amónia e/ou poeiras. À semelhança de outros estudos, este trabalho confirma que os líquenes podem ser usados com sucesso em estudos de biomonitorização, mesmo em locais florestados. Além disso, traz também informações adicionais sobre como a acumulação de elementos pode influenciar a cinética da fluorescência da clorofila *a* em líquenes.

keywords

atmospheric pollution, bark pH, biodiversity, biomonitoring, chlorophyll *a* fluorescence, *Chrysothrix flavovirens,* epiphytic lichens, *Hypotrachyna lividescens, H. pseudosinuosa, Lecanora sorediomarginata, Lepraria elobata, Ochrolechia arborea,* pine forests on sand dunes, pulp mill.

abstract

This thesis focuses on the biodiversity of epiphytic lichens of Portuguese pine forests on sand dunes, and on the use of lichens as biomonitors of atmospheric pollution in this habitat. Mata Nacional das Dunas de Quiaios (Figueira da Foz) was the starting location of the biodiversity studies undertaken during this thesis, but some were extended to most of the Portuguese coast. As a result a new species to science, *Lecanora sorediomarginata* Rodrigues, Terrón & Elix was discovered epiphytic on *Pinus pinaster* Aiton and *P. pinea* L, in most of the coast. It is characterised morphologically by a crustose whitish-grey to greenish thallus developing soralia from small, marginal warts and chemically by the presence of 3,5-dichloro-2'-*O*-methylnorstenosporic acid [major], 3,5-dichloro-2'-*O*-methylanziaic acid [minor], 3,5-dichloro-2'-*O*-methylnordivaricatic acid [minor], 5-chloro-2'-*O*-methylanziaic acid [trace], atranorin [minor], chloroatranorin [minor], and usnic acid [trace]. It is chemically similar to *L. lividocinerea* Bagl., to which it shows phylogenetic affinities based on ITS rDNA sequence analysis, and to *L. sulphurella* Hepp. Additionally, *Chrysothrix flavovirens* Tønsberg and *Ochrolechia arborea* (Kreyer) Almb, were also found epiphytic on *P. pinaster* and *P. pinea* in several pine forests along the coast, representing new records for Portuguese lichen flora, as well as that of *Lepraria elobata*, which was found epiphytic on *P. pinaster* only at Dunas de Quiaios. Furthermore, *Hypotrachyna lividescens* (Kurok.) Hale e *H. pseudosinuosa* (Asahina) Hale were found epiphytic on *P. pinaster* and other phorophytes at Dunas de Quiaios, and were new records for the lichen flora of the Iberian Peninsula. These results indicate the importance of pine forests on sand dunes has habitats for epiphytic lichens. In a study conducted in a pine forest on sand dunes (Mata do Urso, Figueira da Foz), impacted by a pulp mill at its border, between January and July 2008, lichen transplants of the species *Flavoparmelia caperata* (L.) Hale were used to evaluate the accumulation of thirty-three elements putatively emitted by paper and pulp mill industry. Chlorophyll *a* fluorescence kinetics studies were performed in the transplanted lichens, through the analysis of the parameters F_v/F_m, F_0, F_m, q_P, NPQ, Φ_{PSII}, and Φ_{Exc} in order to evaluate the effect of elemental accumulation on lichen vitality. It was intended to evaluate if elemental accumulation and chlorophyll *a* fluorescence kinetics varied significantly with site and period of exposure, taking into account the results from transplants performed in a reference location (Dunas de Quiaios) during the same period of time. Most elements — Al, B, Ba, Ca, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, P, S, Sb, Sc, Sr, Ti and V — were found in significantly higher concentrations in the transplants exposed at 500 m of distance from the point source. Nearly half of the elements — B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V — were also found in significantly higher concentrations in the transplants exposed during 180 days. (*Continues on the verse*)

abstract

Soil was identified as a partial source for most elements. The chlorophyll *a* fluorescence kinetics parameters F_v/F_m, F_m, Φ_{PSII}, and Φ_{Exc} varied significantly with site and/or period of exposure. F_v/F_m and F_m were significantly decreased in the transplants exposed at 500 and 1000 m from the pulp mill and in those exposed during 135 and 180 days. Both, Φ_{PSII} and Φ_{Exc} decreased significantly after 180 days of exposure. Significant negative correlations were identified between these parameters and the accumulation of elements: F_v/F_m: B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb, and Zn; F_m: Ba, Co, Hg, Mn, Mo, N, P, S, Sb, and Zn; Φ_{PSII}: N and P; Φ_{Exc}: Mn, N, P, and S. Lichen diversity studies performed in the same locations where lichen transplants were placed at the impacted location revealed a lower lichen diversity value at the 500 m, which was also the only site were nitrophylous species were found, what could be due to the deposition of ammonia and/or dust. Similarly to other studies, this work confirms that lichens can be successfully used in biomonitoring studies, even in forested locations. Furthermore, it provides additional information on how chlorophyll *a* fluorescence kinetics of lichens can be influenced by elemental accumulation.

Index

Index of figures

Index of tables

Chapter 1

General introduction and objectives

Figure on the previous page: *Pinus pinaster* Aiton in a pine forest on sand dunes.

Lichen diversity

Lichens are an ecological group of organisms classically regarded as consisting of fungi living in symbiosis with photosynthetic partners (mostly green algae and/or cyanobacteria), which provide the fungi with carbohydrates (Wolseley & Hawksworth 2009, Hill 2009). As lichens are taxonomically classified as fungi, they are also known as lichenized fungi or lichen-forming fungi (Hawksworth 2001, Sipman & Aptroot 2001), and the photosynthetic partners (photobionts) are classified independently (Wolseley & Hawksworth 2009).

Recent studies however, indicate that non-photosynthetic bacterial communities may also be part of the lichen symbiosis (e.g., Bates et al. 2001, Grube & Berg 2009, Grube et al. 2009). Lichens host highly structured bacterial communities belonging to several phyla (see references in Bates et al. 2011), which apparently are species-specific (Bates et al. 2011, Grube et al. 2009). Bacteria associated with lichens may have a role in nitrogen fixation, in the solubilization of phosphate compounds, in the production of compounds with antibiotic and hormonal activities, and may possess lytic activity, enhancing nutrient mobilization (Bates et al. 2001, Grube & Berg 2009, Grube et al. 2009).

The vast majority of lichens are Ascomycota, although only few orders are exclusively lichenized, and only few species are Basidiomycota or anamorphic fungi (Kirk et al. 2008, Nash 2008). About 20 % of all Fungi and 40 % of Ascomycota are lichenized (Kirk et al. 2008). Lichenization is thought to be an obligate nutritional mode for most of the fungal partners of the lichen symbiosis, due to the slow growth rate of aposymbiotically grown mycobionts, that is likely to render them low probabilities of survival in the free-living state due to competition with other fungi or consumption by other organisms (Nash 2008). However, recent findings showed that at least in the *Stictis-Conotrema* complex, lichenization and saprotrophy are optional nutritional modes, allowing different individuals to colonize distinct niches during forest succession (Wedin et al. 2004).

Lichenization is an ancient mode of nutrition that was gained independently in different groups of Ascomycota (Lutzoni et. al 2001, 2004). This form of nutrition is at different states of evolution in several groups, as some are evolving to be more strongly lichenized, while in others the reverse in occurring (Kirk et al. 2008).

Most lichens have green algae and/or cyanobacteria as photosynthetic partners (photobionts), but a lichen symbiosis with a brown algae is also known (Moe 1997). *Trebouxia* and *Trentepohlia*, both green algae, and *Nostoc*, a cyanobacteria, are the most represented genus (Friedl & Büdel 2008), but most lichens (approximately 20%) have *Trebouxia* as a photobiont (Hill 2009, Nash 2008). About 10 % of lichen species contain cyanobacteria as exclusive photobionts, although a number of lichen species have green algae as a primary photobionts and cyanobacteria secondary ones (Friedl & Büdel 2008).

Although it has been postulated that mycobionts associate with any available algae or cyanobacterium that allows their survival (Hill 2009), it is known that mycobionts are generally specific for photobionts at the genus level, but not at the species level, at least in what regards trebouxoid and trentepohlioid photobionts, but possibly also in cyanobacteria (Lücking et al. 2009). However, a high degree of specificity towards the photobiont was detected in some cyanolichens, what was associated with the mostly asexual mode of reproduction of those lichens, which involves the vertical transmission (co-dispersal) of the photobiont, and the specialization to narrow environmental conditions (Otálora et al. 2010).

Horizontal photobiont transfer is thought to occur in lichen guilds (ecologically similar and coexisting lichens), and that may be the cause for which some photobionts exist apparently exclusively lichenized, as the recently described genus of cyanobacteria *Rhizonema* (Lücking et al. 2009). The same phenomenon may occur with the green algal genus *Trebouxia* that although having been reported to occur in the free-living state (Bubrick et al. 1984, Hedenås et al. 2007), is thought by some authors to occur only in lichens (Ahmadjian 2004, Friedl & Büdel 2008).

Lichens are within the better-known groups of Fungi (Schmitt & Muller 2007). A global checklist of lichens and lichenicolous fungi includes around 17,320 lichen species described (Feuerer & Hawksworth 2007). Previously, Hawksworth et al. (1995) and Sipman & Aptroot (2001) indicated the existence of 13,500 accepted species, not including orphaned species, that is species that were not or only rarely recorded a second time after their initial description. Nonetheless, estimates of the number of extant lichen species in world reach the number 20,259 (Galloway 1992, Sipman & Aptroot 2001, Schmitt &

Muller 2007). A minimum number of 3,875 species are estimated to occur in Europe (Schmitt & Muller 2007).

Sipman & Aptroot (2001) estimated that about 4,000 lichen species are still undiscovered, mostly in the primary tropical forests, but also in the southern hemisphere. The authors also indicate that in cooler zones in the North Hemisphere considerable numbers of undescribed species may also occur, namely crustose lichens that rarely produce spores.

The estimation of current lichen species numbers is hampered by i) the lack of knowledge on "orphaned species", which taxonomic status needs to be reassessed; ii) the possible occurrence of undescribed cryptic species, that is species considered to represent only one taxonomic entity, but that latter may be found to represent more than one species based on molecular methods, and eventually previously ignored morphological characteristics; iii) the need to clarify the taxonomic rank accorded to chemical races of some species, but also of species with disjunctive continental distributions, species pairs, and phycosymbiodemes (fungi that form morphologically distinct lichens with different photosynthetic partners, green algae or cyanobacteria) (Feuerer & Hawksworth 2007, Sipman & Aptroot 2001).

Recently, the global structure of the biodiversity of lichens was found to be divided in four main biogeography units: holarctic, subantarctic and Australian, pantropical, and Oceanian (Feuerer & Hawksworth 2007). The holoarctic unit includes two Takhtajan's floristic regions existing the in Portuguese territory, the Macaronesian, and the Mediterranean (Feuerer & Hawksworth 2007). Nonetheless, it is possible that these floristic regions become more similar, with an increase in the knowledge regarding the distribution of lichen species (Feuerer & Hawksworth 2007).

Lichen diversity in Portuguese coastal pine forests

Pine forests on sand dunes cover a large extension of the Portuguese west coast, despite in some areas only small patches occur. They are abundant in the centre of the country where they form an almost continuous strip from Cortegaça (Ovar) to Valado dos Frades (Nazaré), only separated by Ria de Aveiro (Aveiro) and the Mondego estuary (Figueira da Foz). However, in the north and south of the country they are patched and

present in only some localities. Most of these pine forests were planted, and in the majority *Pinus pinaster* Aiton is the main phorophyte present, but may be accompanied by *P. pinea* L., invasive *Acacia* species, and other phorophytes as *Arbutus unedo* L., and *Myrica faya* Aiton. *Pinus pinea* is the main phorophyte in a small number of those forests, and *P. halepensis* Mill. patches also occur, namely at the Arrabida Natural Park.

Many of these coastal pine forests are classified as Natural Parks or Nature Reserves, while others are national forests or forest perimeters that are owned and/or partially managed by the Portuguese State (AFN 2012, ICN 2012). Many are included in Natura 2000 sites (ICN 2006).

Despite their extension in some parts of the coast, pine forests on sand dunes are poorly studied in what regards their lichen diversity. This situation is similar to that of most pine forests in the Iberian Peninsula, what has been attributed to the doubtful naturalness of many of the pine forests and to a poor floristic diversity when compared to oak forests (Aragón et al. 2006). Nonetheless, records of mostly epiphytic lichens occurring epiphytic on pine in Portuguese coastal areas are available, which already provide an account of the lichen diversity that can be found there (Boqueras & Llimona 2003; Carballal et al. 2007; Catarino et al. 1985; Giralt 2001; Jones 2002; Paz-Bermúdez & Carballal 2005; Paz-Bermúdez & López de Silanes 2007; Paz-Bermúdez et al. 2008; Rodrigues et al. 2007, 2011a,b; Tavares 1945; van den Boom & Giralt 1999; van den Boom et al. 1990; van den Boom 2006). These records, along with records of lichens found epiphytic on other coniferous trees in coastal areas are compiled in Table 1.1, where the authorship of lichen names cited in the following paragraphs can also be found.

Hypotrachyna laevigata [as *Parmelia laevigata*] was collected among other lichens in pine forests in central-littoral Portugal (e.g., Mata do Urso, Figueira da Foz) by Tavares (1945) and although he did not indicate the specific substrate where the species was collected, he indicates that it was frequent in pine forests of the north of the country, mostly north of Beira Litoral (inclusively). Tavares also collected *Pertusaria heterochroa* epiphytic on *Pinus halepensis* at Praia do Gincho (Cascais) (Boqueras & Llimona 2003), as well as *Fuscopannaria atlantica* epiphytic on the same phorophyte at an urban park at Cascais, although this species was only recently described (Paz-Bermúdez et al. 2008). *Lecanora lividocinerea* was collected epiphytic on pine near Setúbal by Cordeiro, and was

Table 1.1: Records of lichens epiphytic on pine and other coniferous trees found at the Portuguese coast.

Species	Substrate	Locality	References
Arthonia pruinata (Pers.) Steud. ex A.L. Sm.	*Pinus*	Nazaré	van den Boom and Giralt (1999)
[as *Arthonia impolita* (Hoffm.) Borrer]	"	Aldeia do Meco (Sesimbra)	van den Boom et al. (1999)
Buellia schaereri De Not.	pine	Estefânia (Sintra)	Paz-Bermúdez and Giralt (2010)
Calicium hyperelloides Nyl.	*Cupressus*	Convento dos Capuchos (Sintra)	van den Boom et al. (1999)
Caloplaca aegatica Giralt, Nimis & Poelt	*Pinus*	Nazaré	van den Boom and Giralt (1999)
C. holocarpa (Hoffm.) A.E. Wade [as *C. holocarpa* (Ach.) Wade]	branches of *Juniperus phoenica*	Porto Covo (Alentejo)	van den Booom and Etayo (2000)
Candelariella reflexa (Nyl.) Lettau	*P. pinaster*	S. Bartolomeu (Nazaré)	van den Boom (2006)
Catinaria atropurpurea (Schaer.) Vězda & Poelt	*Cupressus*	Convento dos Capuchos (Sintra)	van den Boom et al. (1990)
Chaenotheca brunneola (Ach.) Müll. Arg.	*P. pinaster*	S. Batolomeu	van den Boom (2006)
Chrysothrix candelaris (L.) J.R. Laundon	base of *Pinus*	"	"
[as *Lepraria candelaris* (L.) Fr.]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
C. flavovirens Tønsberg	"	several locations along the west coast (see chapter 2.2)	Rodrigues et al. (2011b)
	P. pinea	Mindelo (Vila do Conde); Costa de Lavos, Quiaios, Serra da Boa Viagem (Figueira da Foz) (see chapter 2.2)	"
Cladonia coniocraea (Flörke) Spreng. [as *C. coniocraea* auct.]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
C. macilenta Hoffm	"	S. Bartolomeu	van den Boom (2006)
C. ramulosa (With.) J.R. Laundon	Pinus	Orbacém (Caminha)	Jones (2002)
Cliostomum flavidulum Hafellner & Kalb	*P. pinaster*	S. Bartolomeu	van den Boom (2006)
C. griffithii (Sm.) Coppins	*Pinus*	Aldeia do Meco	van den Boom et al. (1999)
	"	Nazaré	van den Boom and Giralt (1999)
Degelia atlantica (Degel.) P.M.Jørg. & P.James	pine	Monte Real (Leiria)	Carballal et al. (2007)
Diploicia canescens (Dicks.) A. Massal.	*Pinus*	Carcavelos (Cascais), Sines	Jones (2002)
Dimerella tavaresiana Vězda	pine	Lisboa	Alvares Andrés & Carballal (2001)
Endohyalina ericina (Nyl.) Giralt, Van den Boom & Elix var. *ericina* H. Mayrhofer & Giralt [as *Rinodina ericina* (Nyl.) Giralt var. *ericina*]	"	Azóia (Sintra)	Giralt (2001)

Table 1.1: *Continued*.

Species	Substrate	Locality	References
Evernia prunastri (L.) Ach.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
Flavoparmelia caperata (L.) Hale [as *Parmelia caperata* (L.) Ach.]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Serra de Santa Luzia (Viana do Castelo)	Jones (2002)
F. soredians (Nyl.) Hale [as *Parmelia soredians* Nyl.]	"	Tanganheira (Santiago do Cacém)	"
Fuscidea lightfootii (Sm.) Coppins & P. James	"	Vila do Bispo	"
Fuscopannaria atlantica P. M. Jørg. & P. James	*P. halepensis*	Cascais	Paz-Bermúdez et al. (2008)
Heterodermia leucomelos (L.) Poelt	"	Serra de Sintra (Sintra)	Jones (2002)
H. obscurata (Nyl.) Trevis.	"	"	"
Hyperphyscia adglutinata (Flörke) H. Mayrhofer & Poelt	"	Tanganheira	"
Hypogymnia physodes (L.) Nyl.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Orbacém	Jones (2002)
	P. pinaster	S. Bartolomeu	van den Boom (2006)
H. tubulosa (Schaer.) Hav.	"	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Serra de Santa Luzia	Jones (2002)
Hypotrachyna laevigata (Sm.) Hale [as *Parmelia laevigata* Ach.]	pine	coastal pine forests in central Portugal	Tavares (1945)
H. lividescens (Kurok.) Hale	*P. pinaster*	Quiaios (see chapter 2.3)	Rodrigues et al. (2007)
H. pseudosinuosa (Asahina) Hale	"	" (see chapter 2.3)	"
H. revoluta (Flörke) Hale [as *P. revoluta* Flörke]	*Pinus*	Serra de Santa Luzia	Jones (2002)
Imshaugia aleurites (Ach.) S.L.F. Mey.	"	Serra da Boa Viagem	"
	P. pinaster	S. Bartolomeu	van den Boom (2006)
Lecanora albella (Pers.) Ach. [as *L. pallida* (Schreb.) Rabenh.]	"	Figueira da Foz – Leiria	Catarino et al. (1985)
L. confusa Almb.	twig of *Pinus*	Aldeia do Meco	van den Boom et al. (1999)
	P. pinaster	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Serra do Risco (Sesimbra)	Jones (2002)
L. horiza (Ach.) Röhl.	"	Montenegro (Faro)	"
L. intricata (Ach.) Ach.	on exposed root of *P. pinaster*	S. Bartolomeu	van den Boom (2006)

Table 1.1: *Continued*.

Species	Substrate	Locality	References
Lecanora lividocinerea Bagl.	pine	Setúbal	Paz-Bermúdez and López de Silanes (2007)
L. rubicunda Bagl.	*Pinus*	Aldeia do Meco	van den Boom et al. (1999)
	"	Nazaré	van den Boom and Giralt (1999)
L. sorediomarginata Rodrigues, Terrón & Elix	*P. pinaster*	several locations along the west and sourthern coasts (see chapter 2.1)	Rodrigues et al. (2011a)
	"	S. Bartolomeu	B. Coppins (pers. comm.)
	P. pinea	Murta, Mata de Valverde (Alcácer do Sal); Quiaios, Serra da Boa Viagem (Figueira da Foz) (see chapter 2.1)	"
L. strobilina (Spreng.) Kieff.	*Pinus*	Nazaré	van den Boom and Giralt (1999)
	twig of *Pinus*	Aldeia do Meco, Sintra	van den Boom et al. (1990)
Lecidea dolliformis Coppins & P. James	*P. pinaster*	S. Bartolomeu	van den Boom (2006)
Lepraria elobata Tønsberg	"	Quiaios (see chapter 2.2)	Rodrigues et al. (2011b)
Loxospora elatina (Ach.) A. Massal. [as *Haematomma elatinum* (Ach.) A. Massal.]	*Pinus*	Pedrógão (Leiria)	Jones (2002)
Melanelixia fuliginosa subsp. *glabratula* (Lamy) J.R. Laundon [as *P. glabratula* Lamy]	"	Melides (Grândola)	"
M. subaurifera (Nyl.) O. Blanco et al. [as *P. subaurifera* Nyl.] as *Melanelia subaurifera* (Nyl.) Essl.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	pine	Moledo do Minho	Paz-Bermúdez and Carballal (2005)
Micarea prasina Fr. s. str.	*Pinus*	Aldeia do Meco	van den Boom et al. (1990)
	P. pinaster	S. Bartolomeu	van den Boom (2006)
M. nitschkeana (J. Lahm ex Rabenh.) Harm.	twig of *Pinus*	Sintra	van den Boom et al. (1990)
Normandina pulchella (Borrer) Nyl.	*Pinus*	Serra da Boa Viagem	Jones (2002)
Ochrolechia alboflavescens (Wulfen) Zahlbr	on exposed roots of *P. pinaster*	S. Bartolomeu	van den Boom (2006)
O. arborea (Kreyer) Almb.	*P. pinaster*	several locations at the west coast (see chapter 2.2)	Rodrigues et al. (2011b)

Table 1.1: *Continued*.

Species	Substrate	Locality	References
Ochrolechia arborea	*P. pinea*	Quiaios, Serra da Boa Viagem (see chapter 2.2)	Rodrigues et al. (2011b)
O. parella (L.) A. Massal.	*Pinus*	Serra de Santa Luzia	Jones (2002)
Opegrapha vermicellifera (Kunze) J.R. Laundon	*P. pinaster*	S. Bartolomeu	van den Boom (2006)
O. vulgata (Ach.) Ach.	*Pinus*	Nazaré	van den Boom and Giralt (1999)
Parmelia sulcata Taylor	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
Parmelinopsis minarum (Vain.) Elix & Hale 1987 [as *P. minarum* Vain.]	*Pinus*	Foz do Minho (Caminha)	Jones (2002)
Parmeliopsis ambigua (Wulfen) Nyl.	"	"	"
P. hyperopta (Ach.) Vain. [as *Foraminella hyperopta* (Ach.) S.L.F. Mey.]	"	Serra da Boa Viagem	"
Parmotrema hypoleucinum (J. Steiner) Hale	"	Nazaré	van den Boom and Giralt (1999)
	P. pinaster	S. Bartolomeu	van den Boom (2006)
[as *P. hypoleucina* J. Steiner]	*Pinus*	Sines, Tanganheira	Jones (2002)
P. perlatum (Huds.) M. Choisy [as *P. perlata* (Huds.) Ach.]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Foz do Minho	Jones (2002)
P. reticulatum	"	Nazaré	Boom and Giralt (1999)
	pine	Lisboa	Paz-Bermúdez and Carballal (2005)
[as *P. reticulata* Taylor]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
"	*Pinus*	Foz do Minho, Tanganheira	Jones (2002)
P. robustum (Degel.) Hale	*P. pinaster*	S. Bartolomeu	van den Boom (2006)
[as *P. robusta* Degel.]	"	Figueira da Foz – Leiria	Catarino et al. (1985)
"	*Pinus*	Foz do Minho, Pedrógão	Jones (2002)
Pertusaria albescens (Huds.) M. Choisy & Werner	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
P. amara (Ach.) Nyl.	"	"	"
P. ficorum Zahlbr.		"	"
P. hemisphaerica (Flörke) Erichsen	*Pinus*	S. Bartolomeu	van den Boom (2006)
P. heterochroa (Müll. Arg.) Erichsen	*P. halepensis*	Praia do Gincho (Cascais)	Boqueras and Llimona (2003)
P. leioplaca DC.	*Pinus*	Vila Nova de Milfontes (Odemira)	Jones (2002)

Table 1.1: *Continued*.

Species	Substrate	Locality	References
Phaeographis dendritica (Ach.) Müll. Arg.	"	S. Pedro de Muel (Marinha Grande)	Jones (2002)
Phaeophyscia hirsuta (Mereschk.) Essl.	"	Gala (Figueira da Foz)	"
Phlyctis argena (Ach.) Flot.	"	Monte Redondo (Leiria)	"
Physcia adscendens (Th. Fr.) H. Olivier	*P. halepensis*	Cascais	Paz-Bermúdez and Carballal (2008)
P. clementei (Turner) Lynge [as *P. clementei* (Sm.) Maas Geest.]	pine	Benfica (Lisboa)	"
Pseudocyphellaria aurata (Ach.) Vain.	*Pinus*	Serra de Sintra	Jones (2002)
Pyrenula macrospora (Degel.) Coppins & P. James	"	Gala	"
Pyrrhospora quernea (Dicks.) Körb.	*P. pinaster*	S. Bartolomeu	van den Boom (2006)
[as *Protoblastenia quernea* (Dickson) Clauz.]	"	Figueira da Foz – Leiria	Catarino et al. (1985)
Ramalina calicaris (L.) Röhl.	"	"	"
Ramalina canariensis J. Steiner	*Pinus*	Tanganhiera, Sines, Quinta do Lago (Quarteira)	Jones (2002)
R. farinacea (L.) Ach.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Guarda de Pedrianes (Marinha Grande), Sines	Jones (2002)
R. fastigiata (Pers.) Ach.	*P. pinaster*	Figueira da Foz - Leiria	Catarino et al. (1985)
	Pinus	Quinta do Lago	Jones (2002)
R. inflata subsp. *australis* G.N. Stevens [as *R. pusilla* Le Prévost]	"	"	"
R. lacera	"	Azinhal (Santiago do Cacém)	"
[as *R. duriaei* (De Not.) Bagh.]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
R. lusitanica H. Magn.	*Pinus*	Azinhal	Jones (2002)
Rinodina anomala (Zahlbr.)	*P. pinaster*	Aldeia do Meco	Giralt (2001)
R. dalmatica Zahlbr	*P. halepensis*	Setúbal	"
R. furfuracea H. Magn.	*P. pinaster*	Santana (Sesimbra)	"
R. pruinella Bagl.	"	Aldeia do Meco	"
R. roboris (Dufour ex Nyl.) Arnold	*Pinus*	Nazaré	van den Boom and Giralt (1999)

Table 1.1: *Continued*.

Species	Substrate	Locality	References
Rinodina roboris	*P. pinea*	Estela (Póvoa do Varzim)	Giralt (2001)
Schismatomma decolorans (Turner & Borrer ex Sm.) Clauzade & Vězda	*Pinus*	Nazaré	van den Boom and Giralt (1999)
Trapeliopsis granulosa (Hoffm.) Lumbsch	on burnt bark of *Pinus*	S. Bartolomeu	van den Boom (2006)
Tuckermanopsis chlorophylla (Willd.) Hale [as *Cetraria chlorophylla* (Willd. Vain.)]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Pedrógão	Jones (2002)
Usnea cornuta Körb.	"	S. Bartolomeu	van den Boom (2006)
[as *U. inflata* Delise]	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
U. esperantiana P. Clerc	*Pinus*	Foz do Minho	Jones (2002)
U. rubicunda Stirt.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
	Pinus	Orbacém	Jones (2002)
U. subfloridiana Stirt.	*P. pinaster*	Figueira da Foz – Leiria	Catarino et al. (1985)
U. subscabrosa Nyl. ex Motyka	*Cupressus*	Convento dos Capuchos (Sintra)	van den Boom et al. (1990)
Xanthoria parietina (L.) Beltr.	*Pinus*	Portimão	Jones (2002)

originally described as *L. manuelina* by J. Harmand (Paz-Bermúdez & López de Silanes 2007).

Furthermore, van den Boom & Giralt (1999) reported the occurrence of *Arthonia pruinata*, *Caloplaca aegatica*, *Cliostomum griffithii*, *Lecanora rubicunda, L. strobilina*, *Opegrapha vulgata*, *Parmotrema hypoleucinum*, *P. reticulatum*, *Rinodina roboris*, and *Schismatomma decolorans* epiphytic on pine near Nazaré.

Giralt (2001), in her monograph on the genera *Rinodina* and *Rinodinella* occurring in the Iberian Peninsula reported the occurrence of *Endohyalina ericina* var. *ericina* [as *Rinodina ericina* var. *ericina*], *R. anomala*, *R. dalmatica*, *R. furfuracea*, *R. pruinella*, and *R. roboris* epiphytic on pine at distinct locations along the coast.

In a floristic survey of lichens at the São Bartolomeu hill (Nazaré), van den Boom (2006) reports lichens epiphytic on pine, some of which were not previously known in Portugal, as *Cliostomum flavidulum*, *Lecidea dolliformis*, *Ochrolechia alboflavescens*, and *Opegrapha vermicellifera*, among other lichens (Table 1.1). Despite the floristic peculiarity of this hill, these lichens may also possibly be found in adjacent areas, as this hill is bordered by a pine forest on sand dunes, Mata do Valado. However, van den Boom (2006) indicates that pines in the surroundings of S. Bartolomeu hill are very poor in lichen diversity.

As a result of epiphytic lichen diversity studies in coastal pine forests, Rodrigues et al. (2011a) recently described a new species, *Lecanora sorediomarginata*, which was found epiphytic both on *Pinus pinaster* and *P. pinea* through most of the countries' coast. Additionally, Rodrigues et al. (2011b) found that *Chrysothrix flavovirens* and *Ochrolechia arborea* are also widely distributed along the coast, although *O. arborea* was not found south of the region of Estremadura. Despite that, these species were not previously known in Portugal. Furthermore, another species unreported from Portugal, *Lepraria elobata*, was found in a pine forest on sand dunes in central-littoral Portugal (Dunas de Quiaios, Figueira da Foz) (Rodrigues et al. 2011b), as well as two species that were not previously known in the Iberian Peninsula *Hypotrachyna lividescens* and *H. pseudosinuosa* (Rodrigues et al. 2007).

In the context of a biomonitoring study, Catarino et al. (1985) reported the presence of several lichens epiphytic on *Pinus pinaster* in the area comprehended between Figueira da Foz and Leiria (Table 1.1). Jones (2002) published records of the distribution of mostly

epiphytic lichens in the country, based on collections made over the course of over 30 years. Those include lichens epiphytic on pine from several locations along the coast (Table 1.1).

The terricolous lichen flora of Portuguese coastal pine forests is also scarcely studied, but some records can be found in the literature (Burgaz 2009, Burgaz and Martínez 2008, Burgaz et al. 1999, van den Boom 2006).

In the rest of the Iberian Peninsula the lichen flora of coastal pine forests also appears to be scarcely studied. Nonetheless, Carballal et al. (2001) report lichens epiphytic on *Pinus pinaster* in a sand dune area in Galicia (Spain). Furthermore, records of lichens epiphytic on pine in coastal areas are included in some articles (e.g., Alonso & Egea 1999; Alvares Andrés & Carballal 2001, Atienza & Segarra Morages 2002, 2006; Giralt & Gómez Bolea 1990; Longán et al. 2000; López de Silanes & Carballal 1987; Torrente & Egea 1984). The terricolous lichen flora of those forests appears not to have been studied, but there are records of terricolous lichens sand dunes (Carballal et al. 2001, Gallego Fernández & Díaz Barradas 1997, Paz-Bermúdez et al. 2003).

Despite the abundance of pine forests on sand dunes in the European coast (EU 2011) with *Pinus pinaster* and/or *P. pinea* as main phorophytes, studies on the epiphytic lichen flora of those habitats are hardly found in the literature. Some records are possibly included in Masson (2001, 2005), although the author only mentions that samples were collected on *P. pinaster* but does not link that information to the locations where samples were retrieved. There are however, records of epiphytic lichens on *P. sylvestris* occurring in sand dune areas (e.g., Fałtynowicz & Wojtyłla-Kuchta 1995, Kösta & Tilk 2008, Suija et al. 2010), and in other coastal areas (e.g., Tibbel 2007, Tønsberg 1994). Records of coastal epiphytic lichens occurring on other pine species (e.g., Garofalo et al. 2010) and other coniferous trees (e.g., Giralt et al. 1995, Tretiach & Haffelner 1998) are also available. On the contrary, several studies have been performed in European coastal dunes addressing the terricolous lichen flora of coastal sand dunes (e.g; Aptroot et al. 2001, 2007; Fałtynowicz & Wojtyłla-Kuchta 1995; Ketner-Oostra & Sýkora 2004; Ketner-Oostra & van der Loo 1998; Ketner-Oostra et al. 2006; Kösta & Tilk 2008; Magnusson 1982; Rhind & Jones 1999; Suija et al. 2010; Zedda et al. 2010).

Effects of atmospheric pollution on lichen diversity

The effects of atmospheric pollution on lichen diversity are dependent on the nature of atmospheric pollutants and their concentrations in the atmosphere (e.g., Hawksworth & Rose 1970, Larsen et al. 2007, van Herck et al. 2003). The interaction between distinct pollutants occurring in the atmosphere may be an important factor affecting lichen diversity (Balaguer et al. 1997, Loppi et al. 2002). Several atmospheric pollutants have been shown to affect lichen diversity, sometimes in different ways. Until recent years, SO_2 was the most important pollutant affecting lichen diversity, but its atmospheric levels have decreased greatly in many areas of the world. The effects of the deposition of other pollutants as hydrogen sulphide (H_2S) have also been studied, mostly around point sources. More recently, the deposition of nitrogenous air pollution has gained importance, namely that of ammonia (NH_3) and NO_x. Lately, focus has also been drawn to the effects of the deposition of particulate matter and of specific elements on lichen diversity. The current knowledge regarding the effects of these pollutants on epiphytic lichen diversity is reviewed in the following sub-sections. They are introduced here as they are emitted to the atmosphere by pulp mills (E-PRTR 2012; Bordado & Gomes 1997, 2002a,b; NCASI 1995), and during this thesis a study was conducted to evaluate the effects of the deposition of atmospheric pollution putatively emmited by one of such industries on epiphytic lichens of a costal pine forest (Chapter 3).

Sulphur compounds

Sulphur dioxide (SO_2)

The first reports on the effects of atmospheric pollution on lichen diversity occurred in nineteenth century. Afterwards, SO_2 was identified as the main pollutant leading to declines in lichen diversity, species luxuriance and fertility (Bates 2002). High SO_2 levels were linked to a patterned distribution of lichen species in urban areas. Three zones could generally be distinguished with decreasing levels of atmospheric SO_2: the lichen desert near the pollution source, where none or very few lichens (but not foliose or fruticose ones) occurred; the struggle or transition zone where foliose and fruticose lichens

appeared, but were poorly developed; and the normal outer zone were lichen diversity was the same as in unpolluted areas (Hawkswoth & Rose 1976).

The occurrence of different epiphytic lichen species was linked with mean winter SO_2 levels in England and Wales (Hawksworth & Rose 1970). Distinct lichen species were found to have different sensitivities to this pollutant, with cyanolichens followed by *Usnea* species being included in the most sensitive species, and *Lecanora conizaeoides* Nyl. ex Cromb. as a species occurring in the more polluted areas (Hawksworth & Rose 1970). Several other studies addressed the problematic of loss of lichen diversity and abundance with high levels of SO_2 pollution (e.g., Case 1980, De Wit 1976, Halonen et al. 1993, Holopainen 1983, Newberry 1974, Sheridan et al. 1976, Taylor & Bell 1983). Furthermore, the physiological sensitivity of lichens to SO_2 has been shown in various experiments (e.g., Deltoro et al. 1999, Häffner et al. 2001, Kong et al. 1999, Silberstein et al. 1996a, Tarhananen et al. 1996).

SO_2 emissions declined in recent years, and recolonisation of areas previously devoid of lichens was observed in several studies (e.g., Isocrono et al. 2007, Lisowska 2011, Munzi et al. 2007, Ranta 2001). The distribution of *Lecanora conizaeoides*, a species which growth is favoured by high SO_2 levels or some closely associated chemical factor, also decreased in a number of cities (Bates et al. 2001, Gombert et al. 2004, Lisowska 2011). However, the decrease in atmospheric SO_2 concentrations has not been followed by an establishment of the normal epiphytic lichen flora, and the occurrence of other species typical of eutrophicated environments was noted, and was related to the high levels of atmospheric ammonia (NH_3) (van Dobben & ter Braak 1998).

Historical levels of SO_2 may still affect the European lichen flora, as the distribution of some species known to be affected by this pollutant sometimes does not follow current SO_2 concentrations in the atmosphere (Bates et al. 2001, van Herk et al. 2003). Strong bark acidification due to long-term exposure to SO_2 has been proposed as a cause (Bates et al. 2001).

Nevertheless, present day effects of SO_2 on lichen distribution still exist (e.g., Bjerke et al. 2006; Geiser & Neitlich 2007; Giordani 2007; van Dobben & ter Braak 1998, 1999; van Dobben et al. 2001; van Herk 2001; van Herk et al. 2003). A threshold of 8 t/year of SO_2 was modelled, above which lichen diversity is significantly affected in the Genova region, Italy (Giordani 2007). van Herk et al. (2003) found that the European

distribution of *Chaenotheca chrysocephala* (Turner ex Ach.) Th. Fr., *Cladonia digitata* (L.) Hoffm., *Evernia prunastri*, *Lecanora pulicaris* (Pers.) Ach. and *Phlyctis argena* was negatively correlated with atmospheric SO_2 concentrations.

The critical level of SO_2 for cyanolichens is 10 μg m^{-3} year $^{-1}$ (annual mean) (UNECE 2011).

Hydrogen sulphide (H_2S)

H_2S has a similar effect on the epiphytic lichen flora to that of SO_2, promoting a decrease in lichen diversity in the vicinity of its sources (Loppi & Nascimbeni 1998, Tretiach & Ganis 1999). Loppi et al. (2006) found that threshold of 8 μg m^{-3} H_2S lied between a low and a moderately altered lichen flora, based on the scale of environmental alteration proposed by Loppi et al. (2002a). In addition, the authors calculated a threshold of 15 μg m^{-3} H_2S above which lichen diversity would indicate a high environmental alteration.

Nitrogenous compounds

Ammonia (NH_3)

High atmospheric NH_3 concentrations may not lead to decreased total lichen diversity, but to shift in lichen species composition from acidophyle to acidophobous and nitrophylous species (Loppi & Nascimbene 2010, van Dobben & ter Braak 1999), or from neutrophylous-nitrophylous to strictly nitrophylous species in Mediterranean environments (Frati et al. 2007). However, Wolseley et al. (2006) found that lichen diversity on twigs was negatively correlated with modelled ammonia concentrations, with lichens absent from sites with high atmospheric NH_3 levels.

The change in lichen community composition associated with elevated levels of atmospheric NH_3 is likely the result of higher substrate pH caused by the dry deposition of NH_3, than on the nitrogen supply itself (Bates 2002; Frati et al. 2006, 2007; van Dobben & ter Braak 1998; van Herk 1999, 2001). However, NH_3 itself may have an effect on lichens, as nitrophytic species do not occur in areas not affected by NH_3, but where substrates have

high pHs (van Herk 2001). In addition, bark pH varies with the species and age of the tree, whereas the shift in lichen communities occurs on all tree species subjected to atmospheric ammonia (Larsen Vilshom et al. 2009).

NH_3 is known to promote nitrophyte lichen abundance and diversity (Jovan & McCune 2005, Sparrius 2007, van Dobben & ter Braak 1998, van Herk 2001). Nonetheless, the response of nitrophyte species to atmospheric NH_3 may vary, as some may prefer intermediate rather than high concentrations (van Dobben & ter Braak 1999). Furthermore, van Dobben and ter Braak (1999) found that nitrophytes respond more rapidly to falling SO_2 levels than to NH_3. On the contrary, NH_3 negatively affects the abundance of acidophyte lichens, and these are pointed disappear at approximately 35 µg m^{-3} of atmospheric NH_3 (van Herk 2001). The loss of acidophyte species occurs before the increase in nitrophyte species (Larsen Vilsholm et al. 2009, Wolseley et al. 2006), and acidophyte lichen species may be completly replaced by nitrophytic ones in the vicinity of NH_3 emission sources (Pinho et al. 2011).

Recently, a reduction in NH_3 atmospheric concentrations over 8 years in a moderately polluted area in the Netherlands was correlated with a decrease in the abundance of nitrophyte and neutrophyte lichens (Sparrius 2007). Neutrophyte lichens require medium concentrations of NH_3, showing optimum relationships with NH_3 on acid-barked trees, and die massively with decreases of atmospheric NH_3 levels (Sparrius 2007).

NH_3 effects on lichen diversity appear to be dependent on its atmospheric concentration. Gadson et al. (2010) found that canopy NH_3 concentrations were not related neither with total nor nitrophyte lichen cover on canopies of acid barked terries, and pointed the low concentrations measured (0.6–1.4 µg m^{-3}) as a possible explanation, in addition to the distinct requirements for NH_3 of different lichen species. Wolseley et al. (2006) found that atmospheric NH_3 above 2–3 µg m^{-3} promoted an increase in nitrophyte lichens on trunks of acid barked tress, but that lichen diversity remained the same, or even increased. However, with NH_3 concentrations above 3–4 µg m^{-3} a loss of diversity occurred. Despite that, in areas not previously affected by SO_2 and where lichen communities well established on ancient trees trunks, sensitive species were able to withstand ammonia inputs. The relative long-life of lichens and the resilience of bark properties, following changes in the environment, may help explain this fact (Larsen Vilsholm et al. 2009, Wolseley et al. 2006). Wolseley et al. (2006) additionally found that

the diversity of lichens epiphytic on twigs decreased with increasing NH_3 concentrations, and that lichens on twigs responded more rapidly to recent changes in NH_3 concentrations, than those on trunks. Pinho et al. (2009) found critical levels of 1.4 and 1.7 μg m^{-3} of atmospheric NH_3 for acidophytic and nitrophytic lichens, respectively, in a Mediterranean environment.

The effects of NH_3 deposition are limited to the close vicinity of the emission source, since it is deposited within distances of about 200–300 m (Frati et al. 2007, Olsen et al. 2010, Wolseley et al. 2006).

Currently the critical level of ammonia for lichens and mosses in Europe is 1 μg m^{-3} (annual mean) (UNECE 2011).

Ammonium (NH_4^+) and nitrate (NO_3^-)

NH_3 in the atmosphere may react to form NH_4^+ as $(NH_4)_2SO_4$ (ammonium sulphate) or NH_4NO_3 (ammonium nitrate), and can reach distances of 100–1000 Km downwind of the source. Unlike NH_3, the deposition of NH_4^+ does not increase bark pH, inclusively it can contribute to bark acidification in case it is nitrified into NO_3^- (van Herk et al. 2003), although nitrification may not be an important process occurring on tree bark (van Dobben & ter Braak 1998). Bark NH_4^+ was found in some studies to be positively correlated with atmospheric NH_3 (van Dobben & ter Braak 1998), but not in others (Frati et al. 2007), while bark NO_3^- was discovered to be positively correlated with atmospheric NO_2 by van Dobben & ter Braak (1998).

The effect of NH_4^+ on nitrophyte lichens is in need of further study. European studies apparently indicate that this ion, either in the air or bark, does not have a significant effect on the occurrence of these species, as it does not or only slightly, affect bark pH (Frati et al. 2008, van Herck 1999). However, Geiser & Neitlich (2007) found that the occurrence of nitrophyte-dominated communities was correlated with wet deposition of NH_4^+ in the U.S. Pacific Northwest, and that incipient community changes occurred at NH_4^+ concentrations between 0.04 and 0.08 mg L^{-1}.

Acidophyte lichens are negatively affected by NH_4^+ and NO_3^- (Hauck & Runge 2002; van Herck 1999, 2001). van Herk et al. (2003) found that long-range transport of nitrogenous air pollution affects the distribution of acidophyte lichen species on an

European scale, constituting a threat to natural populations. A decrease of the probability of occurrence of *Bryoria capilaris* (Ach.) Brodo & D. Hawksw., *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., *Chaenotheca ferruginea* (Turner ex Sm.) Mig., *Imshaugia aleurites*, *Usnea hirta* (L.) Weber ex F.H. Wigg. and *Vulpicida pinastri* (Scop.) J.-E. Mattsson [as *Cetraria pinastri* (Scop.) Gray] was calculated at concentrations of 0.3 mg L^{-1} of either NH_4^+ or NO_3^- in precipitation. *Bryoria capillaris*, *B. fuscencens*, *C. ferruginea* and *I. aleurites* were absent when NH_4^+ concentrations reached 1.0 mg L^{-1}. On the other hand, due to the much smaller range of concentrations at which community changes were observed in the U.S. Pacific Northwest, Geiser and Neitlich (2007) argued that the levels of NH_4^+ observed in European remote locations by van Herck et al. (2003), already altered the European lichen community composition. As an example, they point the extinction of *B. capillaris*, *U. cornuta*, and *U. filipendula* Stirt., and the critical condition of *B. fuscescens* in the Netherlands, which are considered clean air indicators in the U.S. Pacific Northwest. These studies disagree with that of van Dobben & ter Braak (1998) who reached the conclusion that neither NH_4^+ nor NO_3^- had a significant effect on lichen vegetation, contrarily to NH_3 and SO_2.

van Herk et al. (2003) suggested that NH_4^+ and/or NO_3^- may have a phytotoxic effect on acidophyte lichens, as these were affected by N at long distances from the sources and at low concentrations, and when nitrophytic lichens were not yet present.

UNECE's critical load of N established for European forest habitats is 10–15 Kg ha^{-1} y^{-1} and is based on algal and epiphytic lichen diversity (Geiser et al. 2010)

Nitrogen oxides (NO_x)

Nowadays nitrogen oxides are among the most important atmospheric pollutants in urban environment (Bates et al. 2001). They are though to be a major factor preventing the recolonization of the original epiphytic lichen flora in locations previously impacted high SO_2 levels (Bates et al. 2001), and are pointed as a factor determining the occurrence of nitrophytic species in urban environments, where SO_2 levels in the atmosphere decreased (Davies et al. 2007, Gadson et al. 2011, Gombert et al. 2004, Isocrono et al. 2007, Larsen et al. 2007, Lisowska 2011, Loppi and Corsini 2003, Llopi et al. 2004, Purvis et al. 2003).

However, significant effects of NO_x and SO_2 on the occurrence of those lichens were not found by Pinho et al. (2008b) and van Herk (1999).

Recent studies address specifically the impact of NO_x on lichen diversity, as many studies before noted that lichen diversity was poorer in areas impacted by this pollutant (Cristofolini et al. 2007, Fuentes and Rowe 1998, Giordani 2007, Loppi et al. 2003a, Lorenzini et al. 2003, Shukla & Upreti 2011, van Dobben & ter Braak 1998, van Dobben et al. 2001), but others failed to reach to that conclusion (Frati et al. 2006; Gombert et al. 2004, 2006).

Gombert et al. (2006) and van Dobben & ter Braak (1999) report scales on the sensitivity of some lichen species to NO_2, in which some are dependent, including some nitrophytes, while others are sensitive. Yet, the estimated sensitivity of lichen species to NO_2 sometimes differs among studies (Gombert et al. 2006, van Dobben & ter Braak 1999, van Dobben et al. 2001).

Apparently, as with NH_3, the degree in which NO_x affects lichen diversity is dependent on its concentrations in the atmosphere. Davies et al. (2007) reported that concentrations of NO_x above 70 $\mu g\ m^{-3}$ and NO_2 above 40 $\mu g\ m^{-3}$ were related to a decline in lichen diversity in London. On the other hand, Giordani (2007) estimated a decrease in lichen diversity with atmospheric concentrations of NO_x higher than 40 $\mu g\ m^{-3}$. Gadson et al. (2011) found that the proportion of nitrophytic lichens epiphytic on canopies was positively correlated with canopy NO_2 concentrations, but these were negatively correlated with total lichen cover. Additionally, the authors also found a dominance (cover > 50%) of nitrophytes on canopy with a mean concentration of 26 $\mu g\ m^{-3}$ of NO_2.

Lower NO_2 concentrations may or not have an effect on lichen diversity, as indicated by Frati et al. (2006) and Pinho et al. (2008b). The first authors reported that NO_2 concentrations between 7.3 and 16.6 $\mu g\ m^{-3}$ did not affect lichen diversity in a study conducted near a highway in Italy (Fratti et al. 2006). Pinho et al. (2008b) found that NO_2 concentrations measured during their study were not related to changes in the diversity of nitrophytic macrolichens. Contrarily, the authors discovered that the diversity of acidophytic macrolichen species decreased exponentially with increasing concentrations of NO_2 (annual average concentrations of 2.9 and 7.4 $\mu g\ m^{-3}$ in rural and industrial areas, respectively).

High NO_x concentrations negatively affect nitrophytic species as indicated by Larsen et al. (2007), who reported a decrease in number and frequency of nitrophyte species in areas of central London where the highest peak NO_x concentrations (possibly above 1500 μg m^{-3}) were found.

It is therefore apparent that the concentrations and the spatial scale at which NO_x affects lichens may vary under different environmental conditions (Cristofolini et al. 2008). Nevertheless, a phytotoxic effect of NO_x on lichens has been suggested (Davies et al. 2007, Gadson et al. 2010, Larsen et al. 2007).

Currently, a critical level of 30 μg m^{-3} of NO_2 (annual mean) has been established to protect all vegetation types (UNECE 2011).

NO_x is often correlated with SO_2 (Geiser & Neitlich 2007, Jovan & McCune 2005, van Dobben & ter Braak 1999, van Herk 2001), and synergistic effects of both pollutants may occur (Cristofolini et al. 2008, Loppi et al. 2002b), making it difficult to differentiate the effects of these pollutants on lichen diversity.

Particulate matter

Particulate matter, including PM_{10}, has been associated with reduced lichen diversity (Lorenzini et al. 2003, Saipunkaew et al. 2007, Svoboda et al. 2010). Species found in areas with high PM_{10} in London were generally the same pollution tolerant species associated with NO_x and SO_2 (Davies et al. 2007). In Northern Thailand, PM_{10} are thought to be the major factor negatively affecting lichen diversity, possibly through the promotion of an increased bark pH (Saipunkaew et al. 2007). High atmospheric particulate levels may be a factor that increases the limitation of lichen colonization promoted by NO_x (Purvis et al. 2003).

Other elements, including metals

The accumulation of different elements in lichen thalli, bark samples, or tree leaves has been related to a loss in epiphytic lichen diversity. Paoli et al. (2011) found that lichen diversity was negatively correlated the content of Pb, V, and Zn measured in transplanted thalli of *Evernia prunastri* in an industrial area of southern Italy. Loppi et al. (2006)

reported a decrease in lichen diversity correlated with increasing Hg concentrations measured in thalli of *Xanthoria parietina* collected in situ in the vicinity of geothermal power plants, but excluding areas where Hg levels were highest. Herzig et al. (1989) reported a positive correlation between lichen diversity and Ca concentrations measured in samples of *Hypogymnia physodes* collected in situ, and a negative correlation with concentrations of Al, Cd, Cl, Co, Cr, Cu, Fe, K, Li, Mg, P, Pb, S, and Zn in a study undertaken in Switzerland.

Hauck (2003) refers that in mountain coniferous forests a decrease in lichen abundance is observed with increasing Mn concentrations in bark or stemflow. The author additionally refers that high Cu concentrations in bark may have a limiting effect on lichen diversity. A similar conclusion was found by Branquinho et al. (1999), who found that lichen diversity on oak trees was decreased around the centre of a copper mine and in the direction of the prevailing winds. The authors further found that the absence of *Ramalina fastigiata* near the mine was related to toxic levels of Cu containing dust. Additionally, Bjerk et al. (2006) found that Cu, as well as Ni, measured in birch leaves were negatively correlated with total lichen cover in boreal forests impacted by nickel-copper smelters.

In a range of trace elements studied, van Dobben et al. (2001) found that As, Br, Ca, and Sb analysed in bark samples had a significant effect on the occurrence of lichen species in the Netherlands. Ca was positively correlated with bark pH, and both were positively correlated with the abundance of nitrophytic species and negatively with that of acidophytic species. The concentration of Br, which was negatively correlated with distance from the coast, was positively correlated with species richness. Higher levels of As and Sb were correlated with a higher occurrence of *Lecanora conizaeoides*, but Sb was negatively correlated with occurrence of 5 other species, including nitrophytes, in total of 72 species studied. Pinho et al. (2008b) reported a decrease in the diversity of acidophytic macrolichens associated with Mg, Ti, and Zn determined in thalli of *Xanthoria parietina* collected in situ. A positive relationship between the occurrence of nitrophytes and high levels of Fe, Mg, and N was also found by these authors.

Jeran (2002), however, did not find direct and negative influences of trace elements on epiphytic lichen diversity.

Bioindication and biomonitoring of atmospheric pollution with lichens

The sensitivity of lichens to atmospheric pollution has granted these organisms a wide use bioindicators and biomonitors, terms used here in the sense of Market (2007). Despite that, the term biomonitoring may also refer to the evaluation of temporal changes in lichen distribution, and be used even before repeated sampling is complete (Will-Wolf et al. 2002).

As bioindicators lichens provide information regarding the quality of the environment, as is the case of studies on lichen diversity. Alternatively, lichens can be used as biomonitors, giving quantitative information regarding the quality of the environment, due to their known capacity to bioaccumulate several pollutants. Reviews on the use of lichens as bioindicators and/or biomonitors can be found in Conti and Cecchetti (2001), Falla et al. (2000), Garty (2001), Nimis et al. (2002), Szczepaniak & Biziuk (2003), and Wolterbeek (2002, 2010).

Lichen diversity studies

Studies on lichen diversity have been widely used to assess air quality (e.g., Fernández-Salegui et al. 2007, Gombert et al. 2004, Geiser & Neitlich 2007, Käffer et al. 2011, Loppi & Frati 2006, Loppi & Nascimene 2010, Loppi et al. 2006, Pinho et al. 2004, Svoboda et al. 2010), but also to evaluate temporal changes in air quality (e.g., Frati & Brunialti 2006, Isocrono et al. 2007, Larsen Vilshom et al. 2009, Loppi & Corsini 2003, Loppi et al. 1998, 2003, 2004, Munzi et al. 2007, Purvis et al. 2010, Sparrius 2007, Vokou et al. 1999). Not only studies on lichen diversity can be used to bioindicate atmospheric pollution, but also the distribution of particular indicator species, with a known response to pollutant concentrations (e.g., Bates et al. 2001).

Bioindication of atmospheric pollution through the study of lichen diversity has been performed through various approaches, mostly using epiphytic lichens, which often lead to the construction of maps indicating air quality (e.g., Davies et al. 2007, Fernández-Salegui et al. 2007, Ferreti et al. 2004, Geiser & Neitlich 2007, Jeran et al. 2002, Nimis et al. 1990, Pinho et al. 2004).

Studies on lichen diversity are nowadays mostly performed using quantitative methods, which present the opportunity for more powerful statistical testing of lichen diversity data (Kricke & Loppi 2002). Recently, the LDV method was proposed in order to reduce the objectiveness of former ones (Asta et al. 2002), and is now an European Guideline that is becoming increasingly used (e.g., Isocrono et al. 2007, Larsen et al. 2007, Nali et al. 2007, Giordani 2007, Paoli et al. 2011, Svoboda et al. 2010, chapter 3 of this thesis). This and other alternative methods have also been used in lichen diversity studies, which involve only the partial study of the lichen flora: either sets of specific lichen species and genera (Blasco et al. 2008, Mayer et al. 2009), or only lichen morphotypes, as crustose, foliose, and fruticose (Jeran et al. 2002), or excluding crustose lichens (e.g., Geiser & Neitlich 2007, Pinho et al. 2004). Some of these methods facilitate the use of lichens as bioindicators, as they may not require a profound knowledge of the lichen flora (Geiser & Neitlich 2007, Mayer et al. 2009, Jeran et al. 2002).

Quantitative methods based on total lichen diversity estimations may not be appropriate for indicating atmospheric pollution (Giordani 2007). As NH_3 is known to promote the diversity and abundance of neutrophyte and nitrophyte lichens (see sub-section on NH_3 in this chapter), it may lead to increased values of estimated total lichen diversity if species of these groups are included (Cristofolini et al. 2008, Fernández-Salegui et al. 2007, Llopi & Nascimbene 2010, van Herck 2001). Therefore, some authors have suggested the exclusion of nitrophyte species in bioindication studies, when studying acidic air pollution in the presence of NH_3 emissions (Loppi & Nascimbene 2010, van Herck 2001). However, nitrophyte species are useful indicators of NH_3 and NO_x deposition (see sections on these pollutants in this chapter).

Most bioindication studies focus on the lichen flora growing on trunks, but recently species growing on twigs of deciduous trees also began being addressed (Wolseley & Pryor 1999). These were found to be more strongly correlated with atmospheric NH_3 than those on trunks, suggesting that they better reflect current environmental conditions (Wolseley et al. 2005, 2006). Older surfaces as trunks may support relict communities that established in former and possibly different environmental conditions (Larsen Vilsholm et al. 2009, Wolseley et al. 2005). A further advantage of studying lichens on twigs is that this method can provide yearly information, as the occurrence of lichens is measured on twig annual increments separated by girdle scars, in the case deciduous tree are studied

(Wolseley 2002). Despite that, the usage of this method is restricted to accessible twigs of lower branches, with twigs of canopies not being useful, as they are not exposed to the same conditions as the first ones, namely light regime, drought and exposure to increased air turbulence, apart from being more difficult to study (Larsen Vilsholm et al. 2009).

The role of environmental variables may need to be assessed in bioindication studies, as these may co-vary with pollution and have synergistic or antagonistic effects on lichen communities, and those effects may not remain constant under different ecological conditions or spatial scales (Cristofolini et al. 2008; Giordani 2007; Pinho et al. 2008a,b; Svoboda et al. 2010).

Lichens as bioaccumulator organisms

Lichens are also widely used in biomonitoring studies as bioaccumulators of atmospheric deposition. Lichens retrieve nutrients from the atmosphere, absorbing gases and water with dissolved substances through most of their surface that is in contact with the atmosphere. Therefore the chemical composition of lichens largely reflects that of the atmosphere, including the content in pollutants (Bargagli & Mikhailova 2002). Resistant lichens may concentrate high levels of persistent atmospheric pollutants, what is due to their slow growth rate and longevity, high surface-to-mass ratio and lack of protective structures characteristic of higher plants, as cuticles (Mikhailova 2002).

Although not replacing instrumental recording, lichens nonetheless provide a cheaper accessory method, and allow for the evaluation of atmospheric deposition over large and remote areas (Bargagli & Mikhailova 2002). Moreover, biomonitoring though the study of lichen accumulation may be used to select areas of higher risk where instrumental monitoring devices should be placed (Nimis et al. 2000). Furthermore, lichens provide a means for monitoring atmospheric pollution over long periods, while air sampling may only provide information relative to short study periods (Blasco et al. 2006).

The use of lichens as biomonitors allows not only for a local evaluation of air quality trends, but also regional (e.g., Brunialti & Frati 2007, Vieira et al. 2007) and national ones (e.g., Freitas et al. 1999, Zeran et al. 2002).

Biomonitoring has been mostly performed with foliose and fruticose lichens, but some studies point to the applicability of crustose lichens to biomonitoring (e.g., Bačkor et

al. 2003; Bajpai et al. 2009, 2010; Satya et al. 2012; Sawidis et al. 2010), although their small biomass hampers their wide use in most cases (Bačkor & Loppi 2009).

Lichens have been widely used to monitor the deposition of wide range of elements (e.g., Bergamaschi et al. 2007, Fuga et al. 2008, Giordano et al. 2009, Godinho et al. 2008a, Jeran et al. 2007, Smodiš & Bleise 2002, Vieira et al. 2004, Wolterbeek 2002, chapter 3 of this thesis). Some studies focus particularly on the accumulation of metals and metalloids (e.g., Adamo et al. 2007, Aprile et al. 2010, Ayrault et al. 2007, Bajpai et al. 2010, Brunialti & Frati 2007, Conti et al. 2004, Cuny et al. 2004, Garty et al. 2001, Giordano et al. 2005, Pirintos et al. 2006, Rusu et al. 2006), nitrogen and sulphur (e.g., Frati et al. 2006, Gombert et al. 2006, Olsen et al. 2010, Vingiani et al. 2004, Wadleigh 2003), radioelements (e.g., Adamo et al. 2004; Daillant et al. 2004, 2009; Di Lella et al. 2004; Kirchner & Daillant 2002; Loppi et al. 2003, 2004; Purvis et al. 2004; Seaward 2002), and saline elements (e.g., Figueira et al. 2001, 2002).

Lichens have also been applied to monitor the accumulation of polycyclic aromatic hydrocarbons (PAHs) (e.g., Augusto et al. 2010, Blasco et al. 2008, Satya et al. 2012, Shukla & Upreti 2009) and organochlorides (PCDD/Fs) (e.g., Augusto et al. 2004, 2007, 2009). Lichens may also be used to monitor the accumulation of particulate matter (Adamo et al. 2008, Williamson et al. 2004), and pesticides (Calvelo & Liberatore 2004).

The analysis of the chemical composition of lichens collected in situ has been used to ascertain the air quality in urban, industrial and rural areas (e.g.; Augusto et al. 2004, 2010; Bajpai et al. 2010; Branquinho et al. 2008; Conty et al. 2004; Cuny et al. 2004; Fuga et al. 2008; Gombert et al. 2006; Hissler et al. 2008; Loppi & Corsini 2003; Loppi & Frati 2006; Loppi et al. 2004, 2006; Minganti et al. 2003; Satya et al. 2012; Shukla & Upreti 2009). In addition, it has also been used in forested environments (Jeran et al. 2007, Loppi & Pirintsos 2003, Otnyukova 2007, Purvis et al. 2005) and in remote and pristine locations (Bergamaschi et al. 2002, 2004; Conti et al. 2009; Zhang et al. 2002), where they can signal changing air quality conditions, sometimes pinpointing the enrichment in elements derived from long-range transport (Bergamaschi et al. 2002, 2004; Conti et al. 2009; Loppi & Pirintsos 2003).

Lichen transplants have been used as an alternative to the collection of native lichens. This method allows the performance of biomonitoring experiments in areas with scarce lichens or without suitable substrata, and in a highly standardized way (Bargagli &

Mikhailova 2002). Further advantages are the possible use of lichens free from their substrate and the knowledge regarding the initial contents in pollutants (Cercasov et al. 2002). The lichen transplant technique allows testing for the effects of period and spatial conditions of exposure, as well as of climatic variables on bioaccumulation dynamics (e.g., Adamo et al. 2003; Ayrault et al. 2007; Baptista et al. 2008; Branquinho et al. 2008; Conti et al. 2004; Cuny et al. 2001; Figueira et al. 2002; Giordano et al. 2005, 2009; Godinho et al. 2008a,b; Ljubič Mlakar et al. 2011, Pacheco et al. 2008; Pirintsos et al. 2006; Rusu et al. 2006; Tretiach et al. 2011; chapter 3 of this thesis). Recently, lichen transplants were also applied as biomonitors in indoor environments (Canha et al. 2012).

In biomonitoring experiments with lichens, the identification of pollution sources is generally performed by means of statistical analysis (e.g., Alvarez et al. 2006, Pirintsos et al. 2006, Rusu et al. 2006, chapter 3 of this thesis), or through the calculation of enrichment factors, mostly for ascertaining the contribution of soil particles to pollutant accumulation (e.g., Adamo et al. 2008, Bergamaschi et al. 2002, Carignan et al. 2002, Cuny et al. 2001, Minganti et al. 2003, Vieira et al. 2004), or by the analysis of the isotopic composition of elements accumulated in lichens (e.g., Carignan et al. 2002; Hissler et al. 2008; Purvis et al. 2004, 2005; Wadleigh 2003).

The relationship between the concentrations of pollutants in atmospheric deposition and those found in lichens is poorly investigated (Adamo et al. 2007). Nonetheless, this issue has been addressed, and lichen transplants have been used to relate the observed accumulation with measured atmospheric deposition/emission of several elements (e.g., Figueira et al. 2002; Freitas and Pacheco 2004; Godinho et al. 2008b; Ljubič Mlakar et al. 2011; Pacheco et al. 2008; Reis et al. 1999, 2002). The accumulation of Hg in lichens collected in situ has been used to estimate emission rates of that metal by geothermal power plants (Loppi et al. 2006).

The accumulation of pollutants by lichens may not be linearly dependent on pollutants deposition, as it is affected by physiological conditions (e.g., Bačkor & Loppi 2009, Bergamaschi et al. 2007, Godinho et al. 2008b). Furthermore, the accumulation process results from an equilibrium between the intake and discharge of the pollutant from and into the environment (e.g., Reis et al. 1999, Wolterbeek 2002), and an acclimatization behaviour in lichens transplanted for long periods of time has been detected (Godinho et al. 2008b). Moreover, lichens were shown to have different remembrance times for distinct

elements (Reis et al. 1999, Walther et al. 1990), what may have implications on the period of exposure that is actually reflected by the elemental content in transplanted lichen thalli (Bergamaschi et al. 2007, Godinho et al. 2008b).

Effects of the exposure to pollutants on lichen physiology

Despite recent evidences indicate that the capacity of lichens to accumulate pollutants is affected by their physiological condition, the inverse relationship, that the accumulation of pollutants affects lichen physiology, is vastly reported in the literature. Therefore, it has been suggested that at least some physiological activities may be used as an early warning system for environmental contamination, as these respond before the appearance of morphological symptoms on lichens, changes in lichen community structure and species abundance (Paoli & Loppi 2008, Riga-Karandinos & Karandinos 1998).

The accumulation of pollutants induces oxidative stress in lichens due to the increased production of reactive oxygen species (ROS), which include singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Cuny et al. 2002). These can damage cells and may attack nucleic acids, lipids and proteins (Cuny et al. 2002). As a cellular means of protection, ROS are scavenged by antioxidants, as ascorbate and reduced glutathione, and enzymes, as catalase, peroxidases and superoxide dismutase (Cuny et al. 2002). Lichen substances may also have a role in the protection of oxidative stress, as some possess antioxidant activity (e.g., Cuny et al. 2002, Silberstein et al. 1996b)

Regarding metals exposure, cell wall immobilization, chelation by gluthatione and phytochelatins, and vacuolar compartmentalization are thought to be first line of defence in lichen photobionts (Sanità di Toppi et al. 2008). Second line defence mechanisms are thought to involve the production of stress proteins and the activation of enzymatic antioxidants (Sanità di Toppi et al. 2008). In lichen mycobionts, which contribute substantially to detoxification owing to their large biomass in the lichen thallus, cell wall immobilization and high constitutive levels of reduced glutathione, have been observed as means of metal detoxification, particularly of Cd (Sanità di Toppi et al. 2008).

Increased production of reduced glutathione, and increased activities of catalase, glutathione reductase, peroxidase, and superoxide dismutase were observed in lichens

exposed to atmospheric pollutants (e.g., Deltoro et al. 1999, Kong et al. 1999, Silberstein et al. 1996b). Decreased contents in reduced glutathione and decreased activities of enzymatic antioxidants have also been reported for some species, and that was related to their sensitivity to atmospheric pollutants (Deltoro et al. 1999, Silberstein et al. 1996b). Nonetheless, the low production of such enzymes in some lichen species may signify that lichens are more resistant to that type of stress (Schlee et al. 1995).

When scavenging processes are not completely efficient, ROS can damage membrane lipids and proteins and inactivate membrane-bound enzymes, thereby affecting membrane integrity and permeability. Increased membrane lipid peroxidation and membrane permeability, as indicated by means of electrical conductivity studies, were observed in lichens exposed to atmospheric pollutants (e.g., Carreras & Pignata 2001; Carreras et al. 2005; Dzubaj et al. 2008; Garty et al. 1997, 1998, 2003; Godinho et al. 2004; Häffner et al. 2001; Paoli & Loppi 2008; Riddell et al. 2008; Tarhanen et al. 1996).

Furthermore, decreased photosynthetic and respiration rates have observed in lichens exposed to atmospheric pollutants, what may be due to membrane damage (e.g., Deltoro et al. 1999, Djubaj et al. 2008, Eversman and Sigal 1987, Garty et al. 2001, Häffner et al. 2001, Riddell et al. 2008, Silberstein et al. 1996a).

The effects of the exposure to pollutants on lichen photobionts, and indirectly on photosynthesis, have also been addressed by the analysis of photosynthetic pigment concentrations. Most studies report decreases in pigments concentrations (mostly chlorophylls *a* and *b* and carotenoids) with exposure to pollutants, and increases in chlorophyll degradation (phaeophytinization) (e.g., Kardish et al. 1987, Kong et al. 1999, Garty et al. 1997, Pisani et al. 2011, Riddell et al. 2008, Silberstein et al. 1996a). There are however reports of increasing pigments concentrations with exposure to pollutants, including N, what has been attributed to a fertilizing effect of some pollutants (e.g., Carreras & Pignata 2001, Carreras et al. 1998, Ochoa-Hueso & Manrique 2011).

Decreased content in ATP (e.g., Kardish et al. 1987, Silberstein et al. 1996a), increased ethylene production (e.g., Garty et al. 2003), and increased content in amino acids in lichens exposed to atmospheric pollution have also been observed in lichens exposed to atmospheric pollution (Silberstein et al. 1996a).

Chlorophyll fluorescence

The photosynthetic performance of lichens exposed to atmospheric pollutants has also been evaluated through studies on the kinetics of chlorophyll *a* fluorescence. These studies evaluate changes in photosystem II (PSII) photochemistry, based in a model in which photochemistry competes with the processes of fluorescence and heat loss for excitation energy in the pigment antenna of PSII (Baker 2008). Chlorophyll fluorescence studies involve the measurement and calculation of several parameters in both dark- and light-adapted samples. These provide information on the processes occurring within PSII, and also on how these may have been altered by the accumulation of atmospheric pollutants.

In chlorophyll fluorescence studies with lichens exposed to atmospheric pollutants, the most used parameter is F_v/F_m, a relative measure of the maximum quantum efficiency of PSII photochemistry, which is considered a rapid and simple way to monitor stress (Baker 2008, Maxwell & Johnson 2000). F_v/F_m is calculated as $(F_m-F_0)/F_m$, F_m being the maximum fluorescence, and F_0 the minimal fluorescence of dark-adapted samples (Baker 2008). Despite decreases in F_v/F_m indicate exposure to stress, this may not mean that the efficiency of photosynthetic performance under ambient light has been compromised, as this parameter is measured in dark-adapted samples (Baker 2008).

Decreases in F_v/F_m in different lichen species have been found with short-term exposure to several elements, mostly metals, such as Cd, Cu, Cr, Hg, Ni, and Pb in laboratorial conditions (e.g., Bačkor et al. 2010; Branquinho et al. 2011, 1997a,b; Dzubaj et al. 2008; Pisani et al. 2011; Unal et al. 2010). F_v/F_m also decreased in photobiont cultures exposed to Cd and Cu (e.g., Bačkor et al. 2007, Pióvar et al. 2011). Field experiments also point to decreases in F_v/F_m correlated with the accumulation of Ba, Ca, Cu, Ni, S, and Sr in transplanted or in situ collect lichens (Garty et al. 2000, 2002, 2003; Odasz-Albrigtsen et al. 2000). Furthermore, Garty (2004) refers that decreases in F_v/F_m were also coincident with the accumulation of Cr, Fe, Hg, Mn, Pb, V, and Zn in transplanted lichens. However, F_v/F_m was found to be positively correlated with K content in transplanted lichens (Garty et al. 2000), and Matos et al. (2011) found reductions in F_v/F_m concomitant with reductions in intracellular K, but also with increases of intracellular Na, subsequent to the incubation of lichen thalli in saline water. Decreases in

F_v/F_m have also been observed in lichens environmentally exposed to NH_3, O_3, SO_2 (Calatayud et al. 1996, Fernández-Salegui et al. 2006, Odasz-Albrigtsen et al., 2000, Paoli et al. 2010), and were confirmed by laboratorial experiments for SO_2 (Deltoro et al. 1999). Fumigation experiments with O_3 indicate that F_v/F_m decreases with exposure in some studies (Balaguer et al. 1996, Scheidegger & Schroeter 1995), but not in others (Calatayud et al. 2000). Fumigation experiments also point to a decrease in F_v/F_m with exposure to CO_2 (Balaguer et al. 1996). The effects of NH_4^+ exposure in laboratorial conditions also point to its negative effect on F_v/F_m in some studies (Munzi et al. 2011, Pirintsos et al. 2009), but no significant variation was observed in lichen exposed in environmental conditions and supplied with NH_4NO_3 (Gaio-Oliveira et al. 2005). However, Ochoa-Hueso & Manrique (2011) refer a critical load of above 40 Kg ha^{-1} yr^{-1} of nitrogen for F_v/F_m, below which F_v/F_m is unaffected in the lichen *Cladonia foliacea* (Huds.) Willd. Decreases in F_v/F_m were also observed in lichens incubated in solutions containing fluoranthene, a PAH (Kummerová et al. 2007).

Nonetheless, care must be taken in the interpretation of F_v/F_m values in samples exposed in field conditions, as climatic conditions may induce decreases in F_v/F_m (Baruffo & Tretiach 2007, Fernández-Salegui et al. 2006, Piccotto et al. 2011). These decreases are thought to be a consequence of a long-term adaptation at physiological and structural levels to changes in macro- and microclimatic conditions, but also of photoinhibition resulting from exposure to excess light, but only in the case it leads to photodegradation (Baruffo & Tretiach 2007).

The parameters F_0 and F_m, used in the calculation of F_v/F_m, have also been directly studied for testing the effects of atmospheric pollution on photosynthetic performance. Deltoro et al. (1999) found that both parameters decreased in lichens fumigated with SO_2, although F_m was more affected than F_0. F_m was also reduced in lichens exposed to ambient NH_3 (Paoli et al. 2010). Lichens incubated with fluoranthene-containing solutions also showed increases in F_0 (Kummerová et al. 2007). Both parameters, F_0 and F_m, were negatively correlated with ambient NO_x in transplants of *Flavoparmelia caperata* (Tetriach et al. 2007). However, as F_v/F_m, these parameters are influenced by environmental factors, as temperature (Baruffo & Tretiach 2007, Tretiach et al. 2007).

The study of fluorescence quenching is necessary for the evaluation photosynthetic performance (Baker 2008). Quenching may occur thorough two processes, one of which is

photochemical and the other non-photochemical. Photochemical quenching, q_p, gives information on the transference of electrons from the reaction centres in PSII to a primary quinone (Q_A) acceptor, and the second occurs when there is energy loss as heat (Baker 2008). Decreases in q_P have been documented in lichens exposed to SO_2 and O_3 (Calatayud et al. 1996, Deltoro et al. 1999, Fernández-Salegui et al. 2006), but Calatyud et al. (2000) did not find effects of exposure to O_3 on this parameter. Paul & Hauck (2006) report different responses of q_p among chloro- and cyanolichens incubated in a Mn counting solution.

Non-photochemical quenching has been mostly studied through the parameter NPQ designated precisely non-photochemical quenching, and to a less extent by the parameter *qN*, the non-photochemical quenching of variable fluorescence (Tretiach et al. 2007). The last in an older parameter, which was found to be insensitive to quenching at high values (Maxwell & Johnson 2000). Reductions of NPQ with exposure to atmospheric pollutants have also been reported in lichens with exposure to O_3, NO_x, SO_2 (Calatayud et al. 1996, Deltoro et al. 1999, Fernández-Salegui et al. 2006, Tretiach et al. 2007), but not in other studies for SO_2 or O_3 (Calatayud et al. 2000, Tretiach et al. 2007). Tretiach et al. (2007) found that *qN* was negatively correlated with ambient NO_x levels in transplants of *Flavoparmelica caperata*. Both NPQ and *qN* were increased in lichens incubated in an Mn-containing solution, but only at high light intensities (Paul & Hauck 2006).

Non-photochemical quenching is a major photoprotective mechanism (Adams et al. 2008, Baker 2008, Maxwell & Johnson 2000), and its decrease with exposure to pollutants may indicate that photoprotective mechanisms are exhausted (decrease in xanthophylls cycle) or that there has been damage to PSII antennas (Calatayud 2007). However, reductions in non-photochemical quenching may also be due to stress induced into other physiological systems diminish the rate of consumption of NADPH and ATP produced during photosynthesis (Baker 2008). Nonetheless, it has been postulated that for some lichens photochemistry is the main form of energy dissipation and not non-photochemical quenching (Piccotto & Tretiach 2010).

The chlorophyll fluorescence parameter F_v/F_m is determined in dark-adapted samples. However, chlorophyll fluorescence studies may also be used to evaluate photosynthetic efficiency in light-adapted samples (Baker 2008). One of the studied parameters is the PSII operating efficiency (Φ_{PSII}). It estimates the efficiency at which the

light absorbed by PSII is used for quinone (Q_A) reduction, the primary electron acceptor of PSII reaction centres (Baker 2008, Maxwell & Johnson 2000). Changes in Φ_{PSII} are caused by alterations in non-photochemical quenching, and/or in the capacity of excited reaction centres to promote electron transport (q_p) (Baker 2008). Decreases in Φ_{PSII} have been reported in lichens exposed to O_3 and SO_2 (Balaguer et al. 1996, Deltoro et al. 1999, Fernández-Salegui et al. 2006), but in other studies exposure to O_3, NO_x, and SO_2 were not found to lead to decreases in Φ_{PSII} (Calatayud et al. 2000, Tretiach et al. 2007). Furthermore, decreases in Φ_{PSII} were also reported chloro- and cyanolichens incubated with an Mn-containg solution (Paul & Hauck 2006). Incubation with fluoranthene-containg solutions also induced decreases in Φ_{PSII} in lichens (Kummerová et al. 2007).

The maximum efficiency of PSII (Φ_{Exc}), also known as the excitation capture efficiency of PSII, or the intrinsic PSII photochemical energy, is another parameter studied in light-adapted samples (Calatayud et al. 1996). It represents the quantum yield of open PSII reaction centres, that is the efficiency of excitation energy transfer from the light harvesting complexes (LHCII) to the PSII reaction centres (Rosenquist & van Kooten 2003), but also of how efficiently open PSII centres reduce Q_A (Calatayud et al. 1996). It is nonlinearly correlated to NPQ, with higher Φ_{Exc} values being found with low NPQ values, and linearly correlated with Φ_{PSII}, since $\Phi_{Exc} = \Phi_{PSII} / q_P$ (Rosenquist & van Kooten 2003). Calatayud et al. (1996) and Fernández-Salegui et al. (2006) report increases in this parameter with exposure of lichens to SO_2 and O_3, although Deltoro et al. (1999) found decreases in lichens fumigated with SO_2.

The effects of atmospheric pollutants on lichen's chlorophyll fluorescence kinetics has also been studied by means of the JIP-test, which involves an analysis of fast chlorophyll fluorescence signals with a an elevated temporal resolution, and allows the analysis of the energy fluxes occurring in PSII (Paoli et al. 2010). Among other findings, Paoli et al. (2010) reached the conclusion that the parameter $PI_{ABS,}$ which combines in a single expression the three functional steps of the photosynthetic activity (light absorption, excitation energy trapping, and conversion of excitation energy to electron transport), is a much more sensitive parameter of lichens exposure to NH_3 than F_v/F_m.

Objectives

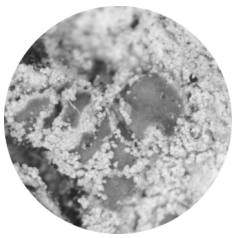
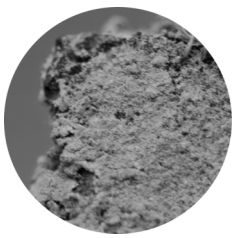
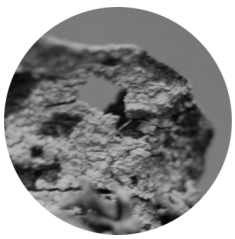
The lack of knowledge regarding the epiphytic lichens occurring in Portuguese pine forests, lead to the establishment to two main objectives in this work. The first involved the study of biodiversity the epiphytic lichen flora of Portuguese pine forests on sand dunes with focus on the centre of the country. The second aim of this thesis was to evaluate if epiphytic lichens of these forests were affected by the presence of nearby polluting industries.

The biodiversity study was primarily undertaken at a selected pine forest in central Portugal, Dunas de Quiaios (Figueira da Foz). However, subsequent studies were extended to most of the Portuguese coast. The results of this work are addressed in chapter 2, but are also included in chapters 3 and 4, and in annexes I and II).

In order to fulfil the second objective of this work, a study was undertaken at a coastal pine forest also in the centre of the country (Mata do Urso, Figueira da Foz), which is impacted by the presence of a Kraft pulp mill at its border. One of the aims of this study was to evaluate the accumulation of several elements putatively emitted by pulp and paper mills on lichen transplants, as little information regarding their emissions from Portuguese facilities is available. The second objective of this study was to test if distance and period of exposure had a significant effect on elemental accumulation. Thirdly, it was intended to evaluate if elemental accumulation had a significant effect on lichen physiology, by means of chlorophyll *a* fluorescence kinetics analysis also testing for the effects of distance and period of exposure on selected chlorophyll *a* fluorescence parameters. The fourth objective of this study was to evaluate if the epiphytic lichen flora was affected by the atmospheric emissions of the pulp mill. The results of this study are presented in chapter 3.

The work undertaken for this thesis is resumed and generally discussed in chapter 4, where the concluding remarks of this work are also presented.

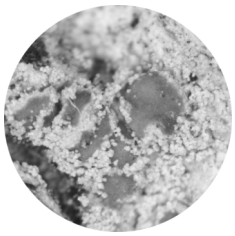


Chapter 2

Epiphytic lichen diversity in Portuguese coastal pine forests

Figures on the previous page: from top to bottom, *Chrysothrix flavovirens* Tønsberg, *Hypotrachyna lividescens* (Kurok.) Hale, *H. pseudosinuosa* (Asahina) Hale, *Lecanora sorediomarginata* Rodrigues, Terrón & Elix, *Lepraria elobata* Tønsberg, and *Ochrolechia arborea* (Kreyer) Almb. The images of *L. elobata* and *O. arborea* were kindly provided by Prof. Tor Tønsberg.



Chapter 2.1

Lecanora sorediomarginata, a new epiphytic lichen species discovered along the Portuguese coast

Figure on the previous page: *Lecanora sorediomarginata* Rodrigues, Terrón & Elix.

Lecanora sorediomarginata, a new epiphytic lichen species discovered along the Portuguese coast

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Abstract

Lecanora sorediomarginata Rodrigues, Terrón & Elix sp. nov., described as new to science from Portugal, is characterised morphologically by a crustose whitish-grey to greenish thallus developing soralia from small, marginal warts and chemically by the presence of 3,5-dichloro-2'-*O*-methylnorstenosporic acid [major], 3,5-dichloro-2'-*O*-methylanziaic acid [minor], 3,5-dichloro-2'-*O*-methylnordivaricatic acid [minor], 5-chloro-2'-*O*-methylanziaic acid [trace], atranorin [minor], chloroatranorin [minor], and usnic acid [trace]. It is chemically similar to *L. lividocinerea,* to which it shows phylogenetic affinities based on ITS rDNA sequence analysis, and to *L. sulphurella*. *Lecanora sorediomarginata* is epiphytic on *Pinus pinaster* and *P. pinea*, in pine forests on sand dunes along the Portuguese coast.

Keywords: ITS rDNA, *Lecanoraceae*, pines forests, sand dunes, taxonomy

Introduction

Lecanora Ach. (*Lecanoraceae*) is a large genus comprising *c.* 800 species and is defined by hyaline and simple spores, *Lecanora*-type asci, green algal photobionts, an usually thalline margin of the apothecium and generally a crustose thallus (LaGreca & Lumbsh 2001, Pérez-Ortega et al. 2010). *Lecanora* s. str. comprises *c.* 300 species and is characterized by the presence of oxalate crystals in the amphithecium and the production of atranorin and/or usnic acid in the cortex (La Greca & Lumbsch 2001). This genus has been divided in several groups, which were until recently circumscribed using morphological, anatomical or chemical characters (Arup & Grube, 1998). In recent years, molecular studies began clarifying the phylogenetic relationships in and between some of these groups. These indicate that *Lecanora* s. str. is a heterogenous assemblage of species (Grube et al. 2004), but the relationships between groups are still largely unresolved (Pérez-Ortega et al. 2010).

The core of *Lecanora* is the *L. subfusca* group, which contains the type species *L. allophana* Nyl., and is identified by the presence of a crustose thallus containing atranorin, either as a major or trace constituent; as well as crystals in the amphithecium and filiform conidia (Lumbsch et al. 2003). So far, no phylogenetic studies were performed in order to assess the phylogenetic relationships of species currently included in this group, despite some were used in studies relative to other groups, namely on the subgenus *Placodium* and the *L. rupicola* and *L. varia* groups. Both the subgenus *Placodium* and *L. varia* groups were found to be heterogenous, and some species considered to belong to these groups were found to group with other *Lecanora* groups (Arup & Grube 1998, Pérez-Ortega et al. 2010). The *L. rupicola* group, previously circumscribed to saxicolous species containing sordidone, was found to be monophyletic and including all species containing sordidone, regardless of their substrate, as the corticolous species of the *L. carpinea* group (Grube et al. 2004). Further, it has been shown that the genus *Rhizoplaca* Zopf is polyphyletic and is nested within several groups of *Lecanora* (Arup & Grube 2000). More studies are therefore needed to clarify the phylogenetic relationships between members of *Lecanora*, what may require more intensive taxon sampling (Pérez-Ortega et al. 2010).

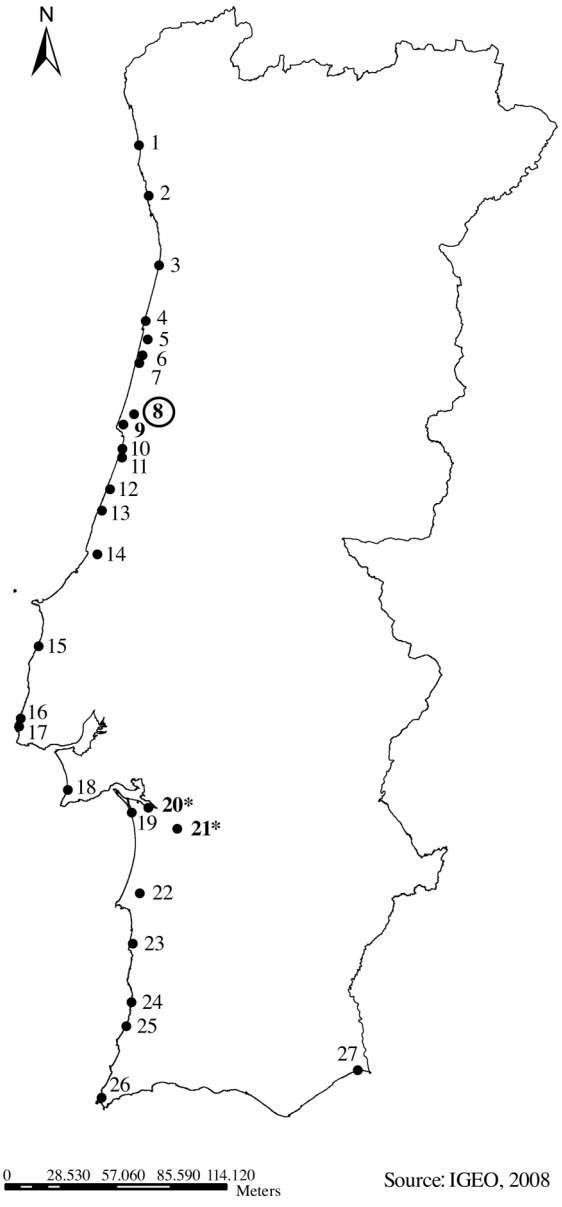
Lecanora sorediomarginata sp. nov. was discovered at Dunas de Quiaios (Figueira da Foz) on the central west coast of Portugal. It is epiphytic on *Pinus pinaster* Aiton and *P.*

pinea L. Dunas de Quiaios is a pine forest on a sand dune area, which was scarcely vegetated until 1924 (Almeida 1997). In that year the Portuguese Forest Services started stabilizing the dunes by sowing *P. pinaster*, the species selected to promote dune stabilization, together with *Acacia longifolia* (Andrews) Willd, *Acacia retinoides* Schltdl., *Corema album* (L.) D. Don, *Myrica faya* Aiton and *Ulex europaeus* L., in the interior dunes. Since the original seeding, *Cistus salvifolius* L., *Cytisus grandiflorus* (Brot.) DC., *Halimium halimifolium* (L.) Willk., *H. calycinum* (L.) K. Koch and *Lavandula stoechas* L. subsp. *sampaiana* Rozeira have spontaneously naturalised and proliferated in the area (Almeida 1997). Other species occurring in secondary dunes include *Acacia melanoxylon* R. Br., *Arbutus unedo* L., *Eucalyptus globulus* Labill. and *P. pinea*, the latter thought by some authors to be the original forest species of these sand dunes (Danielsen 2008). In the depressions and flat surfaces of these dunes hygrophilic species such as *Schoenus nigricans* L. and *Scirpus holoschoenus* L., among others can be detected (Almeida 1997).

This pine forest on sand dunes is rich in epiphytic and terrestrial lichens. The most abundant epiphytic lichens are *Chrysothrix candelaris* (L.) J. R. Laundon and *Pyrrhospora quernea* (Dicks.) Körb., followed by *Hypogymnia physodes* (L.) Nyl., *Flavoparmelia caperata* (L.) Hale, *Parmotrema reticulatum* (Taylor) M. Choisy, *Usnea rubicunda* Stirt. and *U. subscrabosa* Nyl. ex Motyka. Other interesting species have also been found here, including *Hypotrachyna lividescens* (Kurok.) Hale and *H. pseudosinuosa* (Asahina) Hale, species not previously known from the Iberian Peninsula (Rodrigues et al. 2007). *Chrysothrix flavovirens* Tønsberg, *Lepraria elobata* Tønsberg and *Ochrolechia arborea* (Kreyer) Almb. have also been detected in Dunas de Quiaios and are novelties for the Portuguese lichen flora (Rodrigues et al. 2011b).

Pine forests on sand dunes are common along the Portuguese coast. The most famous is probably the pine forest of Leiria known as "Pinhal de Leiria", where seeding was greatly encouraged by king D. Dinis (13–14th century) (Arroteia 2009). Given the large number of pine forests on sand dunes along the coast, surveys were undertaken in other similar areas, ranging from north to south-eastern Portugal (Fig. 2.1.1) in search of *L. sorediomarginata*. In most of the forests the main phorophyte is *P. pinaster*, but in Mata de Valverde (Alcácer do Sal) (Fig. 2.1.1: 21) *P. pinea* is the main phorophyte. Some nearby mountains beyond the sand dunes were also visited, including the Serra da Boa Viagem (Figueira da Foz) (Fig. 2.1.1: 9) and Serra de Sintra (Sintra) (Fig. 2.1.1: 17). The

type locality, Dunas de Quiaios (Figueira da Foz) (Fig. 2.1.1: 8), as well as several of the localities visited are national forests or forest perimeters owned and/or partially managed by Portuguese Forest Services (Fig. 2.1.1: 3, 5–14, 17, 21–22). Some areas are within Natural Parks or Nature Reserves, as is the case of the Natural Parks of Litoral Norte (Fig. 2.1.1: 1), Sintra-Cascais (Fig. 2.1.1: 16, 17), Arrábida (Fig. 2.1.1: 18) and Sudoeste Alentejano e Costa Vicentina (Fig. 2.1.1: 23–26); and of the Nature Reserves of S. Jacinto (Fig. 2.1.1, 4) and of Estuário do Sado (Fig. 2.1.1: 19–20). Most of the areas surveyed belong to the Natura 2000 Network (Fig. 2.1.1: 1, 4, 6–8, 15–20, 23–27), Dunas de Quiaios being part of the Site "Dunas de Mira, Gândara e Gafanhas" (PTCON0055) (ICN 2006).



Materials and methods

The morphology of the thallus was examined under stereomicroscopes and images were taken with a stereomicroscope (Nikon SMZ1500), using the program NIS-Elements (Nikon). The measurement of morphological structures, such as wart size, diameter of apothecia and thickness of the apothecial margin were made with a Leica MS5 stereomicroscope. The thickness of the thallus as well as the size of consoredia and soredia were made in samples mounted in lactophenol cotton blue and viewed under a Leitz HM-LUX 3 microscope.

Anatomical observations of the apothecia were performed on hand cut sections and also on microtome sections mounted in K/I and lactophenol cotton blue. For obtaining microtome sections, apothecia were placed in gelatine (Tissue-Tek, Sakura), frozen inside a microtome (Microm HM 505 E) at –20ºC, and 14 µm thick sections cut. Sections for spot tests with K and C were mounted in distilled water. The same procedure was used for viewing the reactions of the epihymenial crystals following the addition of K and HNO_3. Images of the apothecial sections were taken with an epifluorescence microscope (Optihot 2, Nikon), using the imaging program NIS-Elements (Nikon). Images were taken under normal and polarized light and under an UV excitation filter (EX 330-380 nm, DM 400 nm and BA 420 nm).

SEM imaging of both hand made and micrototome sections of apothecia and of consoredia and soredia was performed with a Scanning Electron Microscope (JSM-6480LV, JEOL). For that, apothecial sections were mounted on SEM holders and covered with gold in a Sputter Coater (SCD 004, Balzers).

Figure 2.1.1: Locations where *Lecanora sorediomarginata* was found along the Portuguese coast. 1- Fão (Esposende), 2- Parque de Campismo de Angeiras (Matosinhos), 3- Dunas de Ovar (Ovar), 4- Dunas de S. Jacinto (Aveiro), 5- Dunas da Gafanha (Ílhavo), 6- Dunas de Vagos (Vagos), 7- Dunas de Mira (Mira), 8- Dunas de Quiaios (Figueira da Foz), 9- Mata do Prazo de Santa Marinha/Serra da Boa Viagem (Figueira da Foz), 10- Dunas da Leirosa (Figueira da Foz), 11- Mata do Urso (Figueira da Foz), 12-Mata do Pedrógão (Leiria), 13- Pinhal de Leiria (Marinha Grande), 14- Mata do Valado (Nazaré), 15- Praia do Seixo (Torres Vedras), 16- Praia das Maçãs (Sintra), 17- Serra de Sintra (Sintra), 18- Praia do Moinho de Baixo (Sesimbra), 19- Praia da Comporta (Alcácer do Sal), 20- Murta (Alcácer do Sal), 21-Mata de Valverde (Alcácer do Sal), 22- Área Florestal de Sines (Santiago do Cacém), 23- Praia do Malhão (Odemira), 24- Praia do Carvalhal (Odemira), 25- Praia de Vale dos Homens (Aljezur), 26- Pinhal de Vale Santo (Vila do Bispo), 27- Dunas de Vila Real de Santo António (Vila Real de Santo António). Numbers in bold refer to locations where *L. sorediomarginata* was found both on *Pinus pinaster* and *P. pinea*, while numbers in bold and with an asterisk refer to locations where this species was found only on *P. pinea*. The encircled number refers to the type locality.

Specimens were analysed chemically by standardized thin layer chromatographic methods (TLC) (Elix & Ernst-Russell 1993, Orange *et al.* 2001, White & James 1985) and by high performance liquid chromatography (HPLC) (Elix *et al.* 1993).

Confirmation of the identity of *L. sorediomarginata* from distinct localities was performed by morphological and chemical analysis (TLC) of one to three specimens from each localition.

The locations visited were georeferenced using GoogleEarth and maps were plotted using ArcGis version 9.2.

Phylogenetic analysis

For DNA extraction, soredia and apothecia were used from *L. sorediomarginata* and *L. lividocinerea* (Bagl.) respectively; they were separated from thallus with the help of a forceps. Special care was taken to avoid areas with possible contaminating fungi. Samples were extracted using the DNEasy Plant Mini Kit (Qiagen®), following the manufacturers' protocol, with minor modifications (Crespo *et al.* 2001). PCR reactions were prepared for a 25 µl final volume, containing 1.25 µl of each primer (10 µM), 17.5 µl of distilled water and 5 µl of the DNA template; PuReTaq Ready-To-Go PCR beads (GE Health Care, Amersham Biosciences, 2004) were added to the mix according to the manufacturer's instructions. PCR amplifications were carried out in a PTC-100 Peltier® Thermal Cycler, using the following conditions: initial denaturation for 4 min at 94ºC, followed by 3 cycles of 1 min at 94ºC, 1.30 min at 54ºC and 1.45 min at 72ºC; then 30 cycles of 1 min at 94ºC, 1 min at 48ºC and 1.45 min at 72ºC, and final elongation for 7 min at 72 ºC. The following primers were used for PCR amplifications: ITS1F (Gardes & Bruns 1993), ITS4 (White *et al.* 1990), ITS1LM (Myllys *et al.* 1999) and ITS2KL (Lohtander *et al.* 1998). PCR products were purified using QIAquick PCR Purification Kit (Qiagen®) following the manufacturer's instructions. Both complementary strands were sequenced by Secugen (CIB, Madrid), using the BigDye ® Terminator v3.1. Sequence fragments obtained were checked, assembled and edited in SeqMan v.7 (Lasergene®).

Sequence alignment

Amplicons obtained from our samples were aligned with members of the genus *Lecanora* found in Gen Bank, trying to encompass the highest diversity within the genus. For that, members of *L. dispersa*, *polytropa*, *rupicola*, *subfusca*, *symmicta* and *varia* groups, as well as of the *Protoparmeliopsis* group were used in the analysis, corresponding to a total of 47 ingroup and 1 outgroup taxa (*Japewia tornoensis* (Nyl.) Tønsberg) (Table 2.1.1).

Table 2.1.1: ITS rDNA sequences used in the phylogenetic analysis of *Lecanora sorediomarginata* and their GenBank accession numbers (newly produced sequences in bold).

Species	Genbank Accession Number (ITS rDNA)	Species	Genbank Accession Number (ITS rDNA)
Japewia tornoensis	EF495163	*Lecanora lojkaeana*	AY541256
Lecanora albella 1	AY541240	*Lecanora macrocyclos*	AF159933
Lecanora albella 2	AY541241	*Lecanora muralis*	FJ497040
Lecanora albescens	AF070033	*Lecanora nashii 1*	AF159931
Lecanora allophana 1	AF070031	*Lecanora nashii 2*	AY398702
Lecanora allophana 2	AF159939	*Lecanora orosthea*	AY398701
Lecanora bicincta	DQ451664	*Lecanora paramerae*	EF105413
Lecanora bipruinosa	AF159932	*Lecanora perpruinosa*	AF070025
Lecanora caesiorubella	AY541245	*Lecanora polytropa*	DQ534470
Lecanora campestris	AF159930	*Lecanora pulicaris*	AF101274
Lecanora carpinea	AY541249	*Lecanora reuteri*	AF070026
Lecanora cateilea	AY541250	*Lecanora rugosella*	AY398712
Lecanora cenisia	EU558541	*Lecanora rupicola* 1	DQ451669
Lecanora chlorophaeodes 1	AF070029	*Lecanora rupicola* 2	DQ451667
Lecanora chlorophaeodes 2	AY398704	*Lecanora rupicola* 3	DQ451670
Lecanora concolor	AF070037	*Lecanora saligna*	AF189716
Lecanora conizaeoides	AF189717	*Lecanora sorediomarginata* 1	**GU480121**
Lecanora contractula	AF070032	*Lecanora sorediomarginata* 2	**GU480122**
Lecanora dispersa	EU266081	*Lecanora straminea*	AY398700
Lecanora dispersoareolata	AF070016	*Lecanora subcarpinea*	DQ451657
Lecanora epibryon	AY541251	*Lecanora subrugosa*	AY398711
Lecanora flotowiana	AF070034	*Lecanora sulphurea*	AF070030
Lecanora garovaglii	AF189718	*Lecanora swartzii*	DQ451656
Lecanora horiza	AY541252	*Lecanora varia* 1	AF070021
Lecanora hybocarpa	DQ782849	*Lecanora varia* 2	AF070028
Lecanora intricata	AY398703	*Rhizoplaca aspidophora*	DQ534484
Lecanora intumescens 1	AY541253	*Rhizoplaca chrysoleuca*	EU586515
Lecanora intumescens 2	AY541254	*Rhizoplaca huashanensis*	AY530885
Lecanora lividocinerea	**GU480123**		

Alignments were constructed using Muscle v3.6 (Edgar 2004) and subsequently checked and improved by hand. Ambiguously aligned regions were removed from the alignment using Gblocks 0.91b (Castresana 2000). Nucleotide substitution models were

statistically selected with the help of jModelTest (Posada 2008, program available at http://darwin.uvigo.es). Model selection was made according to the Akaike information criterion (AIC, Akaike 1974); the General Time Reversible substitution model (Tavaré 1986) with a proportion of invariant sites and site specific substitution rates following a gamma distribution with six rate categories (GTR+I+G) had the lowest –lnL value according to the AIC. Bayesian analyses were carried out using MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001). The $(MC)^3$ analysis was run for 5000K generations starting from a random tree, employing 8 simultaneous chains and using the default temperature of 0.2. Every 200^{th} trees were sampled and the first 5000 trees were discarded as burn-in. Posterior probabilities of each branch were calculated by counting the frequency of trees that were visited during the course of the B/MCMC analysis. The 50% majority-rule consensus tree was obtained from the remaining trees. Trees were visualized using the program Treeview (Page 1996).

Taxonomic description

Lecanora sorediomarginata Rodrigues, Terrón & Elix sp. nov.
Mycobank MB 518287

Thallus crustaceus, cretaceo-griseus usque ad viridis, verruculosus et cum sorediis. Prothallus cretaceus, ad thalli marginem et inter verrucas visibilis. Soredia verrucularum exorientes, initium sejuncta, sed posterius confluentia ad matura thalli partes. Soredia composita per tenuia soredia et consoredia. Photobiont *Trebouxia*. Apothecia lecanorina, rara, sejuncta vel aggregata, sessilia, 0.37–1.25 mm diam. Discus epruinosus, pallide vel obscure brunneus. Excipulum thallinum flexuosum, cum sorediis. Epihymenium brunneum, propter crystallorum brunneorum praesentiam, inspersum. *Sporae* ellipsoidales, incoloratae, simplices vel monoseptatae, 4.0–8.5 × 6.5–11.5 µm. *Hypothecium* hyalinum, algarum stratum absens. Thallus continens acidum 3,5-dichlorinum-2'-*O*-methylnorstenosporicum [major] et vestigiae plurium acidorum aliorum.

Typus: Portugal, Beira Litoral, Figueira da Foz, Dunas de Quiaios, MGRS: 29TNE1654, 49 m alt., epiphytic on *Pinus pinaster* in a pine forest on sand dunes, 15 December 2006, *S. A. Rodrigues* AVE-L 197 (AVE-L holotypus; LEB- Lichenes 7581 isotypus).

Thallus crustose, corticolous, whitish-grey to greenish, appearing as separate patches in distinct scales of *Pinus* bark, or forming a continuous crust up to 8 cm wide. Margin endosubstratal to very thinly episubstratal, (0) 7.5–(28.0)–52.0 µm thick (n=9) or forming small warts, 0.07–(0.14)–0.25 mm in diam. (n=74) (Fig. 2.1.2A). In certain older areas of the thallus, it may become more obviously episubstratal and up to 38.0–(213.5)–1184.5 µm thick (n=9). *Prothallus* whitish, visible at thallus margin and around warts. A black border line sometimes present between neighbouring thalli, or when in contact with *P. quernea* or *L. lividocinerea*; otherwise not visible. Thallus sorediate; soredia arising from rupture of episubstratal warts. *Soralia* initially isolated and sparse but becoming dense or coalescing in older areas of the thallus (Fig. 2.1.2B), composed of both fine soredia and consoredia. Consoredia greenish in the upper part and whitish grey in the lower part, coarse, somewhat elongate, 22.5–(45.0)–139.0 × 35.0–(54.5)–147.0 µm (n=47), wall indistinct (Fig. 2.1.2C). Fine soredia usually rounded, ±slightly elongated, 16.0–(22.5)–37.0 × 17.0–(25.5)–37.0 µm (n=57). *Medulla* not observed. Photobiont *Trebouxia*, 4.0–(8.5)–11.5 µm in diam.

Apothecia rare, scattered or grouped, lecanorine, sessile, 0.37–(0.76)–1.25 mm diam. (n= 19) (Fig. 2.1.2D). *Disc epruinose*, pale to dark brown; *amphithecium* flexuose, sorediate, concolorous with the thallus, usually persistent, but sometimes consoredia eroded from part of the margin, (0) 0.05–(0.12)–0.22 mm wide. *Epihymenium* brownish due to the presence of fine brown crystals, crystals soluble in KOH, insoluble in HNO_3, *pulicaris*-type (Brodo 1984), ±also present in the hymenium and subhymenium (POL+) (Fig. 2.1.2E), inspersed with small oil droplets. *Hymenium* hyaline, 60.0–(68.0)–82.0 µm high (n=5), I+ blue; inspersed with oil droplets. *Asci* clavate 32.0–(42.5)–50.0 × 12.5–(15.5)–19.0 µm (n=7). *Spores* ellipsoid, hyaline, simple or monoseptate, 4.0–(6.5)–8.5 × 6.5–(10.0)–11.5 µm (n=96) (Fig. 2.1.2 F&G). *Paraphyses* septate, branched at the base of the hymenium or in the subhymenium, not capitate, slightly bent near the tip, leptodermatous type, c.1.1 µm diam. *Subhymenium* 41.0–54.0–65.0 µm thick (n=5). *Hypothecium* hyaline, 140.0–(150.0)–160.0 µm thick (n=4) in the centre; algal layer not present. *Parathecium* hyaline, 23.0–(29.0)–50.0 µm thick at the extremity of the apothecia. *Amphithecium* entire in very early stages, but then completely composed of consoredia, lacking medulla and cortex, with small crystals (POL+), 451.0–(474.0)–526.5 µm thick (n=5) near the parathecium.

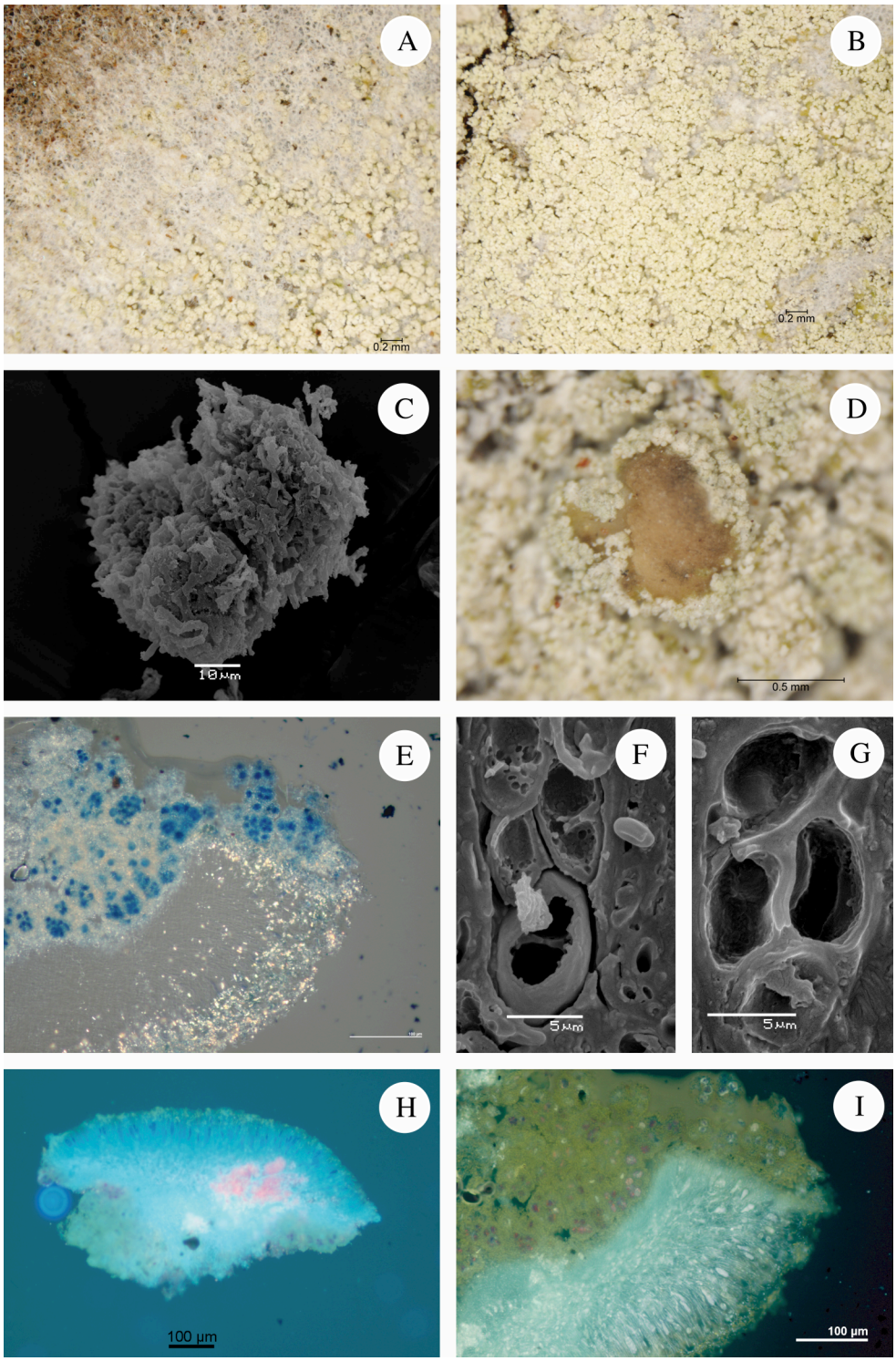


Figure 2.1.2: *Lecanora sorediomarginata*. A, Margin of the thallus, where the thin prothallus is visible as well as warts that lead to soralia; B, older, entirely sorediate part of the thallus; C, consoredium, with three component-soredium; D, apothecia with a consorediate, flexuose margin; E, section of an apothecium under polarized light, crystals are present in the epihymenium, subhymenium, hypothecium, and consoredia; F, ascus with some septate spores, septation observed in both immature and mature spores; G, ascus with simple spores; H, Section of an apothecia seen under UV fluorescence, a substance present in the epihymenium and in the consoredia of the amphithecium fluoresces UV+ yellow, in the subhymenium, hypothecium, parathecium is another UV+ red substance; I: section of an apothecia (*continues on the next page*)

Large droplets of one or several substances that fluoresce red when a UV (330-380 nm) filter is applied were observed in the hypothecium, subhymenium and parathecium (Fig. 2.1.2H). These were larger than the oil droplets present in the hymenium and epihymenium. The presence of one or more additional substances that fluoresce yellow under the same filter was also detected in the epihymenium and amphithecium, as well as in the consoredia beneath the apothecium (Fig. 2.1.2I). These UV+ substances do not entirely coincide with the POL+ crystals, at least not in the epihymenium.

Chemistry: Soralia P–, K– or +yellow, KC+red, C+red. Contains: 3,5-dichloro-2'-O-methylnorstenosporic acid [major], 3,5-dichloro-2'-O-methylanziaic acid [minor], 3,5-dichloro-2'-O-methylnordivaricatic acid [minor], 5-chloro-2'-O-methylanziaic acid [trace], atranorin [minor], chloroatranorin [minor], and usnic acid [trace].

Etymology: The specific epithet "sorediomarginata" refers to the nature of the margin of the apothecia of *L. sorediomarginata*, which is completely sorediate at maturity.

Substratum: Bark of trunks and branches of *Pinus pinaster* and *P. pinea.*

Distribution: Occurs in coastal pine forests, as well as in nearby mountains, along the west coast of Portugal south of Esposende (Fig. 2.1.1: 1), as well as in the south-eastern coast. It was found at varying distances from the sea, from approximately 260 m at Praia de Vale dos Homens (Rogil, Parque Natural do Sudoeste Alentejano e Costa Vicentina) (Fig. 2.1.1: 25) to approximately 22 km at Mata de Valverde (Alcácer do Sal) (Fig. 2.1.1: 21). It was found at approximately 34 m from the Sado Estuary (Fig. 2.1.1: 19). Although surveys were conducted north of Esposende at Praia da Amorosa (Viana do Castelo) and at Mata do Camarido (Caminha), *L. sorediomarginata* could not be found at these localities. Despite that, its presence in these more northern areas cannot be ruled out. At present it is known only from Portugal.

(*Figure 2.2, continued*) seen under UV filter, where the UV+yellow fluorescence is visible in the epihymenium and the consoredia both in the amphithecium and under the apothecium. Scales: A and B = 0.2 mm, C = 10 μm, D = 0.5 mm, F and G = 5 μm, H and I = 100 μm.

Selected specimens examined: **Portugal:** *Minho*: Esposende: Parque Natural do Litoral Norte, Dunas de Ofir/Fão, Fão, epiphytic on *Pinus pinaster* in the border of a pine forest on sand dunes near a road, 2 m alt., MGRS: 29TNF1894, 15/05/2009, *S. A. Rodrigues* (AVE-L 266, LEB-Lichenes 7826). *Douro Litoral*: Matosinhos, Angeiras, Parque de Campismo de Angeiras, MGRS: 29TNF2368, 23 m alt., epiphytic on *P. pinaster* in a small pine stand area used for camping, 15/05/2009, *S. A. Rodrigues* (AVE-L 268). *Beira Litoral*: Ovar, Dunas de Ovar, Cortegaça, MGRS: 29TNF2932, 8 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 25/06/2009, *S. A. Rodrigues* (AVE-L 291, LEB 7827). Aveiro, Reserva Natural das Dunas de S. Jacinto: Dunas de S. Jacinto, S. Jacinto, MGRS: 29TNF2203, 8 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 23/04/2009, *S. A. Rodrigues* (AVE-L 295, LEB-Lichenes 7828). Ílhavo, Dunas da Gafanha, Gafanha do Carmo, MGRS: 29TNE2394, 13 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 26/03/2009, *S. A. Rodrigues* (AVE-L 235, LEB-Lichenes 7829). Vagos, Dunas de Vagos, Gafanha do Areão, MGRS: 29TNE2185, 21 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 26/03/2009, *S. A. Rodrigues* (AVE-L 298, LEB-Lichenes 7830). Mira, Dunas de Mira, Barra de Mira, MGRS: 29TNE1981, 17 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 26/03/2009, *S. A. Rodrigues* (AVE-L 301, LEB-Lichenes 7831). Figueira da Foz, Dunas de Quiaios, Quiaios, MGRS: 29TNE1654, 49 m alt., epiphytic on *P. pinaster*, 05/01/2007, *S. A. Rodrigues* (AVE-L 218), MGRS: 29TNE1554, *id.* (BG-L 88210) MGRS: 29TNE1655, 49 m alt., epiphytic on *P. pinea*, 26/06/2009, *S. A. Rodrigues* (AVE-L 307), MGRS: 29TNE1758, 49 m alt., epiphytic on a branch of *P. pinea* in a pine forest on sand dunes, 26/06/2009, *S. A Rodrigues* (LEB-Lichenes 7832). Figueira da Foz, Mata do Prazo de Santa Marinha/Serra da Boa Viagem, Serra da Boa Viagem, MGRS: 29TNE1149, 205 m alt., epiphytic on *P. pinaster*, 26/06/2009, *S. A. Rodrigues* (AVE-L 306), MGRS: 29TNE1149, 200 m alt., epiphytic on *P. pinea* in a pine forest in a mountainous area, 26/06/2009, *S. A. Rodrigues* (AVE-L 287, LEB-Lichenes 7833). Figueira da Foz, Dunas da Leirosa, Costa de Lavos, MGRS: 29TNE1137, 21 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 25/04/2009, *S. A. Rodrigues* (AVE-L 264, LEB-Lichenes 7834). Figueira da Foz, Mata do Urso, Leirosa, MGRS: 29TNE1032, 28 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 25/04/2009, *S. A. Rodrigues* (AVE-L 262, LEB-Lichenes 7835). Leiria, Mata do Pedrógão, Pedrógão, MGRS: 29S0416, 24 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 25/04/2009, *S. A. Rodrigues* (AVE-L 303, LEB-Lichenes 7836). *Estremadura*: Marinha Grande, Pinhal de Leiria, S. Pedro de Muel, MGRS: 29SNE0004, 49 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 25/04/2009, *S. A. Rodrigues* (AVE-L 256, LEB-Lichenes 7837). Nazaré, Mata do Valado, Valado dos Frades, MGRS: 29SMD9882, 27 m alt., epiphytic on *P. pinaster* in the border of a pine forest on sand dunes, 27/04/2009, *S. A. Rodrigues* (AVE-L 246). Torres Vedras, Casal do Seixo, Praia do Seixo, MGRS: 29SMD6834, 46 m alt., epiphytic on *P. pinaster* in a small pine stand on sand dunes used for recreation, 27/04/2009, *S. A. Rodrigues* (AVE-L 251, LEB-Lichenes 7838). Sintra, Parque Natural de Sintra-Cascais: Colares, Praia das Maçãs, MGRS: 29SMC5996, 25 m alt., epiphytic on *P. pinaster* in a pine stand area on sand dunes heavily used for habitation, 27/04/2009, *S. A. Rodrigues* (AVE-L 239, LEB-Lichenes 7839). Sintra, Parque Natural de Sintra-Cascais, Serra de Sintra, Ulgueira, MGRS: 29SMC5992, 265 m alt., epiphytic on *P. pinaster* in a pine forest in a mountainous area, 27/04/2009, *S. A. Rodrigues* (AVE-L 292). Sesimbra, Parque Natural da Arrábida: Aldeia do Meco, Praia do Moinho de Baixo, MGRS: 29SMC8459, 24 m alt., epiphytic on *P. pinaster* in a

small pine stand on sand dunes, 01/06/2009, *S. A. Rodrigues* (AVE-L 277). *Baixo Alentejo*: Alcácer do Sal, Reserva Natural do Estuário do Sado: Comporta, Praia da Comporta, MGRS: 29SNC1748, 24 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 01/06/2009, *S. A. Rodrigues* (AVE-L 269). Alcácer do Sal, Reserva Natural do Estuário do Sado: Comporta, Murta, MGRS: 29SNC2651, 19 m alt., epiphytic on *P. pinea* in a pine forest on sand dunes, 01/06/2009, *S. A. Rodrigues* (AVE-L 270). Alcácer do Sal, Mata de Valverde, Albergaria, MGRS: 29SNC4140, 79 m alt., epiphytic on *P. pinea* in a pine forest on sand dunes, 31/03/2009, *S. A. Rodrigues AVE-L 234*. Santiago do Cacém, Área Florestal de Sines, Relvas Verdes, MGRS: 29SNC2106, 69 m alt., epiphytic on *P. pinaster* in a pine forest on sand dunes, 03/04/2009, *S. A. Rodrigues* (AVE-L 297). Odemira, Parque Natural do Sudoeste Alentejano e Costa Vicentina: Vila Nova de Milfontes, Praia do Malhão, MGRS: 29SNB1880, 67 m alt., epiphytic on *P. pinaster* in a pine stand on sand dunes near the beach, 30/05/2009, *S. A. Rodrigues Rodrigues* (AVE-L 200, LEB-Lichenes 7840). Odemira, Parque Natural do Sudoeste Alentejano e Costa Vicentina: Brejão, Praia do Carvalhal, MGRS: 29SNB1849, 30 m alt., epiphytic on *P. pinaster* in a small pine stand on sand dunes near the beach, *S. A. Rodrigues* 30/05/2009 (AVE-L 274, LEB-Lichenes 7841). *Algarve*: Aljezur, Parque Natural do Sudoeste Alentejano e Costa Vicentina: Rogil, Praia de Vale dos Homens, MGRS: 29SNB1537, 30 m alt., epiphytic on *P. pinaster* in a pine stand on sand dunes near the beach, 30/05/2009, *S. A. Rodrigues* (AVE-L 308). Vila do Bispo, Parque Natural do Sudoeste Alentejano e Costa Vicentina: Sagres, Pinhal de Vale Santo, MGRS: 29SNB0300, 74 m alt., epiphytic on a branch at breast height of *P. pinaster*, 31/05/2009, *S. A. Rodrigues* (AVE-L 296), epiphytic on *P. pinaster* in a pine forest on sand dunes, 31/05/2009, *S. A. Rodrigues* (LEB-Lichenes 7842). Vila Real de Santo António, Dunas de Vila Real de Santo António, Monte Gordo, Praia do Cabeço, MGRS: 29SPB3515, 7 m alt., epiphytic on *P. pinaster* in the border of a pine forest on sand dunes, 13/06/2009, *S. A. Rodrigues* (AVE-L 284. LEB-Lichenes 7843).

Phylogenetic analysis

The new sequences, two of *L. sorediomarginata* and one of *L. lividocinerea*, aligned with sequences acquired from the GenBank, resulted in a matrix of 455 unambiguously aligned characters after Gblocks analysis. The likelihood parameters of the Bayesian analysis are available from authors upon request. The majority-rule consensus tree based on 20000 trees from the B/MCMC sample is shown in Figure 2.1.3.

In the 50% majority-rule consensus tree, the three specimens of *L. lividocinerea* and *L. sorediomarginata* form a strongly supported clade (PP=1). The two specimens of *L. sorediomarginata* differed in one substitution and one indel of 3 nucleotides in their ITS sequences. On the other hand, the ITS sequence of *L. lividocinerea* diverged in 58 positions compared to *L. sorediomarginata*. The phylogenetic position of the group formed by these two taxa is still unclear. Although the group was sister to the '*subfusca'* group in

the tree obtained from the Bayesian analysis, this relationship was not supported statistically. Likewise, the relationships among the previous groups recognized in the literature (e.g. Arup & Grube 1998, Blaha & Grube 2007, Pérez-Ortega et al. 2010) are not well supported in our analysis, probably due to the low number of molecular characters used in the analysis.

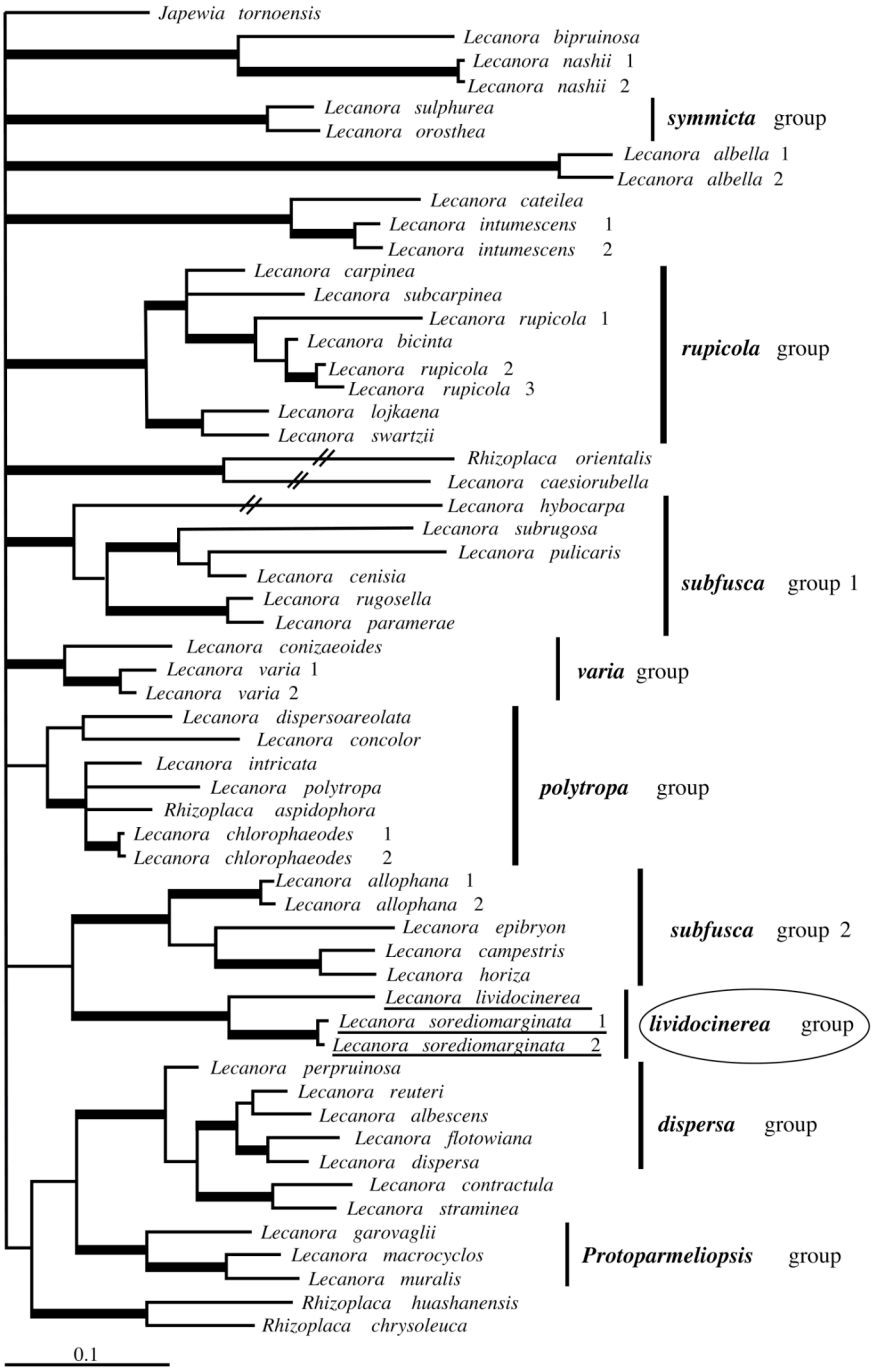


Figure 2.1.3: Phylogenetic tree based on ITS sequences. Bold branches mean posterior probabilities ≥ 0.95.

Discussion

The position of *Lecanora sorediomarginata* within the groups of *Lecanora* so far defined is not clear. In our phylogenetic analysis *L. sorediomarginata* turned out to be more closely related to *L. lividocinerea* than to other members of the '*subfusca*' group, which seems to indicate the importance of chemistry when defining natural groups within the genus. Unfortunately, fresh material of *L. sulphurella*, which is chemically similar to *L. sorediomarginata*, was not available at the time of this study. Further research is necessary to determine whether these taxa actually belong to a "new group" within *Lecanora*. The absence of an amphithecial cortex and medulla at maturity and the absence of usnic acid or atranorin as major compounds make it difficult to place this species in any particular group based on morphological and chemical characters. Some specimens had minute apothecia with entire margins in the beginning of their development. The margin was found to be composed of entangled hyphae with algal cells, without a clearly defined cortex.

The nature of the amphithecium in *L. sorediomarginata*, which is completely sorediate from early juvenile stages, is not unique within *Lecanora*. In this regard it is similar to other sorediate species known to have sorediate apothecial margins, but which have an entire margin in the beginning of the apothecial development. This includes *L. barkmaniana* Aptroot & van Herk, *L. conizaeoides* Nyl. ex Crombie, *L. epanora* (Ach.) Ach., *L. expallens* Ach., *L. farinaria* Borrer in Hook. *L. impudens* Degel., *L. subaurea* Zahlbr. and *L. umbrosa* Degel. (Aptroot & van Herk 1999, Brodo et al. 1994, Edwards et al. 2009, Ryan et al. 2004, Tønsberg 1992).

L. sorediomarginata is a very uniform species in terms of morphology. The thallus always has a granular-sorediate appearance, with warts visible at the margin of the thallus. The older, completely sorediate parts of the thallus may appear as a continuous, cracked granular surface. This may cause it to be confused with *Ochrolechia microstictoides* Räsänen, which also grows in some of the above localities, but the latter has an obvious episubstratal margin with a mean thickness of 54.5 µm (n= 7) in which the algae are uniformly distributed. *L. sorediomarginata*, on the other hand, has an endosubstratal to very thin episubstratal margin, where warts arise and lead to soralia and the algae are not uniformly distributed but only present in the warts, and not in between. Futhermore, the

chemistry of *O. microstictoides* is quite distinct from that of *L. sorediomarginata*, with variolaric acid with satellite and lichesterinic acids present in the thallus (Tønsberg 1992).

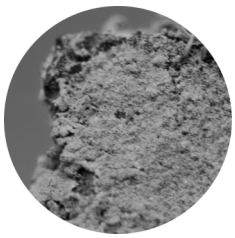
Lecanora sorediomarginata may also be confused with *Ochrolechia arborea* (Kreyer) Almb., a species characterized by a whitish thallus, which is continuous or warted in the periphery, the continuous margin having a mean thickness of 57.0 µm (n=11) and warts with a mean thickness of 79.0 µm (n=18). The soralia are rounded and usually delimited in specimens growing on branches of *Pinus*, but may be confluent in specimens on the trunk of the same phorophyte. Chemically *O. arborea* it contains gyrophoric acid, lecanoric acid (trace) and lichexanthone (Tønsberg 1992), as well as orsellinic acid (Boqueras et al. 1999).

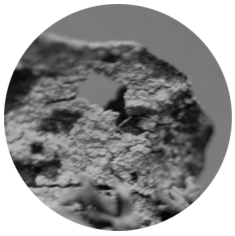
Only two other *Lecanora* species were found at the type locality and are also likely to occur at other areas surveyed: *L. expallens* and *L. strobilina* (Sprengel) Kieffer. *L. expallens* usually has a thin, indeterminate, green or pale yellow, sorediate thallus, with predominantly confluent soralia. The apothecia grow to 1 mm in diam. and the disc varies in colour from dull yellow to dark red, with the margin soon becoming sorediate or excluded. It can be readily distinguished from *L. sorediomarginata* by the colour of the thallus and its secondary metabolites [thiophanic and usnic acids and zeorin as major substances (Tønsberg 1992)]. *L. strobilina* has a granular-warted, greenish to yellowish grey, esorediate thallus. The apothecia have an average diam. of 0.35 mm; and the yellowish-ochre to orange-brown, flat to convex discs have an entire to crenulate margin, which may be persistent or become excluded. This species contains usnic acid, decarboxysquamatic acid and ±zeorin (Printzen 2001).

No additional sorediate, epiphytic species of *Lecanora* are known so far from the type locality. Several other European species have an areolate thallus, with initially discrete soralia that later become contiguous, but in none of these exhibit a C+red thalline reaction. A number of these species including *L. allophana* (Ach.) Nyl. f. *sorediata* (Schaerer) Vainio, *L. barkmaniana*, *L. impudens* and *L. norvegica* Tønsberg contain atranorin or chloroatranorin as a major substance, in addition to other substances (Aptroot & van Herk 1999 and Tønsberg 1992). *Lecanora compallens* van Herk & Aptroot and *L. flavoleprosa* Tønsberg, are also similar in morphology, but contain usnic acid as a major metabolite in addition to other substances (Tønsberg 1992, van Herk & Aptroot 1999). *Lecanora sorediomarginata* only contains atranorin and chloroatranorin in minor amounts

and traces of usnic acid and does not contain any other substances in common with these species. *L. conizaeoides* could also be considered a similar species, not only because it has an aerolate thallus with soralia that become confluent, but also due the ±sorediate thalline exciple (Tønsberg 1992). Nevertheless, the presence of fumarprotocetraric acid as major compound clearly distinguishes this species chemically. *L. variolascens* has a rimose-aerolate thallus, but the soralia are usually well delimited and rarely become confluent; it contains atranorin and psoromic acid as major substances (Lumbsch et al. 1997).

Chemically, *L. sorediomarginata* is similar to the epiphytic *L. lividocinerea* Bagl. and to the saxicolous *L. sulphurella* Hepp. *L. lividocinerea* is characterized by a yellowish-white to whitish-grey thallus, which is esorediate, thin to thick, and with dispersed verrucae or verruculae. The apothecia are sessile, with pale yellow to pale red-brown or grey-brown discs, which may be epruinose or slightly whitish grey-pruinose. The thalline exciple is concolorous with the thallus thin, entire, and ±verrucose to verruculose (Lumbsch & Elix 2004). It contains atranorin [major], 3,5-dichloro-2'-*O*-methylanziaic acid [major], chloroatranorin [minor], 5'-chloro-2'-*O*-methylanziaic acid [minor], 3,5-dichloro-2'-*O*-methylnorhyperlatolic acid [minor] and 3,5-dichloro-2'-*O*-methylnorstenosporic acid [minor] (Elix et al. 1997, Lumbsch & Elix 2004). It is known from coastal localities in Portugal (Carvalho et al. 2002, Paz-Bermúdez & López de Silanes 2007, van den Boom & Giralt 1996) and it occurs in other coastal localities in the Mediterranean (Paz-Bermúdez & López de Silanes 2007) as well as in Australia (Lumbsch & Elix 2004). *L. sulphurella* has a grey to bright yellow, rimose-aerolate thallus with sessile apothecia, which have a slightly pruinose black disc and a persistent margin of the same colour as the thallus (Follmann 1976). It contains atranorin [major], chloroatranorin [major], 3,5-dichloro-2'-*O*-methylanziaic acid [major] and calycin [minor] (Lumbsch & Feige 1992). It is known in the Macaronesian area and from the Iberian Peninsula (Follmann 1976, Llimona & Werner 1975, Lumbsch & Feige 1992).





Chapter 2.2

Chrysothrix flavovirens, Lepraria elobata and *Ochrolechia arborea* new to Portugal

Figures on the previous page: from top to bottom, *Chrysothrix flavovirens* Tønsberg, *Lepraria elobata* Tønsberg, and *Ochrolechia arborea* (Kreyer) Almb. The images of *L. elobata* and *O. arborea* were kindly provided by Prof. Tor Tønsberg.

Chrysothrix flavovirens, Lepraria elobata and *Ochrolechia arborea* new to Portugal

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Abstract

Chrysothrix flavovirens, *Lepraria elobata*, and *Ochrolechia arborea* are reported as new to Portugal, based on surveys carried out in pine forests along the Portuguese coast. Data on the distribution, secondary products and ecology of the species are presented.

Keywords: lichens, epiphytic, sand dunes

Introduction

The epiphytic lichen flora of pine forests in coastal areas in Portugal is poorly studied and is in need of a thorough survey. Pine forests are a common biotope along the Portuguese coast and many of them are classified as Natural Parks or Nature Reserves (Fig. 2.2.1A&B: 3, 8, 18–19, 21–22, 24), while others are national forests or forest perimeters that are owned and/or partially managed by the Portuguese State (Fig. 2.2.1A&B: 1, 7, 9–18, 20, 24). Moreover, many of them are currently classified as Natura 2000 sites (ICN 2006) by the European Commission (Fig. 2.2.1A&B: 1–3, 8, 10, 12, 18–19, 21–22, 24). The only known inventory of epiphytic lichen species in pine forests on sand dunes was generated by Catarino et al. (1985) in the framework of biomonitoring studies.

In the course of our investigations on the epiphytic lichens of these areas — particularly the well-preserved Dunas de Quiaios (Figueira da Foz) (Fig. 2.2.1A&B: 12) — new epiphytic lichen records were reported for the Iberian Peninsula (Rodrigues et al. 2007) and a new *Lecanora* species has been published (Rodrigues et al. 2011a). These discoveries point to the importance of coastal pine forests as habitats for lichens, which is an extra incentive to ensure their future preservation. At Dunas de Quiaios, the tree vegetation, rich in epiphytic lichens, is a planted *Pinus pinaster* forest mixed with *P. pinea*, *Acacia longifolia*, *A. melanoxylon*, and *Eucalyptus globulus*. *Arbutus unedo* and *Myrica faya* are also appropriate supports for lichens (Almeida 1997, Danielsen 2008, Rodrigues et al. 2007, 2011a).

The aim of this work is to report three crustose and sorediate epiphytic lichens growing on *P. pinaster* and *P. pinea*, new to Portugal: *Chrysothrix flavovirens, Lepraria elobata and Ochrolechia arborea.* Data on their distribution along the Portuguese coast, secondary products, and ecology are also provided.

Materials and methods

Unless otherwise stated, all specimens were collected by the first author (SAR) on *P. pinaster* trunks in pine forests on sand dunes along the Portuguese coast (Fig. 2.2.1A&B, see also *Specimens Examined*). Samples were deposited in AVE, BG, and LEB

herbaria. Distribution maps are based on UTM grid projection and coordinates of the locations are given in MGRS. Maps [A] and [B] represent the distribution along the Portuguese coast of *C. flavovirens*, and *O. arborea* respectively. The location where *L. elobata* was found [12] appears on both maps. Visited locations (indicated by numbers on each map) were geo-referenced using GoogleEarth and maps were plotted using ArcGis version 9.2.

Samples were analysed morphologically and chemically, with standard identification methods for lichenized fungi (Nash et al. 2002, Smith et al. 2009) and compared to authentic material deposited in BG herbarium. Morphology of the thallus was examined with a Leica MS5 stereomicroscope and anatomical observations of hand cut sections of apothecia were performed under a Leitz HM-LUX 3 microscope. For that, sections were mounted in distilled water and K/I and the latter was used for measuring anatomical characters. Chemical analyses followed standardized TLC methods (White & James 1985; Orange et al. 2001). Data regarding companion species were retrieved at Dunas de Quiaios (Fig. 2.2.1A&B: 12) during a small biomonitoring campaign, using the method proposed by Asta et al. (2002), in which 9 sampling units with 10 or 12 trees each were studied. Only *C. flavovirens* was detected using this method, and therefore it is the only species for which such data are provided. Only species present in each 10 × 10 cm quadrate where *C. flavovirens* was detected were considered as companion species.

The species

Chrysothrix flavovirens *Tønsberg*

This common species is characterized by a vivid yellowish-green and leprose thallus, which is neither stratified nor lobed. The soralia are discrete to contiguous, forming an areolate or, more often, a thin to thick, continuous thallus. In our material, diffractaic and rhizocarpic acids and ± unidentified substances were detected. Fertile material was found at Mata do Camarido (Caminha) (Fig. 2.2.1A: 1). Fertile specimens had scattered and sessile apothecia, which were 0.3–0.6 mm in diam. (n = 30), yellowish brown in colour, mostly rounded, convex and immarginate, but sometimes surrounded by soredia as also reported by Laundon (1981). Sections of the apothecia reacted K+ violet in

the centre and intensified yellow at the border of the section. The epihymenium, hymenium, and hypothecium were constituted by anastomosing paraphysis, and reacted K/I+ blue. The epihymenium was 2.5–12.5 µm (n = 15) high, the hymenium 38–83.5 µm (n = 8) and the hypothecium 148.5–265 (n = 8). Asci were clavate, sometimes slightly curved, 23–40.5 × 8.5–14 µm (n = 14). Spores were narrowly ellipsoid, some slightly curved, mostly 3-septate, 9.5–12.5 × 2–4 µm (n = 21), although some were found 2-septate and 1-septate, but of smaller sizes. Detailed descriptions can be found in Laundon (1981), Smith et al. (2009), and Tønsberg (1992).

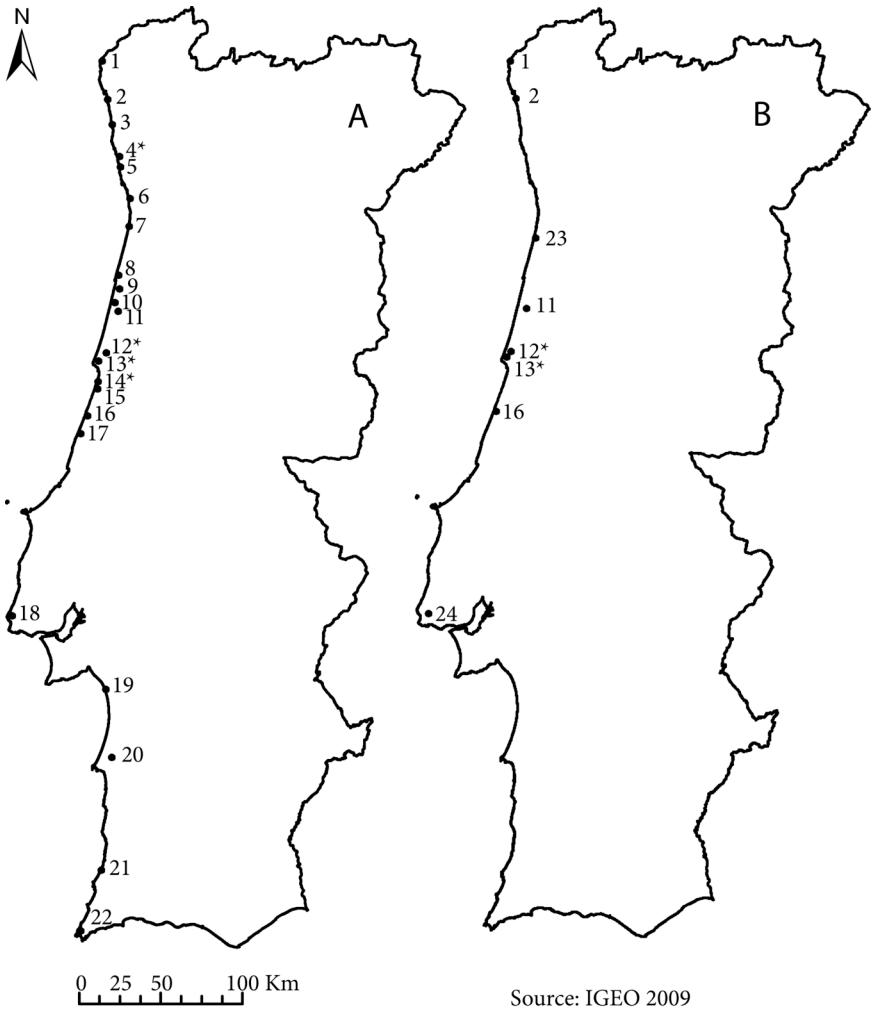

Figure 2.2.1: Distribution of *Chrysothrix flavovirens* [A] and *Ochrolechia arborea* [B] along the Portuguese coast; *Lepraria elobata* [12] appears in both [A] and [B]. Dot numbers correspond to the localities shown in square brackets within the *Specimens examined* sections. Asterisks flag locations where the species were found on both *Pinus pinaster* and *P. pinea*.

Habitat and distribution

Chrysothrix flavovirens may form large patches extending over a large part of the pine trunk or appear as small patches associated with other species. At Dunas de Quiaios (Fig. 2.2.1A: 12) it was found together mostly with *C. candelaris* (L.) J.R. Laundon, *Hypogymnia physodes* (L.) Nyl. and *Pyrrhospora quernea* (Dicks.) Körb., but also with *Trentepohlia* sp., *Usnea rubicunda* Stirt. and *U. subscabrosa* Nyl. ex Motika, and less frequently with *Flavoparmelia caperata* (L.) Hale, *Lecanora strobilina* (Spreng.) Kieff. and *Parmotrema reticulatum* (Taylor) M. Choisy.

This species was frequently found in well-preserved to open coastal pine forests on sand dunes and also in localities with scanty pines, growing on *P. pinaster* and in some sites also on *P. pinea* (Fig. 2.2.1A, see also *Specimens examined*). It was also collected in mountains near the coast at Serra da Boa Viagem (Figueira da Foz) (Fig. 2.2.1A: 13) and Serra de Sintra (Sintra) (Fig. 2.2.1A: 18). Elevations observed ranged from about 2 m at Dunas de Ofir/Fão (Esposende) (Fig. 2.2.1A: 3) to about 260 m at Serra de Sintra. Although *C. flavovirens* is widespread throughout the Atlantic and Mediterranean biogeographic regions of Portugal (ICN 2006), it was not found in surveys at a coastal pine forest in south-eastern Portugal, Dunas de Vila Real de Santo António (Vila Real de Santo António).

This species, which is frequent and of which reports are increasing, is regarded as a preferentially coastal species of acidic bark (Laundon 1981 [as sorediate thalli of *C. chrysophthalma* (P. James) P. James & J.R. Laundon], Kowalewska & Jando 2004, Smith et al. 2009, Tønsberg 1992 [as sorediate thalli of *C. chrysophthalma*]). *Chrysothrix flavovirens* is widely distributed in Europe (Aptroot et al. 2003; Kowalewska & Jando 2004; Laundon 1981; Llimona & Hadlun 2001; Nimis & Martellos 2008; Smith et al. 2009; Søchting & Alstrup 2008; Sparrius et al. 2002; Suija et al. 2009; Tønsberg 1992, 1994) and is also known from North America (Richardson et al. 2009) and Asia (Kowalewska & Jando 2004). A very similar species recently described from eastern North America, *C. chamaecyparicola* Lendemer, is similar to *C. flavovirens*, except that it lacks diffractaic acid (Lendemer & Elix 2010).

Specimens examined (square bracketed numbers refer to localities indicated in Fig. 2.2.1A): **Portugal. Minho:** [1] **Caminha.** Moledo, Mata Nacional do Camarido, 29TNG1133, 14 m, 22.3.2009, AVE-L 337, LEB-Lichenes 7810. [2] **Viana do Castelo.** Chafé, Praia da Amorosa, 29TNG1510, 17 m, 5.9.2009, AVE-L 380, LEB-Lichenes 7811. [3] **Esposende.** Fão, Dunas de Ofir/Fão (Parque Natural do Litoral Norte), 29TNF1894, 2 m, 15.5.2009, AVE-L 358. **Douro Litoral:** [4] **Vila do Conde.** Mindelo, Reserva Ornitológica do Mindelo, 29TNF2275, 24 m, 25.6.2009, AVE-L 376, LEB-Lichenes 7812; *id.*, on trunk of *Pinus pinea* AVE-L 364. [5] **Matosinhos.** Angeiras, Parque de Campismo de Angeiras — in a small pine stand on sand dunes used for camping, 29TNF2368, 23 m, 15.5.2009, AVE-L 366. [6] **Vila Nova de Gaia.** Valadares — in an area with scanty pines, 29TNF2949, 24 m, 15.5.2009, AVE-L 359. **Beira Litoral:** [7] **Ovar.** Cortegaça, Dunas de Ovar, 29TNF2932, 8 m, 25.6.2009, AVE-L 375, LEB-Lichenes 7813. [8] **Aveiro.** S. Jacinto, Dunas de S. Jacinto (Reserva Natural das Dunas de S. Jacinto), 29TNF2302, 2 m, 23.4.2009, AVE-L 349, LEB-Lichenes 7814. [9] **Ílhavo.** Gafanha do Carmo, Dunas da Gafanha, 29TNE2394, 13 m, 9.9.2009, AVE-L 377, LEB-Lichenes 7815. [10] Vagos. Gafanha do Areão, Dunas de Vagos, 29TNE2185, 21 m, 23.3.2009, AVE-L 340. [11] **Mira.** Seixo, Dunas de Mira, 29TNE2280, 24 m, 26.3.2009, AVE-L 341, LEB-Lichenes 7816. **Figueira da Foz.** [12] Quiaios, Dunas de Quiaios, 29TNE1654, 49 m, 4.9.2007, AVE-L 383; *id.*, 29TNE1554, 49 m, 11.1.2007, BG-L 88214, LEB-Lichenes 7817; *id.*, epiphytic on trunk of *P. pinea*, 29TNE1353, 45 m, 26.6.2009, AVE-L 373. [13] Serra da Boa Viagem, Mata do Prazo de Santa Marinha/Serra da Boa Viagem — in a pine forest in a mountainous area, 29TNE1149, 205 m, 26.6.2009, AVE-L 371; *id.*, epiphytic on trunk of *P. pinea*, 29TNE1149, 200 m, 26.6.2009, AVE-L 372. [14] Costa de Lavos, Dunas da Leirosa, 29TNE1137, 21 m, 25.4.2009, AVE-L 356, LEB-Lichenes 7818; *id.*, epiphytic on trunk of *P. pinea*, 29TNE1037, 16 m, 25.4.2009, AVE-L 354, LEB-Lichenes 7819. [15] Leirosa, Mata do Urso, 29TNE1132, 28 m, 25.4.2009, AVE-L 352, LEB-Lichenes 7820. [16] Leiria. Pedrógão, Mata do Pedrógão, 29SNE0416, 24 m, 25.4.2009, AVE-L 344, LEB-Lichenes 7821. **Estremadura:** [17] **Marinha Grande.** S. Pedro de Muel, Mata de Leiria, 29SNE0004, 49 m, 25.4.2009, AVE-L 351. [18] **Sintra.** Serra de Sintra, Ulgueira, Perímetro Florestal da Serra de Sintra (Parque Natural de Sintra-Cascais) — in a pine forest in a mountainous area 29SMC5993, 260 m, 27.4.2009, AVE-L 347. **Baixo Alentejo:** [19] **Alcácer do Sal.** Comporta, Praia da Comporta, 29SNC1748, 24 m, 1.6.2009, AVE-L 367. [20] **Santiago do Cacém.** Relvas Verdes, Área Florestal de Sines, 29SNC2106, 75 m, 3.4.2009, AVE-L 336. **Algarve:** [21] **Aljezur.** Rogil, Praia de Vale dos Homens (Parque Natural do Sudoeste Alentejano e Costa Vicentina), 29SNB1537, 49 m, 30.5.2009, AVE-L 370. [22] **Vila do Bispo.** Sagres, Pinhal de Vale Santo (Parque Natural do Sudoeste Alentejano e Costa Viventina) — epiphytic on branch of *P. pinaster*, 29SNB0300, 74 m, 31.5.2009 AVE-L 369.

Remarks

Recently separated as the sorediate counterpart of *C. chrysophthalma* (Smith et al. 2009, Tønsberg 1994), *C. flavovirens* may be confused with *C. candelaris*, which also occurs on pine trees in the study areas, but developing a more yellowish thallus with

calycin (Elix & Kantvilas 2007, Laundon 1981). The chemical constitution of *C. flavovirens* (Elix & Tønsberg 2004, Laundon 1981, Tønsberg 1992) is variable, as Elix & Kantvilas (2007) refer to the presence of barbatic and conrhizocarpic acids, as well as epanorin, and the absence of atranorin in samples from the United Kingdom and Sweden.

Lepraria elobata *Tønsberg*

This species develops a diffuse, leprose, predominantly non-lobed and non-stratified, bluish-grey thallus, without medulla and with profuse more or less continuous fine soredia (Saag et al. 2009, Tønsberg 1992). Our sterile material of *L. elobata* contained atranorin, zeorin and stictic acid with satellites. Detailed descriptions are provided by Saag et al. (2009), Smith et al. (2009) and Tønsberg (1992).

Habitat and distribution

Only one specimen of *L. elobata* was found epiphytic on *P. pinaster* bark at Dunas de Quiaios (Fig. 2.2.1A&B: 12), at an altitude of about 49 m, where it appears to be uncommon.

This species occurs preferentiality on bark but is also known to grow on soil and siliceous rocks in shady and wet locations (Kukwa 2006). Regarding bark preferences, *L. elobata* seems to prefer deciduous to conifer trees (Saag 2007) and should be expected to occur in other habitats in Portugal.

Lepraria elobata is known from several European countries (Bayerová & Kukwa 2004, Boom et al. 1996, Coppins 2002, Czyżewska & Kukwa 2005, Diederich et al. 2009, Ekman & Tønsberg 2002, Feuerer 2012, Lukošienė & Naujalis 2009, Nimis & Martellos 2008, Osyczka & Stolarczyk 2005, Paz-Bermúdez et al. 2000, Prügger 2000, Tønsberg 1992) and North America (Saag et al. 2009, Tønsberg 2004).

Specimen examined: **Portugal. Beira Litoral:** [Fig. 2.2.1A&B: 12] **Figueira da Foz.** Quiaios, Dunas de Quiaios, 29TNE1554, 49 m, 11.1.2007, AVE-L 390, BG-L 88184.

Remarks

Lepraria nylanderiana Kümmerl. & Leuckert is the most frequent *Lepraria* species at Dunas de Quiaios and is thought to be widespread in pine forests on sand dunes in the central west coast of Portugal. Both *L. elobata* and *L. nylanderiana* have a leprose, bluish-grey thallus, but *L. nylanderiana* has a delimited margin where minute lobes may appear in well-developed specimens. Also, *L. nylanderiana* usually has a whitish medulla, medium to coarse soredia, and thamnolic and roccellic acids and atranorin (Smith et al. 2009).

Lepraria elobata is morphologically similar to *L. caesiella* R.C. Harris and *L. incana* (L.) Ach., which are differentiated chemically by the content in atranorin and zeorin, and divaricatic acid and zeorin, respectively (see Saag et al. 2009). Neither species has been found so far epiphytic on pine at Dunas de Quiaios.

Ochrolechia arborea *(Kreyer) Almb.*

This species is characterized by a whitish grey, sorediate and thin thallus, which may be tuberculate in the centre. The soralia are mostly discrete and may be delimited and orbicular or more or less diffuse, rarely being confluent, but not covering the whole thallus (Boqueras et al. 1999, Tønsberg 1992). Our material was sterile and contained gyrophoric and lecanoric acids, lichexanthone (UV+ orange), and ± atranorin (Tønsberg 1992).

Habitat and distribution

Ochrolechia arborea was found in localities along the western coast from Minho to Estremadura provinces (Fig. 2.2.1B, see also *Specimens examined*) in Portugal's Atlantic and Mediterranean regions (ICN 2006). It was also found in mountainous locations at Serra da Boa Viagem (Figueira da Foz) (Fig. 2.2.1B: 13) and Serra de Sintra (Cascais) (Fig. 2.2.1B: 24), having been recorded at altitudes up to 205 m at Serra da Boa Viagem. Most specimens were found on *P. pinaster*, but some were also found on *P. pinea*.

Contrary to *C. flavovirens*, *O. arborea* is not restricted to maritime habitats and has been reported from inland countries such as Switzerland (Diederich & Sheidegger 1996) and Mongolia (Hauck & Javkhlan 2006). It is expected to occur in inland Portugal on a

wide variety of phorophytes as reported in the literature (Boqueras et al. 1999, Christensen & Svane 2007, Kukwa 2009, Tønsberg 1992). The species has been noted as typical of shady habitats or soft barks (Johansson et al. 2007).

Ochrolechia arborea is currently known from several European countries (Andrianova et al. 2006, Boqueras et al. 1999, Brodo 1991, Christensen & Svane 2007, Coppins 2002, Diederich et al. 2009, Dietrich & Scheidegger 1996, Farkas et al. 2009, Feuerer 2012, Jabłońska & Kukwa 2007, Kukwa 2009, Liška et al. 2008, Nimis & Martellos 2008, Prügger 2000, Roux et al. 2008, Søchting & Alstrup 2008, Tibell 1992, Tønsberg 1992). It is also known from North and South America (Brodo 1991) and Asia (Hauck & Javkhlan 2006, John & Nimis 1998, Koneva 2007).

Specimens examined (square bracketed numbers refer to localities indicated in Fig. 2.2.1B): **Portugal. Minho:** [1] **Caminha.** Moledo, Mata Nacional do Camarido, 29TNG1133, 15 m, 17.5.2009, AVE-L 320, LEB-Lichenes 7822. [2] **Viana do Castelo.** Chafé, Praia da Amorosa, 29TNG1510, 20 m, 17.5.2009 AVE-L 323, LEB-Lichenes 7823. **Beira Litoral:** [23] **Ovar.** Furadouro, 29TNF2823, 9 m, 23.3.2009 AVE-L 310. [11] **Mira.** Seixo, Dunas de Mira, 29TNE2280, 24 m, 26.3.2009, LEB-Lichenes 7824; *id*., on a branch of *Pinus pinaster*, 29TNE2280, 24 m, 26.3.2009, AVE-L 312. **Figueira da Foz.** [12] Quiaios, Dunas de Quiaios, on a branch of *P. pinaster*, 29TNE1353, 42 m, 14.4.2006, AVE-L 389; *id.*, epiphytic on trunk of *P. pinea*, 29TNE1758, 49 m, 26.6.2009, AVE-L 326, LEB-Lichenes 7825. [13] Serra da Boa Viagem/Mata do Prazo de Santa Marinha/Serra da Boa Viagem— in a pine forest in a mountainous area, 29TNE1149, 205 m, 26.6.2009, AVE-L 328; *id.*, on trunk of *P. pinea*, 29TNE1149, 200 m, 26.6.2009, AVE-L 324. [16] Leiria. Pedrógão, Mata do Pedrógão —on a branch of *P. pinaster*, 29SNE0416, 24 m, 25.4.2009, AVE-L 315. **Estremadura:** [24] **Cascais.** Serra de Sintra, Penha Longa, Perímetro Florestal da Penha Longa (Parque Natural De Sintra Cascais) — in a pine forest in a mountainous area, on a branch of *P. pinaster*, 29SMC6490, 149 m, 27.4.2009, AVE-L 317.

Remarks

In our study area, *O. arborea* specimens growing on branches frequently developed roundish and delimited soralia, while those on trunks often generated confluent soralia. Orsellinic acid, detected in Spanish specimens (Boqueras et al. 1999), was not found in our material. *Ochrolechia microstictoides* Räsänen is another very common species in pine forests on sand dune areas in the centre of Portugal. This species, which has confluent soralia towards the centre of the thallus that give it a leprose appearance, contains variolaric and lichesterinic acids (Tønsberg 1992).

Chapter 2.3

The first records of *Hypotrachyna lividescens* and *H. pseudosinuosa* in the Iberian Peninsula

Figures on the previous page: from top to bottom, *Hypotrachyna lividescens* (Kurok.) Hale and *H. pseudosinuosa* (Asahina) Hale.

The first records of *Hypotrachyna lividescens* and *H. pseudosinuosa* in the Iberian Peninsula

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Abstract

Hypotrachyna lividescens and *H. pseudosinuosa* are reported for the first time from the Iberian Peninsula, the second record of these species from Continental Europe. These lichens were found in a coastal area in the centre of Portugal, at Dunas de Quiaios, Figueira da Foz. *H. lividescens* was most commonly found on *Halimium halimifolium*, whereas *H. pseudosinuosa* was found mostly on *Pinus pinaster*. In addition, echinocarpic acid is reported as an accessory substance in *H. lividescens* for the first time.

Keywords: *Hypotrachyna*, Iberian Peninsula, Portugal, Quiaios

Introduction

Hypotrachyna lividescens (Kurok.) Hale and *H. pseudosinuosa* (Asahina) Hale were recently recorded for the first time from continental Europe in France (Masson 2001, 2004). We now report the occurrence of these two species from Dunas de Quiaios (Figueira da Foz), in the central coastal region of Portugal.

Dunas de Quiaios is a sand dune area covered mostly by *Pinus* forest. The Portuguese Forest Service began the pine plantation in 1924 in order to stabilise the sand dunes (Almeida 1997), which at that time had a vegetation mainly formed by scrublands, dune meadows and scattered mixed woods. *Pinus pinaster* Aiton is the main phorophyte but grows together with *Pinus pinea* L., *Acacia longifolia* (Andrews) Willd., *Arbutus unedo* L. and *Myrica faya* Aiton. The shrub vegetation consists mainly of *Cistus salvifolius* (L.), *Corema album* (L.) D.Don, *Cytisus grandiflorus* DC., *C. striatus* (Hill) Rothm., *Halimium halimifolium* (L.) Willk., *H. calycinum* (L.) K. Koch, *Lavandula stoechas* L. subsp. *sampaiana* Rozeira and *Ulex europaeus* L. (Almeida 1997). The mean minimum temperature of the coldest month varies between 4.8–6.4ºC, while the mean maximum temperature of the hottest month ranges between 23.0–24.2ºC (Almeida 1997).

Materials and Methods

Specimens where analysed morphologically and compared with authentic material deposited in CANB. Chemical analyses were performed according to standardized methods of thin layer chromatography (TLC) (White & James 1985; Orange et al. 2001) and high performance liquid chromatography (HPLC) (Elix et al. 2003).

The Species

Hypotrachyna lividescens *(Kurok.) Hale*

Hypotrachyna lividescens was most commonly found on *H. halimifolium*, but it was also collected on *C. salvifolius*, *Cytisus* sp. and *P. pinaster*. The morphology of the specimens (n=38) examined was consistent with the descriptions given by Masson (2005)

and Nash et al. (2002) and was identical with authentic material. The present study also revealed new data regarding the chemistry of this species. In total, 32 samples were analysed by TLC and 9 of these were examined further by HPLC. The majority of specimens contained atranorin (minor), chloroatranorin (minor), olivetoric acid (major) and anziaic acid (minor) but accessory echinocarpic acid (minor) was also detected in some specimens for the first time.

In Europe this species was previously reported from the littoral areas of western and south-central France where it occurs on a variety of substrates (Masson 2005). This species is also known from South Africa, Australia (Elix 1994) and Mexico (Nash et al. 2002).

Selected specimens examined: Portugal: Beira Litoral, Figueira da Foz: Mata Nacional das Dunas de Quiaios, epiphytic on *Halimium halimifolium* in a shrubby area surrounded by pine vegetation, 58m, 15 September 2005, S. Rodrigues (AVE-L 92, LEB 6805 Lichen).

Hypotrachyna pseudosinuosa *(Asahina) Hale*

Hypotrachyna pseudosinuosa was collected growing on *P. pinaster* and on *H. halimifolium*. The morphology and chemistry of the specimens examined were consistent with descriptions given by Louwhoff & Elix (2002a,b), Masson (2005) and Nash et al. (2002) and was identical with authentic material. Specimens were analysed by TLC (n=12) and HPLC (n=3), which confirmed the presence of atranorin (minor), chloroatranorin (minor) and protocetraric acid (major).

This species has previously been reported for continental Europe (Masson, 2001) from littoral areas of western France, where it was also found to occur on *P. pinaster*. This species is also known from Macaronesia, with reports from the Azores and Canary archipelagos (Hafellner 1995). The paucity of information regarding the distribution of this species led Sérusiaux (1989) to include it in the Red List for Europe as a vulnerable species. *Hypotrachyna pseudosinuosa* exhibits a broad world distribution that includes Western Europe, Central, North and South America, the Antilles, Macaronesia, Africa, North Asia (China, Taiwan, Japan), South-East Asia and the Pacific (New Zealand, New Guinea, New Caledonia and Hawaii) (Hafellner 1995; Louwhoff & Elix 2002a,b; Masson 2001, 2005; Nash et al. 2002).

Selected specimens examined: Portugal: *Beira Litoral:* Figueira da Foz: Mata Nacional das Dunas de Quiaios, epiphytic on *Pinus pinaster* in a pine forest, 48m, 28 March 2006, S. Rodrigues (AVE-L 93, LEB-Lichen 6803).

Chapter 3

Biomonitoring atmospheric pollution from pulp mill industry using lichen transplants in central-littoral Portugal

Figures in the previous page: from top to bottom, Celbi, a pulp mill at the border of a pine forest on sand dunes; and transplant of *Flavoparmelia caperata* (L.) Hale.

Biomonitoring atmospheric pollution from pulp mill industry using lichen transplants in central-littoral Portugal

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Abstract

Lichen transplants of the species *Flavoparmelia caperata* were used to evaluate the accumulation of thirty-three elements putatively emitted from Kraft paper mill industry, in a study conducted near a point source at Figueira da Foz (Portugal), between January and July 2008. Chlorophyll *a* fluorescence kinetics studies were performed in the transplanted lichens, in order to evaluate the hypothetical effect of elemental accumulation on lichen vitality. F_v/F_m, F_0, F_m, Φ_{PSII}, q_P, NPQ and Φ_{Exc} were the analysed parameters. It was intended to evaluate the effects of distance — 500, 1000, 1500 and 2000 m — and period — 45, 90, 135 and 180 days — of exposure on elemental accumulation and chlorophyll *a* fluorescence kinetics, also taking into consideration the results from transplants placed in a reference location.

Both distance and period of exposure influenced elemental accumulation at the impacted location. Most elements — Al, B, Ba, Ca, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, P, S, Sb, Sc, Sr, Ti and V — were found in significantly higher concentrations in the transplants exposed at 500 m of distance from the point source. Nearly half of the elements — B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V — were also found in significantly higher concentrations in the transplants exposed during 180 days, and actually increased with increasing exposure time for B, Hg, Mo, and S. Soil was identified as a partial source for most elements.

The chlorophyll *a* fluorescence kinetics parameters F_v/F_m, F_m, Φ_{PSII}, and Φ_{Exc} varied significantly with site and/or period of exposure. F_v/F_m and F_m were significantly decreased in the transplants exposed at 500 and 1000 m from the pulp mill and in those exposed during 135 and 180 days. Both, Φ_{PSII} and Φ_{Exc} decreased significantly after 180 days of exposure. Significant negative correlations were identified between F_v/F_m and the

concentrations in lichen transplants of B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb, and Zn; F_m and Ba, Co, Hg, Mn, Mo, N, P, S, Sb, and Zn; Φ_{PSII} and N and P; and Φ_{Exc} and Mn, N, P, and S.

Lichen diversity studies performed in the same locations where lichen transplants were placed revealed a lower lichen diversity value at the 500 m, which was also the only site were nitrophytic species were found, what could be due to the deposition of ammonia, but also dust.

Keywords: Bark pH, bioaccumulation, chlorophyll *a* fluorescence, elements, lichen diversity, lichen transplants.

Introduction

Pulp and paper mills are responsible for the emission of several substances to the atmosphere among which there are chlorine and chlorine containing substances, some of which organic, and also hydrochlorofluorocarbons, cyanides; fluorine; greenhouse gases; metals; particulate matter; nitrogen compounds as ammonia and nitrogen oxides, polycyclic aromatic compounds; pesticides; sulphur oxides, and volatile organic compounds (E-PRTR 2012, NCASI 2005). These pose a threat to human health and to the environment, and many are included in the polluting substances list of the E.U. directive on industrial emissions (E.U. 2010).

Pulp mills can operate through different processes that lead to different atmospheric emissions. The main processes used in chemical wood pulping are Kraft, sulphite, neutral sulphite semi chemical, and soda, the first three having the greatest potential for causing atmospheric pollution (EPA 1995).

Most Portuguese pulp mills operate under the Kraft processes. Atmospheric emissions from Portuguese Kraft pulp mills have been studied regarding the emission of reduced sulphur compounds (H_2S, methylmercaptan, dimethylmercaptan, dimethylsulfide), which pose a problem to local populations — as they are malodorous — although the concentrations at which they were found are negligibly toxic (Bordado & Gomes 2002a,b). Particulate emissions have been identified as the pollutants emitted in higher amounts, followed by NO_x, SO_2 and H_2S (Bordado & Gomes 1997). Despite that, the introduction of electrostatic precipitators in chimneys of several units of the pulp mill installations (Celbi 2008) may have significantly reduced particle emission. Particulate emissions consist mostly calcium and sodium salts ($CaCO_3$, CaO, Na_2CO_3, Na_2S, Na_2SO_4), which are primarily entrained in ashes and other solids that are carried by turbulent fumes (Bordado & Gomes 1997, Gomes 1999).

Metals are emitted together with particles, in a process highly dependent on the metal content of the black liquor (Gomes 1999). Black liquor is the solution resulting from the cooking of the wood chips with sodium hydroxide and sodium sulphide (white liquor), that after being concentrated becomes a combustible that is burned for the production of energy (Celbi 2008). However, metal emissions may also derive from limekilns and smelt dissolving tanks (NCASI 2005). Metals may enter the Kraft cycle through the pulpwood,

make-up water, make-up chemicals, equipment corrosion, and fossil fuels (NCASI 2005). A study regarding atmospheric emissions from Canadian and US pulp and paper mills, points to quite smaller emissions of most metals regarding NO_x, particles, SO_2, total reduced sulphur compounds, and volatile organic compounds (NCASI 2005). Elements may be volatilized due to the elevated temperatures that may occur in pulp mill's boilers, especially Cd and Hg (Nurmesniemi et al. 2008). Scarce data exists on metal atmospheric emissions from Portuguese Kraft pulp mills, with emissions of As, Cd, Cu, Hg, Ni, and Zn being reported in E-PRTR (2011), although several metals and other elements are reported for Canadian and U.S. pulp mills (NCASI 2005).

Biomonitoring experiments of emissions from pulp mills were conducted with lichens, bryophytes and plants, mostly in the years when the emissions of atmospheric sulphur were higher than the present day, due to the upgrading of processes by pulp mills (Pöykiö & Torvela 2001). Halonen et al. (1993) found that the concentrations of Cd, Cu, Cr, K, Fe, Mg, N, Ni, S, Ti, and Zn, were elevated in samples of *Hypogymnia physodes* collected in the vicinity of a Finnish sulphate pulp mill. Kitöma et al. (1995) found high Al contents in *H. physodes* thalli collected near a Finnish sulphite pulp mill, what was related to particulate emissions from the pulp and paper mill and its associated power plant. Despite that, the emissions of particulate matter, and consequently Al, were reduced by 95% after the introduction of electrical filters, after the study was conducted. Holopainen et al. (1993) found that the concentrations of S were higher in transplanted *H. physodes* placed at 200 m from a sulphite pulp mill and increased after 16 weeks of exposure, but decreased in the transplants placed at 4500 m from the pulp mill. Adoli et al. (2011) found increased concentrations of Cd, Cu, Cr, Fe, and Zn in terrestrial moss samples (*Hylocomium splendens*) collected up to 1000 m from a pulp and paper mill in Western Kenya, indicating dry deposition as an important factor for elemental accumulation. In their experiment, distance of collection, up to 1000 m, did not influence elemental content.

Physiological damages to the lichens, either naturally occurring or transplanted to the surroundings of pulp mills, were not found by Sheridan et al. (1976), who instead found an increase in the photosynthetic rate that was attributed to low-level SO_2 fumigation, in a short-term transplantation experiment of *Alectoria fremontii* to the vicinity of a Kraft pulp mill in the U.S. On the other hand, structural and ultrastructural studies do indicate a possible negative influence on photosynthetic activity, as ultrastuctural damages

in lichen photobionts were observed in lichens naturally growing near sulphite pulp mills and also in transplanted ones in Finland, and they were attributed mainly to SO_2 and N compounds (Holopainen 1983, 1984).

The effects of atmospheric emissions from pulp mills on the diversity of epiphytic lichens have also been studied in the past. These studies pointed to an adverse effect either restricted to the vicinity of sulphite pulp mills (Halonen et al. 1993), or to a lichen desert extending for 1.5 Km downwind from a sulphite pulp mill, with morphological symptoms of exposure to pollution being identified in lichen epiphytic flora up to 4 Km downwind (Holopainen 1983). The occurrence of nitrophytic species, reduction of the frequencies of sensitive species (*Bryoria* and *Usnea*), reduction of lichen biomass and zonation in the occurrence of lichen morphological types were also identified in the proximity of pulp or paper mills (Hoffman 1979, Kinnunen et al. 2003, Newberry 1974, Sheridan et al. 1976).

The occurrence of lichen communities on phorophytes is not only influenced by direct toxic effects of pollutants on lichens, but is also largely influenced by bark pH (e.g., Brodo 1973, van Herck 2001). The deposition of pollutants on bark may alter its pH: SO_2, for instance has acidifying properties (e.g., Bates et al. 2001, Larsen et al. 2007), while NH_3 has the opposite effect (e.g., Frati et al. 2007, van Herck 2001).

This study intends to provide an evaluation of elemental deposition, mostly metals, on transplants of the lichen *F. caperata* placed in the vicinity of a Kraft pulp mill located central-littoral Portugal. Additionally, it aims at understanding the effects of that deposition on lichen transplant vitality, by means of chlorophyll *a* fluorescence kinetics analysis, and also on the diversity of epiphytic lichens in the proximity of the pulp mill.

The lichen *F. caperata* is widely used in biomonitoring experiments, including transplant experiments (Aprile et al. 2010; Baptista et al. 2006, 2008; Cuny et al. 2001; Frati et al. 2007; Godinho et al. 2008a,b, 2009; Machado et al. 2006; Pacheco et al. 2007, 2008).

Materials and methods

Study area

This study was conducted between January and July 2008 at Figueira da Foz in central littoral Portugal, where an industrial park is located that includes two Kraft pulp mills. This experiment was carried out in the vicinity of one of them, Celbi (Celulose da Beira Industrial), located at the border of Mata do Urso, a pine forest on sand dunes (Fig. 3.1). Lichen transplant experiments and lichen diversity studies were performed at this location as well as at a reference location, Dunas de Quiaios, a pine forest on sand dunes located approximately 16 Km north of the impacted location (Fig. 3.1). At Dunas de Quiaios there are no known point sources of atmospheric pollution, and this forest is part of the classified Natura 2000 SCI "Dunas de Mira, Gândara e Gafanhas", PTCON0055 (ICN 2006). The most abundant tree species in both forests is *Pinus pinaster* Aiton, but *P. pinea* L. and *Acacia* species also occur.

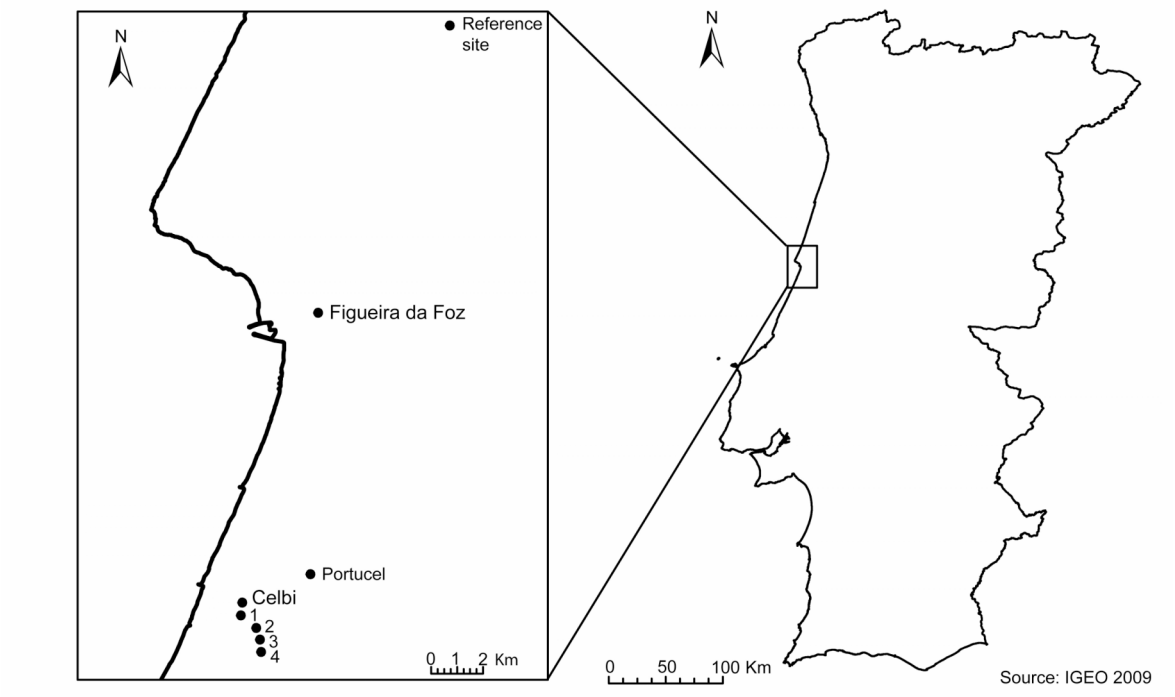

Figure 3.1: Location of the study area. Both the impacted and reference locations are depicted. Celbi and Portucel-Soporcel are two pulp mills located near the impacted location. Dot numbers correspond the sites where transplant experiments and lichen diversity studies were performed at the impacted location, at increasing distances from Celbi: 1, 500 m; 2, 1000 m; 3, 1500 m; and 4, 2000 m.

Meteorological conditions observed during the study period are presented in Fig. 3.2 and Table 3.1 (data supplied by Instituto de Meteorologia, Portugal, obtained from a weather station located at Vila Verde, Figueira da Foz).

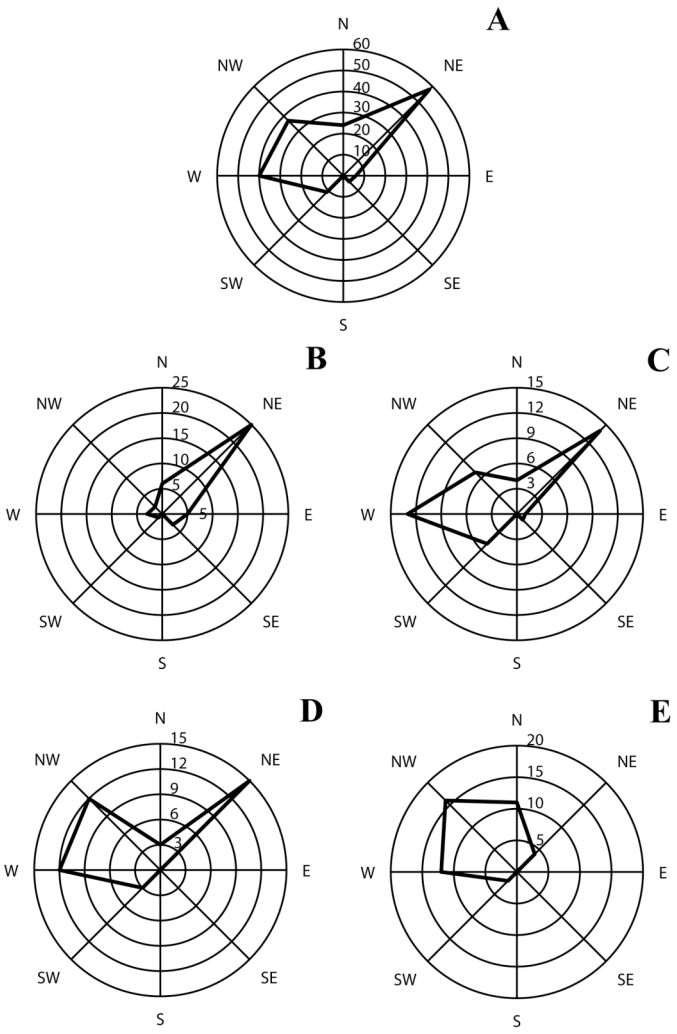

Figure 3.2: Prevailing winds and wind frequency (in number of days) measured between January and July 2008 at the Vila Verde weather station near the impacted (Mata do Urso) and reference (Dunas de Quiaios) locations. A, entire study period (180 days of transplant exposure); B, first study period (0 to 45 days); C, second study period (46 to 90 days); D, third study period (91 to 135 days); and E, fourth study period (136 to 180 days).

The course of the prevailing winds was most frequent from northeast, but was also frequent from west and northwest considering the entire study period (Fig. 3.2A). During each study period the course of the prevailing winds varied, being more frequent from northeast in the first study period (Fig. 3.2B), from northeast and west in the second (Fig. 3.2C), from northeast, northwest and west in the third (Fig. 3.2D), and from northwest in the fourth (Fig. 3.2E). During the entire study period, temperature was in average 15.2 ºC,

daily precipitation 1.9 mm, and 106 days were observed without precipitation (Table 3.1). Average temperature increased with exposure period, but daily precipitation varied, although it was less abundant in the last study period, in which the number of days without precipitation was higher (Table 3.1).

Table 3.1: Meteorological conditions observed from January to July 2008 at the Vila Verde weather station.

Study period (days)	Average air temperature (ºC)	Average daily precipitation (mm)	Number of days without precipitation
0–180 days	15.2	1.9	106[a]
0–45 days	12.4	1.1	23[b]
46–90 days	13.5	4.2	20[c]
91–135 days	15.8	2.0	24
136–180 days	19.1	0.1	39

Notes: there are no data available for [a]15, [b]14 and [c]1 days of the study period

Lichen transplants

The transplantation experiment at the impacted location intended to evaluate the effects of distance to the pulp mill (point source) and period of exposure on lichen elemental content and vitality. The experiment lasted 6-months, and took place between January and July 2008.

The lichen *F. caperata* was selected for transplantation due to its wide use in biomonitoring experiments, and to the fact that it is one the most abundant epiphytic lichen species present at the reference location (Dunas de Quiaios), where samples were retrieved for transplantation. Several samples of *F. caperata* epiphytic on *P. pinaster* were collected at distinct sites at the reference location to prepare the transplants. In a previous experiment it was determined that the content in several elements in samples of this species from distinct locations within the forest was not significantly different (data not shown). Transplants were prepared by placing 4–5 thalli (with a minimum width from border to centre of 4 cm) in plastic nets with a 2 cm mesh.

Lichen transplants were placed at the impacted location (Mata do Urso) on *P. pinaster* trees at a height of 1.5 m, at increasing distances from the pulp mill, approximately 500, 1000, 1500, and 2000 m (Fig. 3.1). As this is a forested location with a

large density of trees, transplants in the four sites were placed at the border of forest, in the case of the 500 m site, and at the border of a forest road for the remaining sites, in the direction that would favour most the contact with atmospheric conditions. Transplants were placed facing north-northeast at the 500 m site, northeast at the 1000 m site, northwest at 1500 m site and north at the 2000 m site. Sets of 24 transplants were placed at each of the exposure sites, and 6 replicate transplants were collected after 45, 90, 135 and 180 days of exposure for elemental analysis and studies on lichen vitality. As a control, a set of 24 lichen transplants was also placed at the reference location, in similar conditions to the transplants placed at the impacted location, and collected after the same exposure periods.

Lichen sample treatment

After collection, lichen transplants were air dried in the laboratory and the constituting lichen thalli were carefully cleaned and detached from pine bark with tweezers, from the border to 4 cm in direction of the centre of the thallus, and placed together in falcon tubes. Samples were then lyophilised (Snijders Scientific 2040 lyophilizer) and kept at –20ºC until homogenization. Homogenization was performed with refrigerated porcelain mortar and pestle, carefully acidly and basically decontaminated between different sample grinding. Samples were kept at –20ºC until elemental analysis.

Soil sampling

In order to evaluate the contribution of sandy soil particles to the element concentration in transplanted lichens, 4 samples of soil were collected at each site where transplants were exposed, both at the impacted and reference locations, for analysis of the same elemental content as lichens. Soil collection was made with a stainless steel hand trowel, which was decontaminated between sample collections. In the laboratory, samples were air-dried and sieved using a sieve with 0.5 mm mesh, in order to remove most of the organic material and larger sand grains. The 4 samples of soil collected at each exposure site were then combined into composite samples (5 g each) for chemical analysis.

Chemical analysis

Lichen and soil samples were analysed for all elements (except Hg) by Laboratorio de Técnicas Instrumentales — Instalación Radioactiva (Universidad de León, Spain). A range of 33 elements was analysed. These elements were selected for analysis based on the study of NCASI (2005) and the data available in E-PRTR (2012).

Al, Ca, Fe, K, Mg, Mn, Na, P, S, and Si were analysed by ICP-AES (Optima 2000 DV, Perkin Elmer); while As, B, Ba, Be, Bi, Cd, Co, Cr, Cu, Li, Mo, Ni, Pb, Sb, Sc, Se, Sn, Sr, Ti, V and Zn were analysed through ICP-MS (Varian). Prior to elemental analysis, 300 mg of each sample were digested with 10 ml 65 % HNO_3 in a microwave oven (MarsXpress, CEM) firstly for 10 min at 100ºC and finally, for 25 min at 200ºC.

N was analysed by the Kjeldahl method. For that, 400–500 mg of composite samples of the 6 transplant replicates collected in the same exposure conditions of site and period of exposure were used, due to insufficient sample mass.

Hg was determined through pyrolysis atomic absorption spectrometry and was analysed directly without sample pre-treatment, using an AMA254 Advanced Mercury Analyzer (LECO). Quality control was performed analyzing 2–3 sub-samples of each lichen sample and 4–5 sub-samples of soil composite samples, and by the analysis of replicate blanks.

The accuracy of the results was verified through the analysis of certified reference material IAEA-336, and also BCR-482 in the case of Hg analysis. Values of the analysed elements were within the confidence intervals reported in the reference sheet for those materials (Quevauviller et al. 1985, Stone et al. 1995).

Lichen vitality

Vitality of lichen transplants was evaluated by means of the analysis of chlorophyll *a* fluorescence kinetics. Measurements of the modulated fluorescence of chlorophyll *a*, were performed with a portable fluorometer PAM-200 (Walz, Germany) in a lobe randomly selected in each transplant before transplantation and after exposure. The lobes were separated from the lichen thalli in the laboratory, after the transplants were air-dried.

Chlorophyll *a* fluorescence kinetics was studied through the analysis of the parameters: F_v/F_m, maximum quantum efficiency of photosystem II (PSII) photochemistry, F_v representing the variable fluorescence ($F_m - F_0$); F_0, minimum fluorescence; F_m, maximum fluorescence; NPQ, non-photochemical quenching; q_P, photochemical quenching; Φ_{PSII}, the operating efficiency of PSII, and Φ_{Exc}, the maximum efficiency of PSII. A detailed description of the analysis procedure undertaken for estimating these parameters is reported in Fernández-Salegui et. al (2006).

Lichen diversity

Lichen diversity was evaluated in the same sites where lichen transplants were performed at the impacted location, using the method proposed by Asta et al. (2002). For that, 12 trees were selected at each site, and lichen diversity in each was evaluated through counts of lichens present in nets constituted by 5 vertically consecutive quadrates of 10 × 10 cm, which were placed in the four cardinal directions at a height of 1.5 m in each tree. From those counts, lichen diversity values (LDVs) were calculated for each site. Selected trees had minimum girth of 60 cm.

At the reference location, LDV was determined from data of a previous experiment. This was calculated using the procedure as for the impacted location, but from 7 randomly chosen sampling units.

Bark pH

Possible alterations in bark pH resulting from the deposition of atmospheric pollutants were also studied at the impacted location and compared to results obtained at the reference location.

Bark pH was measured in bark samples collected in the same trees (n=12) and sites where the diversity studies were undertaken at the impacted location. At the reference location, bark samples were collected in five randomly chosen sampling sites, and in each of those 4 randomly chosen trees were sampled. Samples of bark were collected at a height of 1.5 m, in the four cardinal directions.

In the laboratory, bark samples were cut into round pieces with an area of approximately 46.3 mm^2 with a cork borer. Three disks per sample were weighted before their lower surface was dipped into yellow beeswax (VWR). This was done in order to promote the dissolution of protons only from the upper surface — the one in contact with the atmosphere and that serves as substrate for lichens (Kricke 2002).

The disks were then placed overnight in closed falcon tubes containing 5 ml 0.25 M KCl, with the upper surface, not waxed, facing the solution. After disk removal, the solution pH was measured with a pH electrode (WTW, SenTix 21).

Statistical Analysis

The Anderson-Darling test was used to evaluate data normality and both Bartlett's and Levene's tests were used to verify homoscedasticity. When necessary, data were transformed to normality using the Johnson transformation. Despite that, for some variables normality and/or homoscedasticity were not achieved, and non-parametric alternatives to the tests were used.

The evaluation of the effects of exposure period and site on elemental accumulation and chlorophyll *a* fluorescence kinetics was performed by means of General Linear Model (GLM) analysis. For some variables the assumptions of normality and homoscedasticity could not be met, and a non-parametric alternative was used, based on the Scheirer-Ray-Hare extension of the Kruskal-Wallis test as indicated by Dytham (1999). For this, the adjusted sums of squares regarding the factors and their interaction were used in the calculations. When significant results were found, Tukey's (parametric) or Dunn's post-tests (non-parametric) were performed for post-hoc comparisons. One-way Anova or Kruskal-Wallis tests were used to test for differences between the samples exposed at the reference location regarding period of exposure, and were followed by Tuckey's post-tests or Dunn's post-tests, respectively.

The effects of direction and site of collection on bark pH were also tested by means of GLM. Data regarding the reference location were tested separately, as site of collection was a random factor, contrarily to the impacted location. A Kruskal-Wallis test, followed by a Dunn's post-test, was also used to test for differences between bark pH measured in

samples retrieved at each site studied of the impacted location versus that of samples collected at the reference location.

Spearman rank correlation tests were undertaken to test for correlations in the accumulation of the different elements with that of Sc, the element chosen to provide a reference to the accumulation of soil particles; and also to detect correlations between the fluorescence parameters studied and element accumulation in lichen transplants.

The chlorophyll *a* fluorescence kinetics parameters q_P, NPQ and Φ_{PSII}, Φ_{Exc} were analysed using the values obtained after 14 min of induction, when the process was already steady state.

All statistical analyses were performed with Minitab version 15, but the Scheirer-Ray-Hare test is not available in this package, and therefore the necessary calculations were performed as indicated by Dytham (1999). For all statistical tests the significance level used was P = 0.05, unless otherwise stated.

Results

Element accumulation in lichen transplants

Of the 33 elements analysed in lichen transplants from the impacted and reference locations, As was bellow the detection limits of analysis (400 μg Kg^{-1}) in the majority of the lichen samples; and Be, Bi, Se and Sn were bellow the detection limit in all lichen samples analysed (100 μg Kg^{-1} for Be and Bi, and 500 μg Kg^{-1} for Se and Sn).

Chemical analysis of transplants exposed at the impacted and reference location revealed significant differences in the accumulation of all the elements studied, except N (Fig. 3.3–3.6, Tables 3.2 and 3.3). The site of transplantation was the only factor that determined significant differences in the accumulation of Al, Ca, Cd, Co, Cu, Li, Ni, Sc, Si, Sr, Ti and Zn (Fig. 3.3–3.6). K was the only element for which exposure time was the sole factor significantly affecting its accumulation (Fig. 3.4). The accumulation B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V was varied significantly with both factors, period and site of exposure (Fig. 3.3–3.6). An interaction between both factors was observed for Ca (Fig. 3.3).

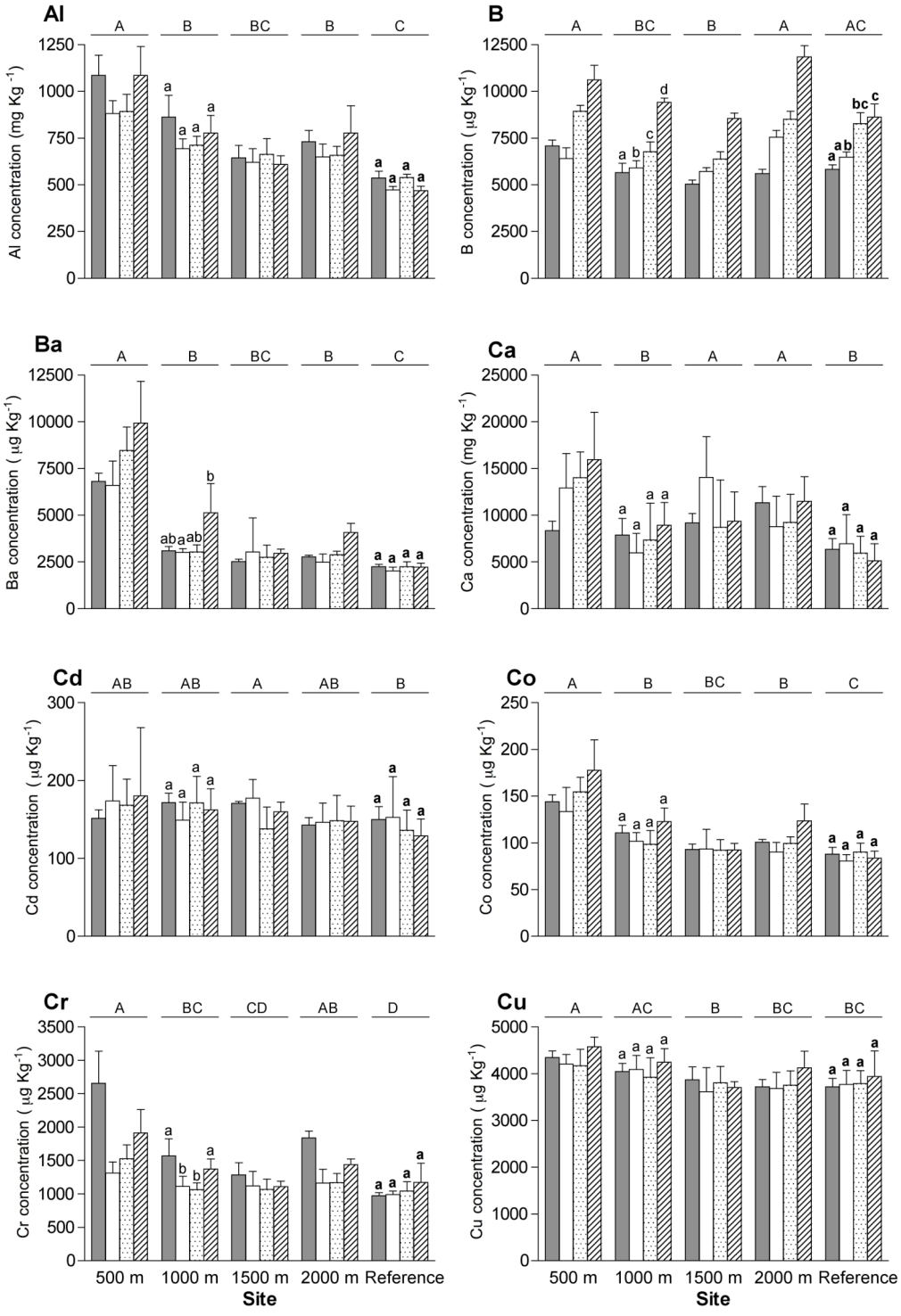

Figure 3.3: Mean concentrations of Al, B, Ba, Ca, Cd, Co, Cr, and Cu measured in transplants exposed at the impacted, at 500, 1000, 1500 and 2000 m from the point source; and reference locations, at increasing time intervals of exposure, 45 ■, 90 □, 135 ⬚, and 180 ▨ days. Data are mean values ± 1SE (n = 6). Different uppercase (factor: exposure site), or lower case (factor: exposure period) letters above the bars represent significant differences ($P < 0.05$) element concentration in transplants exposed at the impacted and reference locations. Bold letters above bars represent the same, but only for transplants at the reference location. Groups sharing letters are not significantly different.

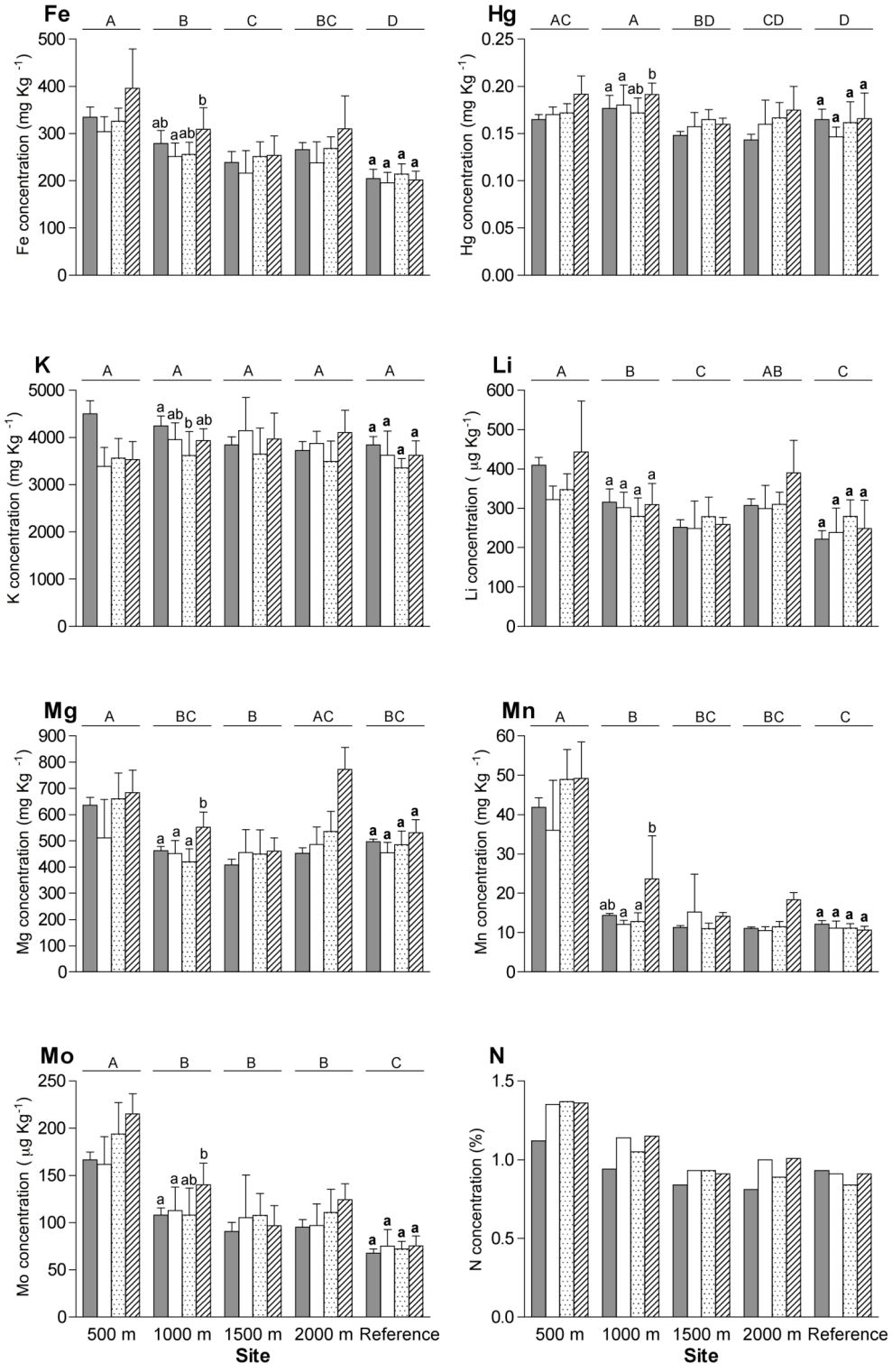

Figure 3.4: Concentrations of Fe, Hg, K, Li, Mg, Mn, Mo, and N, measured in transplants exposed at the impacted location, at 500, 1000, 1500 and 2000 m from the point source; and at the reference location; both at increasing time intervals of exposure, 45 ■, 90 □, 135 ⬚, and 180 ▨ days. Data are mean values ± 1SE (n = 6), except for N, which represent the % found in composite samples of transplants exposed in the same conditions. For further details refer to Fig. 3.3.

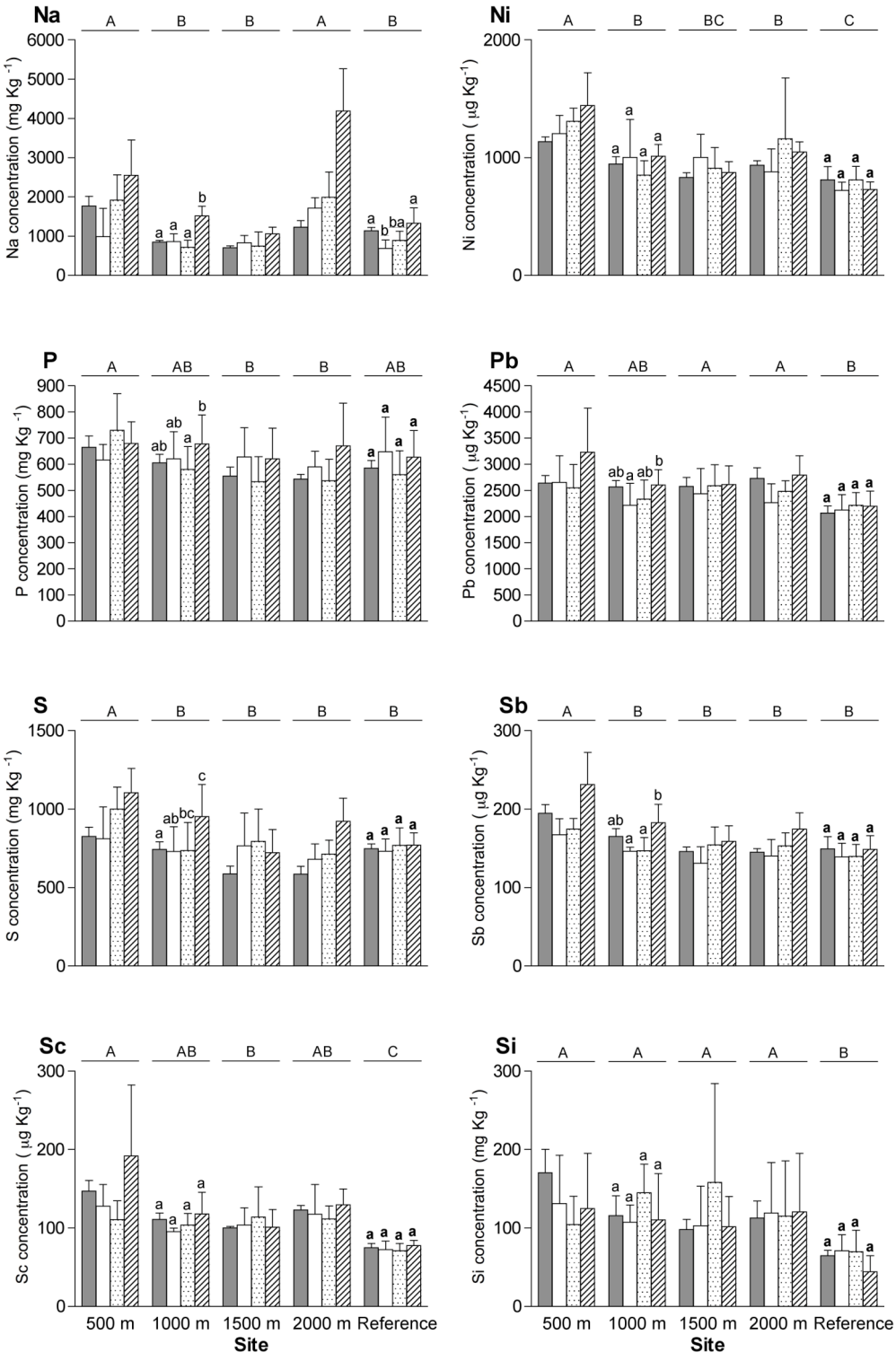

Figure 3.5: Concentrations of Na, Ni, P, Pb, S, Sb, Sc, and Si, measured in transplants exposed at the impacted location, at 500, 1000, 1500 and 2000 m from the point source; and at the reference location; both at increasing time intervals of exposure, 45 ■, 90 □, 135 ⬚, and 180 ▨ days. Data are mean values ± 1SE (n = 6). For further details refer to Fig. 3.3.

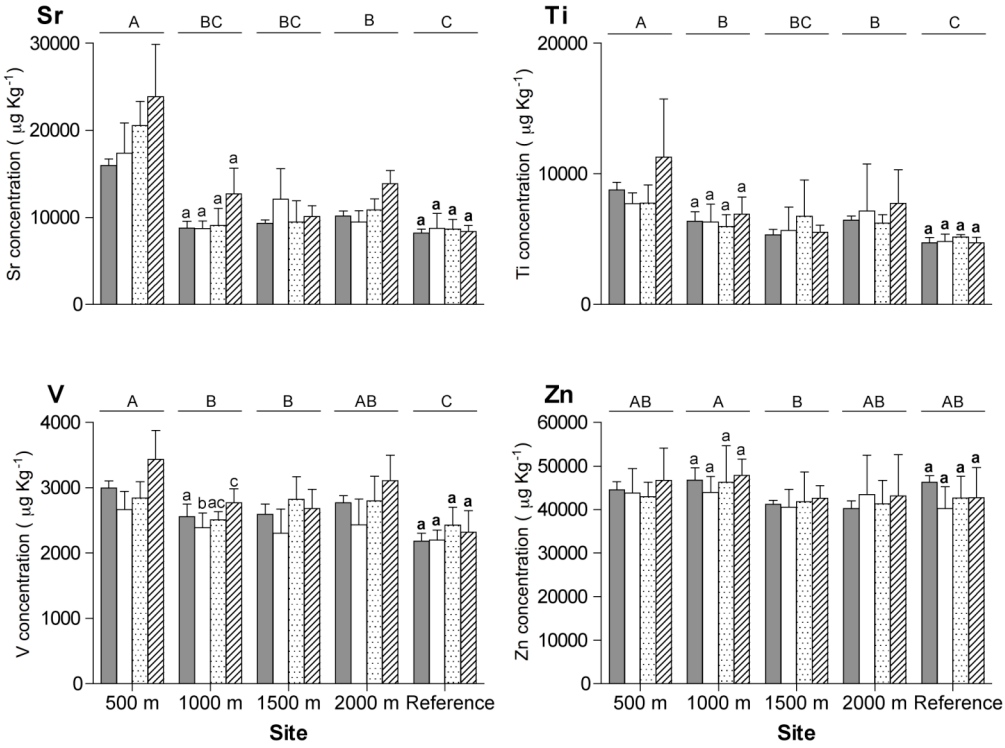

Figure 3.6: Concentrations of Sr, Ti, V, and Zn measured in transplants exposed at the impacted location, at 500, 1000, 1500 and 2000 m from the point source; and at the reference location; both at increasing time intervals of exposure 45 ■, 90 □, 135 ⬚, and 180 ▨ days. Data are mean values ± 1SE (n = 6). For further details refer to Fig. 3.3.

The accumulation pattern of most elements — Al, B, Ba, Co, Cr, Cu, Fe, Li, Mg, Mo, N, Na, Ni, P, S, Sb, Sc, Si and Ti — was similar regarding distance of exposure at the impacted location (Fig. 3.3–3.6). The concentrations of these elements in the transplants placed at 500 m from the point source were in average higher than the ones observed at the other exposure distances, decreasing with increasing distance of exposure, except at 2000 m, where a small increase was observed for most those elements. The concentrations of these elements, except N and Si (Fig. 3.4–3.5), were significantly higher in the transplants exposed at 500 m regarding all or some of the other exposure distances.

The concentrations of Cd and Mn decreased in lichen transplants with increasing distance of exposure at the impacted location (Fig. 3.3–3.4). The differences observed were not significant for Cd, however the concentrations of Mn were significantly higher in the transplants at 500 m versus the ones exposed at 1000, 1500 and 2000 m from the point source.

Table 3.2: GLM for the factors exposure period and site, and their interaction on the accumulation of 14 elements in transplants exposed at the impacted and reference locations ($df_{Exposure\ period}$=3, $df_{Exposure\ site}$=4, $df_{Interaction}$=12, df_{Error}=98, df_{Total}=117).

	F	**P**		***F***	**P**		***F***	**P**
Al			*K*			*S*		
Period	1.55	0.207	Period	5.85	**0.001**	Period	9.68	**0.000**
Site	18.22	**0.000**	Site	1.84	0.128	Site	8.14	**0.000**
Interaction	0.14	0.996	Interaction	1.85	0.050	Interaction	1.76	0.065
B			*Li*			*Si*		
Period	64.36	**0.000**	Period	1.89	0.136	Period	1.06	0.372
Site	13.04	**0.000**	Site	18.85	**0.000**	Site	6.34	**0.000**
Interaction	1.77	0.065	Interaction	1.16	0.321	Interaction	0.62	0.774
Ca			*Mo*			*V*		
Period	0.97	0.411	Period	5.74	**0.001**	Period	10.46	**0.000**
Site	14.60	**0.000**	Site	59.06	**0.000**	Site	15.50	**0.000**
Interaction	1.86	**0.049**	Interaction	0.81	0.641	Interaction	1.25	0.263
Cu			*P*			*Zn*		
Period	2.58	0.058	Period	3.32	**0.023**	Period	0.78	0.507
Site	9.50	**0.000**	Site	3.32	**0.014**	Site	2.63	**0.039**
Interaction	0.54	0.881	Interaction	0.78	0.666	Interaction	0.48	0.925
Fe			*Pb*					
Period	6.03	**0.001**	Period	3.87	**0.012**			
Site	29.93	**0.000**	Site	7.75	**0.000**			
Interaction	0.56	0.866	Interaction	0.69	0.760			

Note: significant results in bold

Table 3.3: Scheirer-Ray-Hare tests for the factors exposure period and site, and their interaction on the accumulation of 13 elements in transplants exposed at the impacted and reference locations ($df_{Exposure\ period}$=3, $df_{Exposure\ site}$=,4, $df_{Interaction}$=12, df_{Total}=117; except Hg, $df_{Exposure\ period}$=3, $df_{Exposure\ site}$=4, $df_{Interaction}$=12, df_{Total}=111).

	H	**P**		***H***	**P**		***H***	**P**
Ba			*Mg*			*Sc*		
Period	8.45	**0.038**	Period	19.71	**0.000**	Period	3.82	0.282
Site	81.57	**0.000**	Site	32.57	**0.000**	Site	57.45	**0.000**
Interaction	4.00	0.983	Interaction	16.45	0.171	Interaction	4.66	0.968
Cd			*Mn*			*Sr*		
Period	1.40	0.706	Period	11.22	**0.011**	Period	6.15	0.105
Site	13.37	**0.010**	Site	64.35	**0.000**	Site	64.11	**0.000**
Interaction	10.10	0.607	Interaction	13.21	0.354	Interaction	10.32	0.588
Co			*Na*			*Ti*		
Period	5.32	0.150	Period	24.20	**0.000**	Period	0.92	0.821
Site	68.68	**0.000**	Site	45.48	**0.000**	Site	56.65	**0.000**
Interaction	7.41	0.829	Interaction	15.89	0.196	Interaction	3.27	0.993
Cr			*Ni*					
Period	16.30	**0.001**	Period	1.43	0.699			
Site	44.40	**0.000**	Site	58.48	**0.000**			
Interaction	9.41	0.668	Interaction	7.87	0.795			
Hg			*Sb*					
Period	12.05	**0.007**	Period	18.79	**0.000**			
Site	21.71	**0.000**	Site	34.29	**0.000**			
Interaction	8.23	0.767	Interaction	5.43	0.942			

Note: significant results in bold

The accumulation of Ca, Hg, and Zn varied with distance of exposure at the impacted location. Ca was significantly inferior in the transplants exposed at 1000 m regarding those placed at the remaining distances (Fig. 3.3), and Hg was significantly inferior in the transplants exposed at 1500 m regarding those placed at 500 and 1000 m, as well as in those exposed at 2000 versus 1000 m (Fig. 3.4). Zn concentrations were significantly higher in the transplants exposed at 1000 m versus the ones exposed at 1500 m (Fig. 3.6).

Furthermore, the concentrations of Pb, Sr, and V decreased between 500 and 1000 m of exposure and increased after that, although significant differences were not found for Pb with distance of exposure at the impacted location (Fig. 3.5). Sr concentrations were significantly higher in the transplants exposed at 500 m regarding those exposed at 1000, 1500 and 2000 m from the point source (Fig. 3.6). Also, V concentrations were significantly higher in the transplants exposed at 500 m versus the ones exposed at 1000 and 1500 (Fig. 3.6).

Elemental accumulation regarding background conditions was investigated through the analysis of lichen transplants performed at the reference location. Statistical analysis showed that the accumulation of Al, Ba Ca, Co, Cr, Fe, Li, Mo, Ni, Pb, Sc, Si, Ti and V in lichen transplants was significantly higher at all or most of the exposure distances tested at the impacted location when compared to those exposed at the reference location (Fig. 3.3–3.6, Tables 3.2 and 3.3). The accumulation of K, P, and Zn did not vary significantly between the impacted and the reference locations, although significant differences were found in the accumulation of those elements in the transplants exposed at the impacted location when considering distance of exposure, except for K (Fig. 3.4–3.6, Tables 3.2 and 3.3).

Many of the elements — B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V — were found in significantly higher concentrations in the transplants exposed during 180 days considering the transplants exposed during all or some of the other exposure periods (Fig. 3.3–3.6, Table 3.2 and 3.3). Despite that, the accumulation of Cr in the transplants exposed during 180 days was inferior to that of the transplants exposed during 45 days (Fig. 3.3).

A similar average accumulation pattern was identified for some elements — Al, Ba, Co, Cr, Cu, Fe, Li, Mg, Mn, Na, Pb, Sb, Ti, V, and Zn — with concentrations

decreasing from 45 to 90 days of exposure, but increasing after that (Fig. 3.3–3.6). However, significant differences with period of exposure were not observed regarding the accumulation of Al, Co, Cu, Li, Ti, and Zn (Fig. 3.3–3.4 and 3.6).

The concentrations of B, Hg, Mo, Ni, S and Sr increased with increasing exposure time (Fig. 3.3–3.6), although for Ni and Sr significant differences were not found. The concentration of K decreased consecutively in transplants exposed up to 135 days, but increased in the ones exposed for 180 days, being significantly higher in those exposed during 45 days regarding those exposed for 135 days (Fig. 3.4). On the other hand, the concentrations of Ca, Cd, N, P, and Si varied with exposure time, but only significantly for P (Fig. 3.3–3.5). The accumulation of Sc was similar in the transplants exposed up to 135 days, but increased after 180 days of exposure, but not significantly (Fig. 3.5).

Regarding the transplants placed at the reference location (Fig. 3.3–3.6), there were no significant differences in element concentrations in the transplants exposed for 45, 90, 135 and 180 days, except for B (one-way Anova, $F_{3,19} = 7.80$, P = 0.001) and Na (one-way Anova, $F_{3,19} = 6.14$, P = 0.004). B concentration increased in lichen transplants with increasing exposure time, and was significantly higher in the transplants exposed for 180 days, when compared to those exposed for 45 and 90 days, as well in those exposed during 135 days, regarding those exposed during 45 days (Fig. 3.3). Na concentration decreased between 45 and 90 days of exposure, and increased after 90 days, being significantly inferior in the transplants exposed for 90 days, regarding those exposed during 45 and 180 days (Fig. 3.5).

The contribution of soil particles to element accumulation in lichen transplants was evaluated through correlation tests between the concentrations of Sc, the element chosen to provide a reference of the accumulation of soil particles, and those of the other elements studied in lichen transplants. At the impacted location, Sc concentrations were significantly and strongly ($0.70 < r_s < 0.89$, according to Fowler et al. (1998)) correlated with those of Ti, moderately ($0.40 < r_s < 0.69$) with those of Al, Ba, Co, Cr, Cu, Fe, Li, Mn, Ni, Sb, Si, Sr and V; and weakly ($0.20 < r_s < 0.39$) with B, Mg, Mo, Na, Pb and Zn (Table 3.4). None of the remaining elements were significantly correlated with Sc. At the reference location, Sc concentrations were significantly and moderately correlated with those of Ba, Cu, Mo, Sb, and V (Table 3.4).

Table 3.4: Spearman correlation coefficients between the concentrations of Sc, the element chosen as soil reference, and the remaining elements analysed in lichen transplants exposed at the impacted and at the reference locations.

Impacted location						Reference location					
	n	*Sc*		n	*Sc*		n	*Sc*		n	*Sc*
Al	95	0.637***	*Mo*	95	0.295**	*Al*	23	0.205	*Mo*	23	0.422*
B	95	0.364***	*N*[a]	16	0.312	*B*	23	0.254	*N*[a]	4	0.362
Ba	95	0.501***	*Na*	95	0.353***	*Ba*	23	0.586**	*Na*	23	0.258
Ca	95	0.181	*Ni*	95	0.575***	*Ca*	23	-0.026	*Ni*	23	0.361
Cd	95	0.054	*P*	95	0.141	*Cd*	23	0.064	*P*	23	0.331
Co	95	0.541***	*Pb*	95	0.385***	*Co*	23	0.398	*Pb*	23	0.356
Cr	95	0.461***	*S*	95	0.176	*Cr*	23	0.344	*S*	23	0.306
Cu	95	0.399***	*Sb*	95	0.433***	*Cu*	23	0.482*	*Sb*	23	0.575**
Fe	95	0.602***	*Si*	95	0.546***	*Fe*	23	0.291	*Si*	23	-0.169
Hg	89	0.179	*Sr*	95	0.417***	*Hg*	23	0.281	*Sr*	23	0.117
K	95	0.037	*Ti*	95	0.714***	*K*	23	0.402	*Ti*	23	0.110
Li	95	0.685***	*V*	95	0.544***	*Li*	23	0.062	*V*	23	0.527*
Mg	95	0.301**	*Zn*	95	0.201**	*Mg*	23	0.402	*Zn*	23	0.010
Mn	95	0.400***				*Mn*	23	0.137			

Notes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [a]For N, the concentrations of N in composite samples were correlated with average Sc concentrations of samples in the same exposure conditions.

Element concentration in soil samples

The results of elemental soil analysis in composite samples retrieved from both the impacted and the reference location are given in table 3.5. As with lichen samples, Be, Bi, Se and Sn were bellow the detection limits in soil samples. In addition, in soil samples B, Cd, N, S, and Sb were also bellow detection limits (respectively, 400 μg Kg^{-1}, 20 μg Kg^{-1}, 0.02 %, 100 mg Kg^{-1}, and 20 μg Kg^{-1}) in all samples analysed. Mo was bellow detection limits (10 μg Kg^{-1}) in 3 of the 5 soil samples analysed. Hg in soil samples was found in negligible amounts.

Ca, Co, Mn, Na, P, and Sr were found in higher concentrations at all the distances studied at the impacted than at the reference location, the more pronounced differences being those of Ca and Sr. P concentrations decreased with distance of collection at the impacted location. As, Cr, Fe, Mg, Ti and V concentrations were higher in the soils collected at this location than at the reference location in most of the distances studied. Here, Cu concentration was higher than in the soils collected at the impacted location.

Table 3.5: Concentrations of 33 elements in soil composite samples from the impacted and reference locations.

	Impacted location				Reference location
	500 m	**1000 m**	**1500 m**	**2000 m**	
Al (mg Kg^{-1})	1643.3	1067.9	1357.1	1420.2	1485.5
As (μg Kg^{-1})	603.5	526.9	593.2	659.9	542.8
B (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Ba (μg Kg^{-1})	2963.3	2494.1	3020.7	3273.9	3040.9
Be (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Bi (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Ca (mg Kg^{-1})	1859.8	4155.5	897.1	6272.9	22.4
Cd (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Co (μg Kg^{-1})	177.4	188.2	168.0	168.3	146.8
Cr (μg Kg^{-1})	1080.4	544.4	1183.2	677.8	636.4
Cu (μg Kg^{-1})	979.2	845.0	980.4	941.6	1015.8
Fe (mg Kg^{-1})	867.5	626.8	788.8	845.0	749.6
Hg (mg Kg^{-1})	0.0	0.0	0.0	0.0	0.0
K (mg Kg^{-1})	109.2	86.5	99.7	107.5	101.6
Li (μg Kg^{-1})	1350.7	967.1	1229.6	1152.7	1226.8
Mg (mg Kg^{-1})	94.9	61.5	104.5	89.7	71.6
Mn (mg Kg^{-1})	17.8	6.2	12.3	9.0	5.4
Mo (μg Kg^{-1})	18.4	n.d.	27.8	n.d.	n.d.
N (%)	n.d.	n.d.	n.d.	n.d.	n.d.
Na (mg Kg^{-1})	40.2	70.7	25.3	105.1	13.1
Ni (μg Kg^{-1})	640.1	370.9	916.7	438.1	441.2
P (mg Kg^{-1})	109.0	99.1	95.7	94.2	89.0
Pb (μg Kg^{-1})	1755.1	820.9	1563.6	918.0	1597.8
S (mg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Sb (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Sc (μg Kg^{-1})	68.0	77.9	102.8	100.9	87.6
Se (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Si (mg Kg^{-1})	97.8	94.8	111.7	121.0	114.8
Sn (μg Kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.
Sr (μg Kg^{-1})	8486.2	23117.7	5197.8	35404.2	971.9
Ti (μg Kg^{-1})	20475.0	12065.0	15548.4	17260.8	13212.3
V (μg Kg^{-1})	1138.5	713.1	1151.0	897.6	889.8
Zn (μg Kg^{-1})	3144.0	1640.0	3161.4	1625.4	2563.7

Note: n.d., not detected.

Vitality of lichen transplants: chlorophyll a *fluorescence kinetics*

None of the studied parameters — F_v/F_m, F_0, F_m, q_P, NPQ, Φ_{PSII}, and Φ_{Exc} — revealed significant differences in the transplants prior to exposure (data not shown) and in those exposed at the reference location (Fig. 3.7). Mean values (± SD) of the chlorophyll *a* fluorescence parameters evaluated were similar in the pre-exposed transplants and those exposed at the reference location: F_v/F_m — pre-exposed, 0.689 ± 0.052; reference, 0.690 ± 0.033; F_0 — pre-exposed, 0.249 ± 0.030; reference, 0.287 ± 0.019; F_m — pre-exposed, 0.979 ± 0.150; reference, 0.935 ± 0.093; NPQ — pre-exposed, 0.710 ± 0.286; reference,

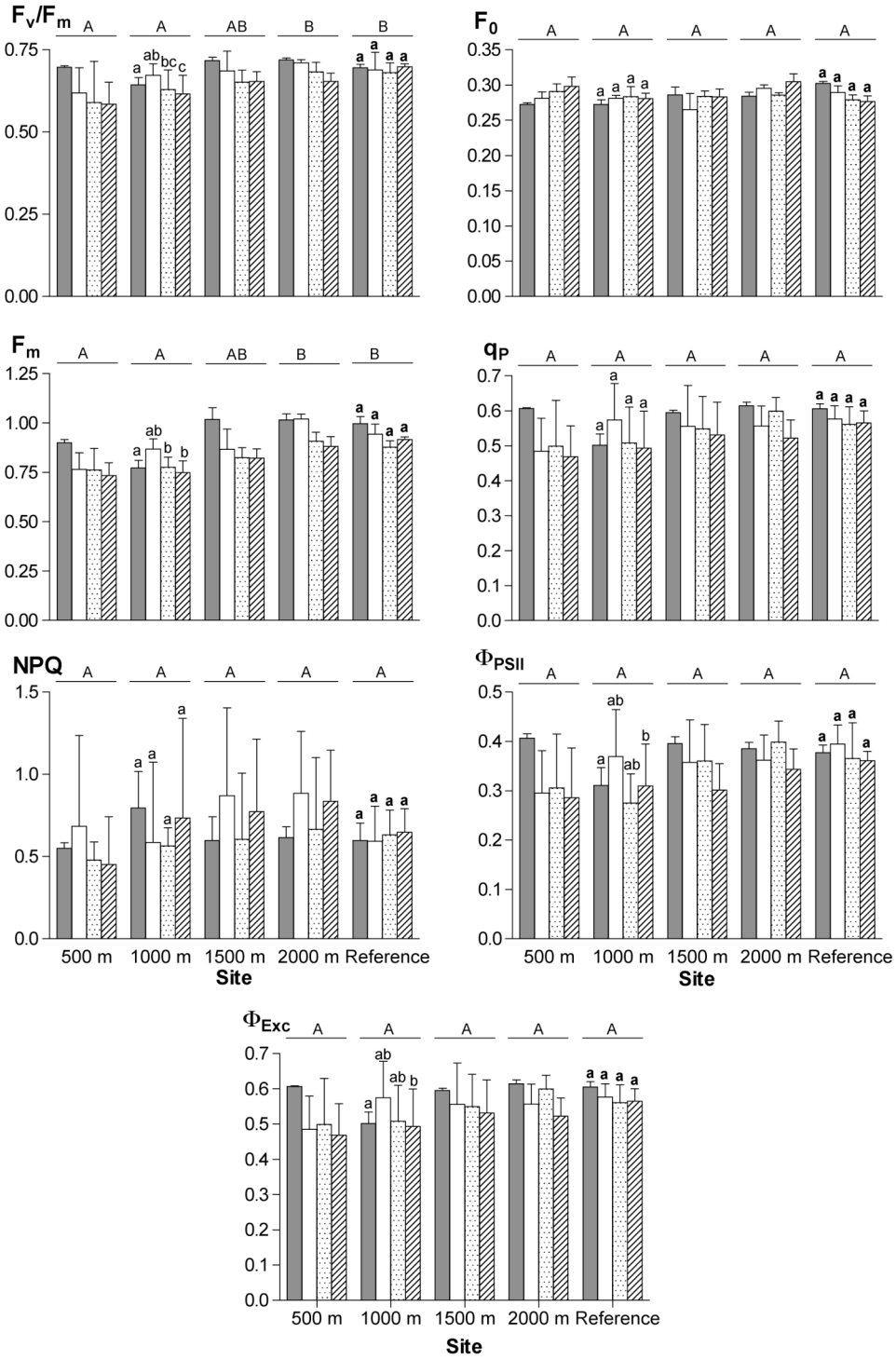

Figure 3.7: Chlorophyll *a* kinetics fluorescence parameters, F_0, F_m, F_v/F_m, q_P, NPQ, Φ_{PSII}, Φ_{Exc} measured in transplants exposed at the impacted location, at 500, 1000, 1500 and 2000 m from the point source; and at the reference location; both at increasing time intervals of exposure, 45 ■, 90 □, 135 ⬚, and 180 ▨ days. Data are mean values ± 1SE (n = 6). For further details refer to Fig. 3.3.

0.609 ± 0.176; q_P — pre-exposed, 0.646 ± 0.041; reference 0.649 ± 0.057; Φ_{PSII} — pre-exposed, 0.371 ± 0.036; reference 0.375 ± 0.046; and Φ_{Exc} — pre-exposed, 0.573 ± 0.043; reference 0.578 ± 0.042.

In the transplants exposed at the impacted and reference locations, results indicate a significant effect of both site and period of exposure on F_v/F_m, but an interaction between both factors was not detected (Table 3.6). F_v/F_m was significantly lower after 180 and 135 regarding 45 days of exposure, and after 180 versus 90 days of exposure. In the transplants placed at the impacted location, F_v/F_m was also significantly higher in the transplants placed at 2000 m regarding those placed at 500 and 1000 m (Fig. 3.7). Furthermore, F_v/F_m was significantly lower in the transplants exposed at 500 and 1000 m at the impacted location, regarding those exposed at the reference location (Fig. 3.7).

F_0 did not vary significantly with either site or exposure period (Table 3.6). On the other hand, F_m varied significantly with both factors, but an interaction between them was not observed (Table 3.6). F_m was significantly higher in the transplants exposed during 45 days, regarding those exposed for 135 and 180 days (Fig. 3.7). Moreover, F_m was also significantly higher in the transplants exposed at 2000 m versus the ones exposed at 500 and 1000 m at the impacted location (Fig. 3.7). F_m was also significantly inferior in the transplants exposed at these two last sites at the impacted location regarding the transplants exposed at the reference location (Fig. 3.7).

Table 3.6: Scheirer-Ray-Hare tests for the factors exposure period and site, and their interaction on the chlorophyll *a* fluorescence kinetics variables in transplants exposed at the impacted and reference locations ($df_{Exposure\ period}$=3, $df_{Exposure\ site}$=4, $df_{Interaction}$=12, df_{Total}=112).

	H	**P**		***H***	**P**
F_v/F_m			*NPQ*		
Period	19.88	**0.000**	Period	1.25	0.741
Site	20.78	**0.000**	Site	6.11	0.191
Interaction	13.17	0.357	Interaction	7.47	0.825
F_0			Φ_{PSII}		
Period	0.80	0.849	Period	9.01	**0.029**
Site	5.86	0.210	Site	7.54	0.110
Interaction	15.16	0.233	Interaction	13.60	0.327
F_m			Φ_{Exc}		
Period	13.13	**0.004**	Period	11.95	**0.011**
Site	23.11	**0.000**	Site	7.10	0.131
Interaction	7.78	0.802	Interaction	14.07	0.296

Note: significant results in bold

q_P (GLM: site, $F_{4,93} = 1.53$, P = 0.201; direction, $F_{3,93} = 0.74$, P = 0.531; interaction, $F_{12,93} = 1.30$, P = 0.222) and NPQ did not vary significantly with either exposure site or period (Table 3.6). Nonetheless, average NPQ increased with increasing distance of exposure at the impacted location (Fig. 3.7).

Exposure period was the only significant factor for Φ_{PSII} and Φ_{Exc} (Table 3.6). Both parameters were significantly higher in the transplants exposed for 45 days versus those exposed during 185 days (Fig. 3.7).

The relationship between the accumulation of each element in lichen transplants at the impacted location and the chlorophyll *a* fluorescence parameters studied was investigated by means of correlation tests. Significant negative correlations were identified between: F_0 and the concentrations in lichen transplants of K and Zn; F_m and Ba, Co, Hg, Mn, Mo, N, P, S, Sb, and Zn; F_v/F_m B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb and Zn; NPQ and Cu and Fe; Φ_{PSII} and N and P; and Φ_{Exc} and Mn, N, P and S (Table 3.7). At the reference location significant correlations between chlorophyll *a* fluorescence parameters and element concentrations in lichen transplants were not found (data not shown).

Significant positive correlations were identified between chlorophyll *a* fluorescence parameters: F_0 and F_m; F_m and F_v/F_m, q_P, Φ_{PSII} and Φ_{Exc}; F_v/F_m and q_P, Φ_{PSII} and Φ_{Exc}; q_P and Φ_{PSII} and Φ_{Exc}; Φ_{PSII} and Φ_{Exc}. Significant negative correlations were also identified between NPQ and Φ_{PSII} and Φ_{Exc} (Table 3.7).

Lichen diversity and bark pH

Thirty-two species were found at the impacted location (Table 3.8). The nitrophytic species *Physcia adscendens* and *Xanthoria parietina* were found only at 500 m from the pulp mill. Lichen diversity values (LDVs) were quite lower at 500 m from the pulp mill than at the other studied sites. *Chrysothrix candelaris*, *Lecanora sorediomarginata*, *L. strobilina*, *Parmotrema perlatum*, *Pertusaria* cf. *heterochroa* and *Pyrrhospora quernea*, were present in all study sites at the impacted location, but were less frequent at the 500 m site. *Usnea* species were absent at this site, with *U. cornuta* being also absent from the 1000 m site, and *U. rubicunda* and *U. subscabrosa* being more frequent at the 2000 m site.

Table 3.7: Spearman correlation coefficients between the values of chlorophyll *a* fluorescence kinetics parameters F_0, F_m, F_v/F_m, q_P, NPQ, Φ_{PSII}, and Φ_{Exc} and the concentrations of 28 elements analysed in lichen transplants exposed at the impacted location (n = 88, except Hg, n = 83 and N, n = 16[a]).

	F_0	F_m	F_v/F_m	q_P	NPQ	Φ_{PSII}	Φ_{Exc}
F_m	0.510***						
F_v/F_m	0.139	0.879***					
q_P	0.104	0.332***	0.359***				
NPQ	0.162	0.000	–0.042	–0.039			
Φ_{PSII}	0.089	0.568***	0.633***	0.836***	–0.032**		
Φ_{Exc}	–0.004	0.625***	0.738***	0.394***	–0.535***	0.786***	
Al	–0.057	–0.112	–0.125	–0.032	–0.141	–0.050	–0.046
B	0.092	–0.200	–0.329**	–0.102	–0.006	–0.195	–0.179
Ba	–0.006	–0.289*	–0.377***	–0.060	–0.153	–0.113	–0.173
Ca	0.069	–0.014	–0.056	0.055	–0.034	0.041	–0.024
Cd	–0.063	–0.009	0.043	0.010	0.067	0.009	0.001
Co	–0.107	–0.214*	–0.254*	–0.023	–0.209	–0.051	–0.063
Cr	–0.111	–0.017	0.001	0.037	–0.026	0.053	0.016
Cu	–0.168	–0.194	–0.200	–0.112	–0.232*	–0.108	–0.078
Fe	–0.040	–0.162	–0.237*	–0.022	–0.215*	–0.029	–0.044
Hg	–0.021	–0.261*	–0.365***	–0.082	–0.147	–0.148	–0.186
K	–0.265*	–0.138	–0.001	–0.022	0.031	–0.071	–0.007
Li	–0.035	–0.114	–0.145	–0.072	–0.111	–0.070	–0.038
Mg	–0.014	–0.189	–0.217*	0.079	–0.057	–0.007	–0.686
Mn	–0.072	–0.322**	–0.405***	–0.087	–0.094	–0.171	–0.224*
Mo	–0.013	–0.245*	–0.302**	–0.091	–0.092	–0.132	–0.168
N	0.184	–0.649**	–0.755**	–0.387	–0.424	–0.531*	–0.690**
Na	0.107	0.049	–0.013	0.139	0.082	0.087	0.021
Ni	–0.005	–0.143	–0.202	0.075	–0.161	0.017	–0.045
P	–0.090	–0.330**	–0.334**	–0.150	0.018	–0.290**	–0.276**
Pb	–0.010	–0.005	–0.004	0.094	–0.154	0.145	0.107
S	–0.013	–0.329**	–0.376***	–0.070	–0.040	–0.172	–0.219*
Sb	–0.054	–0.223*	–0.228**	–0.034	–0.118	–0.060	–0.129
Sc	0.002	–0.029	–0.072	0.015	–0.009	–0.011	–0.077
Si	0.010	–0.048	–0.087	–0.064	–0.034	–0.093	–0.092
Sr	0.027	–0.114	–0.180	0.121	–0.098	0.061	–0.058
Ti	–0.025	–0.149	–0.177	–0.046	–0.126	–0.082	–0.090
V	–0.018	–0.042	–0.080	0.080	–0.143	0.092	0.078
Zn	–0.222*	–0.241*	–0.212*	–0.125	–0.154	–0.149	–0.100

Notes: *P < 0.05, **P < 0.01, ***P < 0.001, [a]For N, the concentrations of N in composite samples were correlated with average chlorophyll *a* fluorescence parameters of samples in the same exposure conditions.

At the reference location fourty-two lichen species were found, and the average LDV was 67.3 (results also given in Annex I, sites A–G).

Bark pH was not correlated with the mass of the disks (data not shown) (Spearman rank correlation, r_s = -0.054, P=0.414). At the reference location, bark pH did not vary significantly with site and direction of collection (GLM: site, $F_{4,60}$ = 2.18, P = 0.132; direction, $F_{3,60}$ = 2.62, P = 0.099; interaction, $F_{12,60}$ = 1.34, P = 0.220). At the impacted location, it varied significantly with both site and direction of collection (Scheirer-Ray-Hare test: site, df = 3, H = 99.24, P=0.000; direction, df = 3, H = 11.49, P=0.009; interaction, df = 9, H = 10.17, P = 0.337). Bark pH was significantly higher at 500 m from

Table 3.8: Mean frequency values of each species found at the impacted and reference locations, in each direction and site were the diversity studies were undertaken. MSFs (mean sum of frequencies) for each cardinal point and LDVs (lichen diversity values) for each site are also provided.

	Impacted location																**Reference location***			
	500 m				**1000 m**				**1500 m**				**2000 m**							
	N	S	E	O	N	S	E	O	N	S	E	O	N	S	E	O	N	S	E	O
Buellia cf. *disciformis*	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0
B. schaereri	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Calicium abietinum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0.1	0.2
Chrysothrix candelaris	1.0	0.2	0.3	0.5	3.2	3.1	2.0	2.0	2.8	2.7	2.9	1.3	3.8	4.3	4.3	3.6	3.5	3.9	3.8	3.5
C. flavovirens	0.3	0	0	0	0	0	0	0	1.3	0.1	1.1	0.2	0.1	0	0	0	0.6	0.6	0.6	0.6
Evernia prunastri	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0.4	0.3	0.3	0.3
Flavoparmelia caperata	0.8	0.1	0.1	0.2	1.8	1.1	1.0	2.2	0.4	0.1	0.4	0.1	1.1	1.3	1.5	0.8	1.7	1.1	1.3	1.4
Hypogymnia physodes	0	0	0	0	1.0	0.1	0.3	0.5	0.5	0.1	0.3	0.1	1.7	0.3	1.1	0.5	3.0	1.0	2.1	1.8
Hypotrachyna lividescens	0	0	0	0	0	0.1	0	0.1	0	0	0	0	0.1	0	0.2	0	0.1	0	0	0
H. pseudosinuosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
H. revoluta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
Lecanora cf. *confusa*	0	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. expallens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0.1	0
L. sorediomarginata	0.1	0	0	0.1	0.4	0	0.3	0.3	0.4	0.6	0.3	0.1	0.7	0.3	0.4	0.1	0	0	0	0
L. strobilina	0	0.3	0.4	0.8	1.1	2.3	1.9	2.3	1.5	2.7	2.2	2.2	0.6	1.4	1.5	2.6	0.2	0.6	0.5	0.6
Lecidea nylanderi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2
cf. *Lecidella scraba*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
Lepraria membranacea	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0
L. nylanderiana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0
Ochrolechia microstictoides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0
Parmelia sulcata	0.1	0	0	0	0.4	0	0	0.1	0.1	0	0	0	0.1	0	0.1	0	0.1	0	0.1	0
Parmotrema hypoleucinum	0.4	0.2	0.1	0.3	1.5	0.3	0.4	0.8	0.4	0.1	0.3	0	0.3	0	0.1	0	0.1	0.2	0.1	0.2
P. perlatum	0.5	0.1	0	0.3	1.8	0.1	0.1	1.1	0.8	0.1	0.3	0.4	1.3	0.3	0.3	0.5	0.6	0.1	0.3	0.2
P. reticulatum	0.3	0	0	0	0.4	0	0	0.4	0	0	0	0	0.3	0	0.2	0	1.2	0.3	0.7	0.5
P. robustum	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pertusaria amara	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1
P. cf. *heterochroa*	0.1	0	0	0	0.3	0	0	0.1	0.4	0	0	0.3	0.1	0	0.1	0.1	0	0	0	0
Physcia adscendens	0.2	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Punctelia subrudecta	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pyrrhospora quernea	0.8	1.1	0.4	2.3	4.8	4.2	4.2	4.8	2.4	3.3	3.3	2.8	3.1	4.6	4.5	4.7	3.3	4.7	3.9	4.5
Ramalina farinacea	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0

Table 3.8: *Continued*.

	Impacted location																Reference location*			
	500 m				1000 m				1500 m				2000 m							
	N	S	E	O	N	S	E	O	N	S	E	O	N	S	E	O	N	S	E	O
Tuckermanopsis chlorophylla	0	0	0	0	0	0	0	0	0.1	0	0	0	0.1	0	0	0	0	0	0	0
Schismatomma niveum	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0
Usnea cornuta	0	0	0	0	0	0	0	0	0.1	0	0	0	0.1	0	0	0	0.7	0	0.2	0.2
U. mutabilis	0	0	0	0	0.1	0	0	0.1	0.1	0	0.1	0	0	0	0.1	0	0.1	0	0.1	0.1
U. rubicunda	0	0	0	0	0	0	0	0	0.1	0	0	0	0.6	0.1	0.8	0.2	2.4	0.8	1.4	1.6
U. subscabrosa	0	0	0	0	0.3	0	0	0	0.3	0.1	0.5	0	0.6	0.7	0.7	0.4	1.0	0.2	0.4	0.6
Xanthoria parietina	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sp. 1	0.2	0	0	0	0	0	0	0	1.8	0	0.2	0.2	1.0	0.1	0	0.2	0	0	0	0
MSF	4.7	1.8	1.4	4.5	18.2	11.5	10.7	15.2	14.3	9.7	11.8	7.5	15.6	13.3	15.8	13.5	19.6	14.3	16.5	16.9
LDV	12.4				55.6				43.3				58.2				67.3			

Note: *Mean frequency values presented for the reference location represent the average of mean frequency values of seven studied sites at this location (for further details see Annex I, sites A–G).

the point source, regarding all other studied distances, and also in the samples collected at 1000 and 2000 m versus the ones colleted at 1500 m (Fig. 3.8). Furthermore, bark pH was significantly higher in the samples collected in the north direction concerning the east and south directions (Fig. 3.8). Also, bark pH was significantly higher in all sites studied regarding the reference location (Kruskal-Wallis test: df = 4, H = 186.40, P = 0.000) (Fig. 3.8).

The increase in pH values was weakly correlated with a decrease in lichen abundance at the impacted location (Spearman rank correlation, r_s = –0.227, P = 0.003), and very weakly correlated (r_s < 0.19, according to Fowler et al. (1998)) with a decrease in number of species (Spearman rank correlation, r_s = –0.158, P = 0.040).

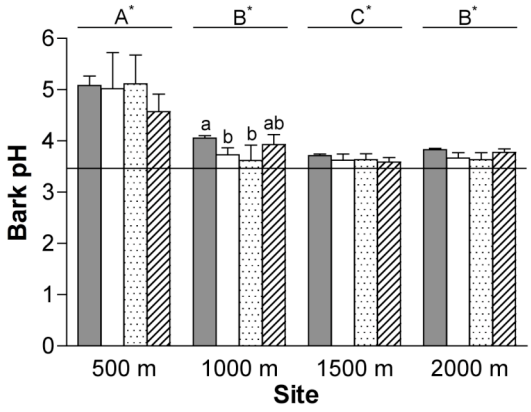

Figure 3.8: Bark pH of *P. pinaster* at the impacted location, measured at 500, 1000, 1500 and 2000 m from the point source (n=12), in the cardinal directions, North (N), South (S), East (E) and West (W). Legend: ■ N, □ S, ⬚ E, ▨ W. Data are mean values ± 1SE. The horizontal line indicates the average bark pH at the reference location. *represents significant differences between bark pH at each site of the impacted location versus bark pH the reference location. For details on notations above bars refer to Fig. 3.3.

Discussion

Element accumulation in lichen transplants and concentration in soil samples

The significantly higher accumulation of most elements — Al, B, Ba, Ca, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, P, S, Sb, Sc, Sr, Ti, and V — in the transplants exposed at 500 m from the point source at the impacted location regarding all or some of the other exposure distances tested, indicates that the point source is the likely source of

those elements. The higher accumulation of N in the transplants exposed at 500 m from the pulp mill also points it as the source of this element.

The accumulation of most metals — Al, B, Ba, Co, Cr, Cu, Fe, Li, Mg, Mo, N, Na, Ni, P, S, Sb, Sc, Si, and Ti — followed the same spatial pattern, being deposited in higher concentrations at the 500 m site and decreasing after that, but increasing again at 2000 m. This indicates not only that distance was a factor that influenced elemental accumulation, but also highlights the particularity of the exposure sites: the 1500 m site has a lower elevation regarding the other sites, in a depression beside the forest road, what may have limited the circulation of air masses reaching the transplants. On the contrary, the 2000 m site was at a higher elevation, and only protected by an area with small trees that may have favoured the contact of transplants with atmospheric masses. This could be the reason why for some elements — B, Ca, Cd, Cr, Hg, Li, Mg, Na, V, and Zn — no significant differences were found in the accumulations observed at 500 and 2000 m, despite their accumulation varied significantly with distance of exposure at the impacted location. Nonetheless, it is also possible that part of the elemental accumulation observed at the impacted location was derived from the deposition of atmospheric emissions from another pulp and paper mill (Portucel-Soporcel) located at approximately 2 Km to East-Northeast from Celbi (in the proximity of which the transplants were placed). Portucel-Soporcel indicated the emission of Cd, N (as N_2O, NH_3, and NO_x/NO_2), Ni, S (as SO_x/SO_2), and Zn, among other pollutants to the atmosphere in 2008 (E-PRTR 2012).

Soil particles also appeared to contribute to the elemental content of lichen transplants. It seems that Ti was accumulated mostly from soil particles, as the content in this element was highly correlated to that of Sc, the element used to evaluate the accumulation derived from soil particles. As the contents in Al, Ba, Co, Cr, Cu, Fe, Li, Mn, Ni, Sb, Si, Sr and V were moderately correlated with that of Sc, and those of B, Mg, Mo, Na, Pb, and Zn were weakly correlated, soil particles may have been at least a partial source of these elements. The accumulation of Ca, Cd, Hg, K, N, P and S in the transplants placed at the impacted location was not correlated with that of Sc, and therefore these elements were not apparently accumulated from soil particles.

It is possible that soil at the impacted location was also a receiver of the elements emitted by the pulp mill, as soil has been shown to be a recipient of atmospheric pollutants (e.g. Bermudez et al. 2010, Hernandez et al. 2003, Saur & Juste 1994, Steinnes &

Friedland 2006). If that was the case, it is possible that in these correlations we are considering elements with an origin in soil, which may actually have an industrial origin. These doubts would be cleared with the results of the soil analysis both at the impacted and reference location, but the samples were collected not long after rainfall events, and the elements may have been leached from topsoil. Nonetheless, the results showed similar elemental contents at the impacted and reference locations, except for Ca, Co, Mn, Na, P, and Sr, which had higher concentrations in soil samples collected at all sites at the impacted location. These could have their source on the atmospheric emissions of the pulp mill, but also in the deposition of sea-spray, mostly for Ca and Sr (Klee & Graedel 2004), which could be accumulated in soil in higher amounts at the impacted location, as this was closer to the sea (approx. 2 Km) than the site at the reference location (approx. 8 Km) were transplants were placed. Additionally, these could have originated in windblown particles from a landfill that receives non-dangerous industrial residues (Celbi, 2008). It is known that Kraft pulp mills residues may contain several elements, among which Ca, Cr, Mn, Na, P and Sr, but also Al, As, Ba, Be, Cd, Cr, Cu, Fe, Hg, K, Mg, Mo, N, Ni, Pb, S, Si, Ti, V, and Zn (Mahmoudkhani et al. 2004; Nurmesniemi et al. 2005, 2008; Pöykiö et al. 2006). These could contaminate the surrounding soils, but also be accumulated by lichen transplants.

Exposure period was a significant factor for the accumulation of most elements, with the concentrations observed at 180 days of exposure being significantly higher than the concentrations observed in all or some of the other exposure periods for B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V. As with accumulation regarding site of transplantation, a pattern was also identified in the temporal accumulation of several elements — Al, Ba, Co, Cr, Cu, Fe, Li, Mg, Mn, Na, Pb, Sb, Ti, V, and Zn: the accumulation of these elements decreased after 45 days of exposure, increasing after that. This decrease may have been due to the higher average daily precipitation observed in the period of exposure of 46–90 days.

Regarding the transplants performed at the reference location, these allowed the validation of the results obtained with the transplantation experiment at the impacted location. Significant differences were found in the concentrations of most elements at all or some sites at the impacted location regarding those exposed at the reference location. The exceptions were K, P, and Zn. B and Na were the only elements for which concentrations

varied significantly with exposure period at the reference location. This leads to the postulation that all or most of the B accumulated was not originated in atmospheric emissions from the pulp mill, although it is pointed by NCASI (2005) as one of the elements emitted in higher concentrations from Canadian and U.S. pulp and paper mills. In the particular case of this pulp mill, if B is emitted, it is either emitted in low concentrations, or it is deposited further away from the sites studied at the impacted location. The differences observed in B accumulation at the impacted location with distance and period of exposure, could be due to: i) small emissions from the pulp mill that would be more visible at 500 m and 2000 m; ii) sea-spray, as the study sites at the impacted location were closer to the sea than the reference site, and B, and also Na, are elements with a marked maritime influence (Klee & Graedel 2004, Rose et al. 2000, Werner et al. 2011) iii) the accumulation of soil particles, as the content in B in lichen transplants at the impacted location was weakly but significantly correlated with that of Sc. However, the content in B in soils of both the impacted and reference locations were below detection limits.

Medium/large range transport from industrial sources could also be hypothesised as sources of B and Na at the reference location. Besides the two pulp mills previously mentioned, the nearest industries are mineral industries to the south, the nearest of which is a ceramics industry approximately 5 Km to the southeast with reported emissions of Cr, Ni and particulate matter to the atmosphere in 2008 (E-PRTR 2012). To the north, the nearest industries are a milk-processing factory (approximately 14 Km to the northeast) that reported the emission of Ni, and pig farm (approximately 16 Km to the northeast) reporting the emission of ammonia to the atmosphere in 2008 (E-PRTR 2012). The apparent absence of industries emitting B to the atmosphere, leads to the hypothesis of its origin being in sea-spray.

There was a clear difference in the accumulation pattern of both elements, with B increasing constantly with time of exposure, also at the impacted location, and Na decreasing after 45 days of exposure and increasing after that in a similar way to other elements. Na may have been leached from the lichen transplants after 45 days of exposure due to the increase in average daily precipitation observed in the 46–90 days exposure period, as previously mentioned. This was not the case of B, what may be related to the way in which these elements are accumulated in lichen thalli. Figueira et al. (1999)

reported that Na is mostly accumulated in the surface of the cellular fraction of *Ramalina canariensis,* with the intracellular concentration remaining constant, and pointed to the possibility of leaching occurring with rain events, for this and other elements occurring at the cell surface.

The form in which B is accumulated by lichen thalli is not known. Despite that, Lavola et al. (2011) indicate that B can be readily complexed with some common carbohydrates, especially mannitol, one of the carbohydrates used by lichen mycobiont cells for C storage (Palmqvist et al. 2008). This could imply that B in lichen cells is in the intracellular fraction, a hypothesis favoured by the finding that boric acid has a high membrane permeability, being taken up passively with water, unless the external concentration is very low (Dannel et al. 2002; Fitzpatrick & Reid 2009). Boric acid is the major form in which B is present in the atmosphere, deriving from sea-salt aerosols (Park & Schlesinger 2002). Nonetheless, it may be possible that B also forms complexes with secondary metabolites produced by *F. caperata*, as depsides (atranorin), depsidones (protocetraric acid), aliphatic acids (caperatic acid), which posses hydroxyl groups to which B may bond (Power & Woods 1997). As B is transferred from the ocean to the atmosphere mostly in the gaseous form (Rose et al. 2000), and has a longer residence time than particulate B (Kot 2009, Rose et al. 2000) and Na, which is transferred to the atmosphere in the aerosol form (Athanasopoulou et al. 2008, Werner et al. 2011), its accumulation by lichens may be increased relative to that of Na.

In the same locations of this study, the impacted and reference locations, Costa et al. (2011) and Norte et al. (2010) also conducted studies on the accumulation of metals in feathers of great tits. Both studies report higher concentrations of Hg in feathers of great tits at the impacted location. Costa et al. (2011) also tested for differences on the accumulation of Cd, Cu, Ni Pb, and Zn in birds from the impacted and reference locations, but the authors did not find significant results. In the present study, of those elements only Zn was not accumulated in significantly higher concentrations at the impacted location. As in the present study, Norte et al. (2010) found very low concentrations of Hg in soil samples from both the impacted and reference location.

Chlorophyll a *fluorescence kinetics in transplanted lichens*

All the parameters analysed varied with exposure distance and period at the impacted location, although only significantly for F_v/F_m, F_m, Φ_{PSII} and Φ_{Exc}.

F_v/F_m is a relative measure of the maximum efficiency of PSII, that is the quantum efficiency if all PSII centres are open (Baker 2008, Maxwell & Johnson 2000). It is considered a rapid and simple way for monitoring stress, as it is known to decrease in the presence of stress (Baker 2008, Maxwell and Johnson 2000), being particularly sensitive to photoinhibition (Maxwell & Johnson 2000, Roger & Weiss 2001). Although decreases in F_v/F_m indicate exposure to stress, this does not necessarily mean that the efficiency of photosynthetic performance under ambient light has been compromised, as this parameter is measured in dark-adapted samples (Baker 2008).

The significant decrease of F_v/F_m in the transplants exposed at 500 and 1000 m from the pulp mill, indicates that some factor(s) associated with it induced stress in lichen transplants. Decreases in F_v/F_m were correlated with increasing concentrations of B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb, and Zn in lichen thalli at the impacted location. All these elements, but N and Zn, were found in significantly higher concentrations at 500 m from the pulp mill, versus some or all of the remaining exposure distances, favouring the hypothesis that atmospheric emissions from the pulp mill were responsible for the stress caused to lichens. Despite that, a contribution of soil particles to this stress may have occurred, as the concentrations of those elements — except Hg, N, P, and S — were moderately correlated with the concentrations of Sc (element chosen for soil reference). But, as mentioned before, the soil content of most of those elements, may have been altered by the deposition of atmospheric emissions, and also by contamination derived from a landfill located in the pulp mill complex. A significant decrease in F_v/F_m with exposure period was also observed, after 135 and 180 days of exposure. This may have been due to the higher accumulation of the previously mentioned elements — except Co, N, and Zn — observed after 180 days of exposure. It is known that the donor side of PSII is inhibited by metal ions, but metal binging sites in light harvesting complexes (LCHs) are also pointed to exist (Boucher & Carpentier 1999), and this frequently leads to a decrease in F_m during fluorescence induction experiments due to the lack of electrons available to provide for the accumulation of photoreduced Q_A (Boucher & Carpentier 1999, Govindjee

1995). This was possibly the case in this study, as F_m was significantly inferior in the transplants exposed at 500 and 1000 m at the impacted location, and those exposed during 135 and 180 days. In addition, significant negative correlations were found between F_m and the content in Ba, Co, Hg, Mn, Mo, N, P, S, Sb, and Zn in the transplants placed at this location.

Decreases in F_v/F_m with Ba, Fe, Hg, Mn, Zn and S accumulation were also observed in other studies (Garty et al. 2000, 2003, 2004). Exposure to N (as NH_3) and S (as SO_2) are also known to lead to decreases in this parameter (Deltoro et al. 1999, Odasz-Albrigtsen et al. 2000, Paoli et al. 2010).

It is known that climatic conditions may proportionate decreases in a species F_v/F_m (Baruffo & Tretiach 2007, Fernández-Salegui et al. 2006, Piccotto et al. 2010), which are thought to be a consequence of a long-term adaptation at physiological and structural levels to changes in macro- and microclimatic conditions, but also photoinhibition resulting from exposure to excess light, but only in the case it leads to photodegradation (Baruffo & Tretiach 2007). Despite that, the changes in weather conditions that occurred at the studied locations apparently did not influence significantly F_v/F_m in the transplants performed at the impacted location, as in the transplants performed at the reference location no significant changes were detected in any of the chlorophyll fluorescence parameters studied. In addition, F_0 did not vary significantly in the transplants placed at the impacted location, and indicates that photoinhibitory damage did not occur (Baruffo & Tretiach 2007).

The significant decreases in F_v/F_m with period and distance of exposure were not related to increases in non-photochemical quenching, despite this is a major photoprotective mechanism (Adams III et al. 2008, Baker 2008, Calatayud 2007, Maxwell & Johnson 2000), as F_v/F_m and NPQ were not significantly correlated in the transplants performed at the impacted location. This may indicate exhaustion of the photoprotective mechanisms (decrease in xanthophylls cycle) or to damage to PSII antennas (Calatayud 2007). In the present study, NPQ did not vary significantly with exposure period or site, although it increased with increasing distance of exposure at the impacted location. Nevertheless, NPQ was significantly and negatively correlated with the contents of Cu and Fe in transplants performed at the impacted location, although not with those of N and S.

Despite that, other studies indicate an effect of SO_2 and NO_x in this parameter (Calatayud et al. 1996, Deltoro et al. 1999, Fernández-Salegui et al. 2006, Tretiach et al. 2007).

PSII operating efficiency, Φ_{PSII}, estimates the efficiency at which the light absorbed by PSII is used for Q_A reduction, the primary electron acceptor of PSII reaction centres (Baker 2008, Maxwell & Johnson 2000). Φ_{PSII} was significantly and negatively correlated with the contents of N and P, indicating a possible adverse effect on the PSII efficiency derived partially from the accumulation of the elements. Furthermore, this parameter was significantly reduced in the transplants exposed during 180 days versus the ones exposed during 45 days, in which the highest average concentrations of N and P were observed, despite the concentrations of N in the transplants exposed for 135 days were also similarly high. Changes in Φ_{PSII} are caused by alterations in NPQ, and/or in the capacity of excited reaction centres to promote electron transport (q_P) (Baker 2008). q_P is related with the fraction of open PSII centres, that are capable of reducing Q_A, but in a non-linear way (Baker 2008). In this study, the decrease in Φ_{PSII} in the transplants exposed for 180 days may have been induced by decreases in q_P and/or by increases in NPQ. Φ_{PSII} was positively correlated with q_P and negatively with NPQ in the transplants placed at the impacted location. The decrease in Φ_{PSII} also may be due to stress induced into other physiological systems, which diminish the rate of consumption of NADPH and ATP produced during photosynthesis (Baker 2008). This could be the case in this study, as that would promote an increase in NPQ as well, and that was observed in the transplants exposed after 135 days (but not significantly). The observed decreases in F_v/F_m and Φ_{PSII}, may be related to oxidative stress, which has recently been found to occur in lichen photobionts of the genus *Trebouxia* (del Hoyo et al. 2011), which is the genus of the photobiont of *F. caperata* (Piccotto & Tretiach 2010).

Φ_{Exc}, the PSII maximum efficiency, also known as the excitation capture efficiency of PSII, represents the quantum yield of open PSII reaction centres, that is the efficiency of excitation energy transfer from LHCII to the PSII reaction centres (Baker 2008, Rosenquist & van Kooten 2003), but also the efficiency with which open PSII centres reduce Q_A (Calatayud et al. 1996). It is nonlinearly correlated to NPQ, with higher Φ_{Exc} values being found with low NPQ values, and linearly correlated with Φ_{PSII}, since $\Phi_{Exc} = \Phi_{PSII}/q_P$ (Rosenquist & van Kooten 2003). As Φ_{PSII}, Φ_{Exc} was also significantly higher in the transplants exposed during 45 days than in those exposed for 180 days. Significant

negative correlations were found between Φ_{Exc} and the contents of Mn, P, and S, indicating that the accumulation of these elements may have led to a lower efficiency of PSII excitation energy capture. Deltoro et al. (1999) found decreases in Φ_{Exc} in lichens fumigated with SO_2, but on the contrary, Calatayud et al. (1996) and Fernández-Salegui et al. (2006) report increases in this parameter with exposure of lichens to this pollutant.

The values observed for chlorophyll *a* fluorescence parameters in transplants before transplantation and those transplanted at the reference location are similar to those reported in the literature for control samples of *F. caperata* (Baruffo et al. 2008, Demmig-Adams et al. 1990, Piccotto & Tretiach 2010, Tretiach et al. 2007), except for Φ_{Exc} which were higher than the ones reported by Tretiach et al. (2007).

Lichen diversity and bark pH

Lichen diversity values (LDVs) were quite inferior at the 500 m site, regarding any of the other studied sites at the impacted and reference locations. That was the only site were the nitrophytic species *P. adscendens* and *X. parietina* were found. These species are favoured by the deposition of ammonia and dust (Nimis & Martellos 2008), and the increased bark pH that results from the deposition of those contaminants (van Herck 2001). This study points to a higher deposition of N in lichen transplants at the 500 m, that could be due to the deposition of ammonia, which was emitted in high amounts by this pulp mill in the year 2008 (E-PRTR 2012). Unlike nitric oxides and their reaction products, ammonia is deposited within a very small distance of the emission source (Wolseley et al. 2006), and could therefore account for the higher N observed in lichen transplants, but also for the significantly higher bark pH observed in the pine trees studied at this site, as well as for the presence of nitrophytic species (Frati et al. 2007, van Herck 1999, 2001; Wolseley et al. 2006). Furthermore, although the abundance of nitrophytic species in pine trunks was low, it was much higher in pine branches between the 500 and 1000 m sites (data not shown), which suggests the atmospheric origin of N and possibly other cations that are known to increase bark pH (Kermit & Gauslaa 2001).

Windblown particles originating in a landfill nearby the 500 m site could also have contributed to increased bark pHs there observed, as it is used to deposit ashes and residues resulting of the production of green liquor, among other wastes of the pulp mill that cannot

be valorised (Celbi 2008). Lime waste for instance, has a high alkaline pH and a high content of CaO (Pöykiö et al. 2006). Other major constituents of solid wastes from Kraft pulp mills are Ca, K, Na, and Mg salts (Mahmoudkhani et al. 2004). The deposition of these particles on nearby tree bark promoted by wind events could also have increased bark pH. The influence of dust deposition on lichens was reviewed by Farmer (1993), who pointed that acid-barked trees near limestone quarries develop a flora typical of calcareous rocks. Soil particles may also have contributed to the increased pH values observed at this site, as Sc (the element chosen for soil reference) was found in significantly higher concentrations in the lichens transplanted to this site. Sea-spray may also have contributed to increased bark pH values (Brodo 1973), but as Na concentrations were not significantly different in the transplants placed at the 500 and 2000 m sites, this was not likely the cause of the increased bark pH values observed at the 500 m site.

The other species found at the 500 m site are generally found in weakly eutrophicated sites — *F. caperata, P. sulcata, P.* cf. *heterochlora,* and *P. quernea* — or in non-eutrophicated or very weakly eutrophicated locations — *C. candelaris, C. flavovirens, L. strobilina, P. hypoleucinum, P. perlatum, P. reticulatum,* and *T. flexuosa,* as well as in very acid to acid substrates (Nimis and Martellos 2008). The recently described *L. sorediomarginata*, which is so far known only from Portuguese coastal pine forests (Rodrigues et al. 2011a), is apparently tolerant to pollution and increased bark pHs, as it was found at this site as well. Despite that, those species were generally found in smaller frequencies at this site than at the other studied sites, except of *T. flexuosa*, pointing to their sensitivity to the environmental conditions at this site. *Usnea* species, which are very sensitive to SO_2 (Hawksworth & Rose 1970) were also not found at the 500 m site and were found in majority at the 2000 m site. Kinnunen et al. (2003) found that the frequency of *Usnea* species was strongly and negatively correlated with the distance of a pulp mill. The decreased occurrence of most species at the 500 m site may also have been due to the increased bark pH in pines at that location, as a significant, negative but weak correlation was found between bark pH and lichen abundance, and a very weak correlation was found with species number. *H. physodes*, which was absent at the 500 m site is known to be very sensitive to ammonia (van Herk 2001).

The LDVs found at the remaining sites are slightly inferior to the average LDV found at the reference location. The lower LDVs observed at the impacted location point to

a lower abundance, but also a smaller diversity of lichens there. There is evidence of the impact of the pulp mostly on the nearby epiphytic vegetation as: i) LDV values are notably lower at the 500 m site; ii) nitrophytic species were detected only at this site on pine trunks; and iii) the abundance of nitrophytic species on pine branches between the 500 and 1000 m sites was visibly higher, than at the 1500 and 2000 m sites, or at the reference location.

Conclusions

This study intended to evaluate the accumulation of several elements putatively emitted by Kraft pulp mills on lichen transplants near a point source located at the border of a pine forested location. It was also aimed to evaluate the effects of the accumulation of the elements studied on lichen vitality and diversity. The pulp mill was identified as the probable main or partial source of most of the elements studied, as they accumulated in higher concentrations in the close vicinity of the pulp mill. However, elemental inputs from another pulp mill located near the impacted area could not be excluded, as well as those from traffic. The analysis of the kinetics of chlorophyll *a* fluorescence revealed that lichens were subject to stress at that site, as indicated by the significant decrease in F_v/F_m in lichens transplanted there. This decrease was correlated with the accumulation of some elements that may have originated from the pulp mill.

The study of lichen diversity study pointed to a local effect on the epiphytic lichen flora, since at 500 m from the pulp mill LDV was clearly lower than at 1000, 1500, 2000 m, and at the reference location. Furthermore, it was only the site where nitrophytic species were found (*P. adscendens* and *X. parietina*) and that was possibly due to the deposition of NH_3 emitted from the pulp mill, that may have lead to an increase in bark pH. However, this might also have been influenced by the deposition of windblown particles from a nearby landfill and soil particles.

The influence of the pulp mill on the epiphytic lichen vegetation is apparently restricted to the close vicinity of the pulp mill. This fact is also mentioned by Halonen et al. (1993). However, in a larger spatial scale there may be effects following the deposition of pollutants further away from the source, as NO_x, and SO_2 (Fernández-Salegui et al. 2006, Wolseley et al. 2006). During the period of this study major winds were mostly from

northeast, west and northwest. Northeast winds would carry atmospheric pollutants to the sea, and those from west to more inland locations. This may imply that the atmospheric emissions from the pulp mill in this particular area would not pose a significant threat to the lichen vegetation of coastal pine forests, as these have a small width, although being extensive along the Portuguese central coast.

Chapter 4

General discussion and concluding remarks

Figure on the previous page: *Pinus pinaster* Aiton in a pine forest on sand dunes.

General discussion

The diversity of epiphytic and terricolous lichens occurring in Portuguese coastal pine forests is not well studied, as is in need of a comprehensive survey. Despite the large number of records of lichens epiphytic on pine in Portuguese coastal areas, some inclusively from pine forests on sand dunes (see chapter 1), surveys aiming at inventorying the lichens occurring in these forests have rarely been conducted and were not thorough (Catarino et. al. 1985). Furthermore, few records of terricolous lichens are available, although pine forests on sand dunes are an excellent habitat for these lichens. The terricolous lichen flora of these forests is largely composed by lichens belonging to *Cladonia* L. subg. *Cladina*, which are included in Annex V of the Habitats Directive (E.C., 1992), attesting the importance of this habitat for lichens.

As a consequence of the lack of knowledge on the diversity of lichens of Portuguese pine forests on sand dunes, this thesis aimed at evaluating the epiphytic lichen diversity in coastal pine forests in Portugal using Dunas de Quiaios (Figueira da Foz), in the centre-littoral, as a specific study area. Additionally, it was intended to evaluate the effect of atmospheric emissions of industrial facilities located near costal pine forests on epiphytic lichens, and for that a case study was conducted at another location (Mata do Urso, Figueira da Foz), which is impacted by a Kraft pulp mill located at its border.

Although studies on epiphytic lichen diversity were undertaken initially at Dunas de Quiaios (see also annexes I and II), some were extended to most of Portuguese coast (Chapter 2). At Dunas de Quiaios a species new to science was initially discovered (Chapter 2.1), as well as three species new to Portugal (Chapter 2.2), and two other species not previously known in the Iberian Peninsula (Chapter 2.3).

The new species, *Lecanora sorediomarginata* Rodrigues, Terrón & Elix was originally found epiphytic both on *Pinus pinaster* Aiton and *P. pinea* L. at Dunas de Quiaios, but was subsequently found in costal pine forests through most of the coast (Chapter 2.1). *Lecanora sorediomarginata* is characterised morphologically by a crustose whitish-grey to greenish thallus developing soralia from small and marginal warts that tend to coalesce, giving the thallus a granular, continuous and cracked appearance in its centre. Fertile specimens were rarely found, with the most striking feature of apothecia being the morphology of the amphithecium. This was completely sorediate from very early stages of

development, and that was the basis of the specific epithet "sorediomarginata" chosen do designate this species.

Of the species occurring in the studied locations *Ochrolechia arborea* (Kreyer) Almb. and *O. microstictoides* Räsänen may be morphologically confused with *L. sorediomarginata*, but those species are chemically distinct.

Lecanora sorediomarginata contains 3,5-dichloro-2'-*O*-methylnorstenosporic acid [major], 3,5-dichloro-2'-*O*-methylanziaic acid [minor], 3,5-dichloro-2'-*O*-methylnordivaricatic acid [minor], 5-chloro-2'-*O*-methylanziaic acid [trace], atranorin [minor], chloroatranorin [minor], and usnic acid [trace]. It is chemically, but not morphologically, similar to *L. lividocinerea* Bagl. and *L. sulphurella* Hepp.

The phylogenetic study preformed based on ITS rDNA sequence analysis showed that *L. sorediomarginata* is more closely related to *L. lividocinerea*, than to other members of the *L. subfusca* group, to which the latter species belongs. The *L. subfusca* group appeared divided in two other groups, besides the one formed by the two first species. It is possible that the *L. subfusca* group is heterogeneous, as has been found for other *Lecanora* groups, including the subgenus *Placodium* and the *L. varia* group (Arup & Grube 1998, Pérez-Ortega et al. 2010). Nonetheless, this phylogenetic study did not resolve the relationships among *Lecanora* groups, what was likely due to the fact that only one gene was analysed. Therefore, more studies are necessary to clarify the phylogenetic relationships between members of the *L. subfusca* group particularly, but also of *Lecanora* (Pérez-Ortega et al. 2010).

Chrysothrix flavovirens Tønsberg, *Lepraria elobata* Tønsberg and *O. arborea* were reported as new records for the Portuguese lichen flora (Chapter 2.2). Although *C. flavovirens* and *O. arborea* were found in most of the western coast, *L. elobata* was only found at Dunas de Quiaios.

Chrysothrix flavovirens is a frequent species in the Portuguese coast, being epiphytic both on *P. pinaster* and *P. pinea*. The distribution of this species in Portugal is similar to that found in other countries, as it is known to occur preferentially at coastal areas and on acidic bark (Laundon 1981 [as sorediate thalli of *C. chrysophthalma* (P. James) P. James & J.R. Laundon], Kowalewska & Jando 2004, Smith et al. 2009, Tønsberg 1992 [as sorediate thalli of *C. chrysophthalma*]).

Ochrolechia arborea was found epiphytic on the same phorophytes *as C. flavovirens*, but showed a less extensive distribution in the coast than this species and *L. sorediomarginata*. However, its presence in more southern regions cannot be ruled out, since not many areas were surveyed and the focus of the surveys undertaken were specimens growing on pine trunks, and not branches, where this species may also be present. Furthermore, the known range of *O. arborea* in Portugal may increase with further studies, as the distribution of this species is not restricted to coastal areas, contrarily to *C. flavovirens*, and it occurs epiphytic on other phorophytes than pine (Boqueras et al. 1999, Christensen & Svane 2007, Kukwa 2009, Tønsberg 1992).

Only one specimen of *L. elobata* was found at Dunas de Quiaios epiphytic on *P. pinaster*, despite the intensive sampling conducted at that location. Most of *Lepraria* existing at Dunas de Quiaios, and presumably also in other coastal pine forests in the centre of the country is *L. nylanderiana* Kümmerl. & Leuckert, a species morphologically similar to *L. ebolata*, since both have a leprose and bluish-grey thallus. *Lepraria elobata* is characterised by a diffuse, leprose, predominantly non-lobed and non-stratified thallus, without medulla and with profuse more or less continuous fine soredia (Saag et al. 2009, Tønsberg 1992). On the contrary, *L. nylanderiana* has a delimited margin where minute lobes may appear in well-developed specimens, as well as a usually whitish medulla and medium to coarse soredia. Morphological distinction of both species may be difficult however, and chemical analysis may be necessary to differentiate both species. Although both species contain atranorin, *L. elobata* additionally contains zeorin and stictic acid with satellites (Tønsberg 1992), while *L. nylanderiana* contains thamnolic and roccellic acids (Smith et al. 2009).

As with *O. arborea*, it is thought that with further studies the known distribution of *L. ebolata* in Portugal may increase, as this species occurs preferentiality on bark but is also known to grow on soil and siliceous rocks, and seems to prefer deciduous to conifer trees, also not being restricted to coastal areas (Kukwa 2006, Saag 2007).

Hypotrachyna lividescens (Kurok.) Hale and *H. pseudosinuosa* (Asahina) Hale were also discovered at Dunas de Quiaios, and were new records for the lichen flora of the Iberian Peninsula (Chapter 2.3). Both species were collected epiphytic on *P. pinaster*, but other phorophytes were also identified as substrates. *Hypotrachyna lividescens* was most commonly found epiphytic on *Halimium halimifolium* (L.) Willk., but was also collected

on *Cistus salvifolius* L. and *Cytisus* sp., while *H. pseudosinuosa* was also collected on *H. halimifolium*. In subsequent studies undertaken at other coastal pine forests, *H. lividescens* was also discovered at Dunas de S. Jacinto (Aveiro), Furadouro (Ovar) and Mata do Urso (Figueira da Foz), and *H. pseudosinuosa* was additionally found Mata do Camarido (Caminha), in all cases epiphytic on *P. pinaster*. Therefore, the known distribution of these species along the Portuguese coast may also increase with further studies, at least at the centre and north of the country. Also, their occurrence in similar habitats in the west and north coasts of Spain should not be excluded, as both species were previously known in Europe from coastal areas in western and south-central France, where they were found epiphytic on a variety of substrates (Masson 2005). The recent finding of *H. lividescens* in North America (Hodkinson 2010) indicates that the world distribution of this species is not well known, and the same may be true for *H. pseudosinuosa*.

Only another species of *Hypotrachyna* was recorded at Dunas de Quiaios, *H. revoluta* (Flörke) Hale. However, it is possible that other *Hypotrachyna* species occur epiphytic on pine, as *H. sinuosa* (Sm.) Hale, and *H. taylorensis* (M.E. Mitch.) Hale that were found in western and south-central France also epiphytic on pine (Masson 2005). *H. taylorensis* are already known in Portugal from Póvoa de Lanhoso (Minho) (Paz-Bermúdez & Carballal 2005). Furthermore, Tavares (1945) that *H. laevigata* (Sm.) Hale [as *Parmelia laevigata* (Sm.) Ach.] is frequent in pine forests of the north of the country (mostly north of Beira Litoral), although it was not found neither at Dunas de Quiaios nor at Mata do Urso in the present study.

Lichen diversity studies undertaken at Dunas de Quiaios were initially meant to serve as a reference, in a case study conducted at another coastal pine forest (Mata do Urso, Figueira da Foz) in order to evaluate the effects of the deposition of atmospheric pollutants on epiphytic lichens of this biotope. Particularly, the objectives of this experiment were to (i) evaluate the deposition of several elements putatively emitted by a Kraft pulp mill located at the border of that forest on lichen transplants (*Flavoparmelia caperata* (L.) Hale), testing for the effects of distance and period of exposure; (ii) estimate the effects of elemental accumulation on lichen vitality by means of chlorophyll *a* kinetics analysis on transplanted lichens; and (iii) investigate if lichen diversity was affected in the vicinity of the pulp mill (Chapter 3).

The accumulation of almost all elements studied was significantly affected by distance and/or period of exposure. As most elements — Al, B, Ba, Ca, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Na, Ni, P, S, Sb, Sc, Si, Sr, Ti and V — were found in significantly higher concentrations in the transplants placed nearest to the pulp mill (500 m), it was implicated as the likely source of these and other elements. Despite soil particles were identified as the partial origin of most of those elements, the possibilities that soil at the impacted location also received atmospheric pollutants from the pulp mill, and that it could be contaminated from wind-blown particles from a landfill situated within the pulp mill complex, put into question whether the accumulation that was related to soil particles was not influenced by soil contamination.

The concentrations of group of elements — B, Ba, Cr, Fe, Hg, Mg, Mn, Mo, Na, P, Pb, S, Sb, and V — were significantly higher in the transplants exposed during 180 days, but only in the case of some these elements it increased with exposure time: B, Hg, Mo, and S. Furthermore, a similar pattern in the accumulation of several elements — Al, Ba, Co, Cr, Cu, Fe, Li, Mg, Mn, Na, Pb, Sb, Ti, V, and Zn — was identified and related to a possible washing effect due to higher precipitation observed during one of the study periods (46–90 days).

Lichen transplants were also concomitantly placed at a selected site at Dunas de Quiaios to serve as a reference for the study conducted at the impacted location. The accumulation of most elements (except K, P, and Zn) was significantly higher in the transplants placed at all or some sites of the impacted location regarding the reference location. The transplants placed at the reference location were also used to test for the influence of period of exposure on elemental accumulation at that site. B and Na were the only elements for which significant differences were found, and both are thought to have originated in sea-spray.

This finding reinforces the need for performing transplantation experiments in reference locations, and is more objective than the use of exposed-to-control ratios (EC ratios, Frati et al. (2005)), sometimes also called enrichment factors (Cercasov et al. 2002, Carreras et al. 2009). Although being useful in investigating the accumulation capacity of the exposed species, this approach does not allow an evaluation of the accumulation of pollutants derived from other sources than the one of study interest, as was the case of sea-spray emissions in this study, or medium/long range transport of pollutants. Also, it does

not allow for statistical significance testing, in what regards the accumulation observed in background conditions.

The analysis of the kinetics of chlorophyll *a* fluorescence revealed that lichens were subject to stress at that the impacted location, as indicated by the significant decrease in F_v/F_m (the maximum efficiency of PSII) in lichens transplanted to the vicinity of the pulp mill (500 and 1000 m), and in those exposed for prolonged periods of time (135 and 180 days). This decrease was correlated with the accumulation of some elements — B, Ba, Co, Fe, Hg, Mg, Mn, Mo, N, P, S, Sb, and Zn — thought to have originated from the pulp mill, possibly with exception of B. The decreases in F_v/F_m were caused by decreases in F_m (maximum fluorescence) observed for the same distances and study periods as for F_v/F_m, and where correlated with the accumulation of the same elements as this last parameter, except, B, Fe, and Mg. The decrease in these parameters, indicate the stress induced to the photosynthetic apparatus derived from the exposure to the emissions of the pulp mill. Although the these parameters are measured in dark-adapted samples, and they not allow stating that photosynthetic performance has been compromised (Baker 2008), the decreases in Φ_{PSII} and Φ_{Exc} in the transplants exposed during 180 days seem to indicate so. The decreases in Φ_{PSII} where correlated with the accumulation of N and P, while those of Φ_{Exc} where correlated with the accumulation of Mn, P, and S, which were found in average higher concentrations in the transplants exposed for 180 days.

The study of lichen diversity study pointed to a local effect on the epiphytic lichen flora, as at 500 m from the pulp mill calculated lichen diversity was visibly lower than at 1000, 1500, 2000 m, and at the reference location (Dunas de Quiaios). Also, it was the only site where nitrophytic species were found, *Physcia adscendens* (Fr.) H. Olivier and *Xanthoria parietina* (L.) Beltr., what was possibly due to the deposition of NH_3 emitted from the pulp mill that may have lead to the observed increase in bark pH. However, this increase may also have been promoted by the deposition of windblown particles from a nearby landfill and soil particles. Pine trees usually have a characteristic acidophytic epiphytic lichen flora, but the occurrence of nitrophytic species has been observed in areas subject ammonia pollution and high bark pH values (Frati et al. 2008).

The influence of the pulp mill on the epiphytic lichen vegetation was apparently restricted to the close vicinity of the pulp mill, up to a distance of approximately 1000 m, based also on the observation of lichens growing in pine twigs. However, there may be

effects in a larger spatial scale following the deposition of pollutants further away from the source, as NO_x, and SO_2 (Fernández-Salegui et al. 2006, Wolseley et al. 2006). Nonetheless, prevailing winds may prevent the deposition of atmospheric pollutants on the pine forest of study and others adjacent to it, in case they carry pollutants to more inland locations or to the sea, at least during partial periods of time.

Concluding remarks

The studies undertaken in this thesis showed that the lichen diversity of Portuguese coastal pine forests is not well studied, not only due to few inventorying studies previously undertaken, but also due to the discoveries of a new species to science and new records for the Iberian and Portuguese lichen floras in this habitat (Chapter 2).

However, the studies presented in this thesis were not thorough, as the epiphytic lichen flora was not fully appreciated in pine forests throughout the country, and the terricolous one was not studied. Additionally, diversity studies were performed only at two locations of the centre of the country: Dunas de Quiaios and Mata do Urso (Figueira da Foz). Nonetheless, the initially studied Dunas de Quiaios, proved to be a good starting point, as all six species reported in this thesis were originally found there.

It is possible that other species not currently known in Portugal or the Iberian Peninsula may still be found in this habitat and may not be unreasonable to expect the occurrence of other species not yet known to science. Portuguese pine forests on sand dunes are in need of a thorough survey, but this situation is similar to that of other European countries.

Studies on lichen diversity are important to understand the conservation status of lichen species, and to elaborate conservation proposals in case they are needed. For instance, *H. pseudosinuosa* was included in the Red List of Macrolichens of the European Community (Sérusiaux 1989), since at that time this species was only known in the laurisilva forest at Tenefire Island (Canary archipelagos), and although the conservation status of the populations was unknown, the rareness and fragility of its habitat, led to the belief that its status was precarious. This species has since been discovered at the Azores (Hafellner 1995) and coastal areas in France (Masson 2005) and Portugal (Chapter 2.3), extending not only the known distribution of this species, but also the number of habitats

in which it occurs. Despite that, the low number of individuals found so far may still qualify this species as vulnerable, according to criterion C for the vulnerable category defined by ICUN (2001). Likewise, Masson (2005) stated that the vulnerable status is appropriate for the French populations. The conservation status of *H. lividescens* may be similar to that of *H. pseudosinuosa*, however at the time that Red List was compiled that species was not known in Europe.

Parmelinopsis horrescens (Taylor) Elix & Hale is also included in that Red List, as an endangered species on in certain areas, but out of danger in others, as is the case of pine forests in the Atlantic coasts of the Iberian Peninsula (Sérusiaux 1989). In the present study, however, it was not detected as an abundant species, at least at Dunas de Quiaios. Also included in that list as a rare species is *Usnea wirthii* P. Clerc, which was found epiphytic on *H. halimifolium*. The occurrence of this and other species (see Annex II), including *H. lividescens* and *H. pseudosinuosa*, on shrubby vegetation in coastal pine forests indicates that it is also an important substrate for lichens.

The conservation status of these and other lichen species, including microlichens in Europe needs to be accessed. While Red Lists of lichens have been compiled for many countries and regions, in Portugal that is still a difficult task as its lichen flora is not well studied. Pine forests on sand dunes are widely distributed along the Portuguese coast and are an important habitat for lichens. The study of epiphytic and terrestrial lichens of this habitat could be an important tool to evaluate conservation status of lichen populations, including of *Cladonia* L. subg. *Cladina* included in annex V of the Habitats Directive (E.C., 1992). Therefore this habitat should not be disregarded as a source of information.

Although the biomonitoring experiment undertaken for this thesis at Mata do Urso was performed on a small spatial scale, the presence of a pulp mill at the border of this forest apparently only has a significant effect on the diversity of epiphytic lichens in close proximity to the pulp mill. In order to fully appreciate the effects not only of the pulp mill studied, but also of other atmospheric pollution sources in the area, studies should be extended to north and northeast, as epiphytic lichens there are apparently less abundant, although that could also the a consequence of the large amount of traffic passing in a national road that crosses the area.

Lichen transplants successfully accumulated elements putatively emitted by the pulp mill. The elemental emission, including metallic, from Portuguese Kraft pulp mills is

largely unknown to the public as few data are reported in the European Pollutant Release and Transfer Register (E-PRTR 2012). However, this study provides only circumstantial evidence on what regards their emission from this pulp mill and data regarding their emission from the industrial facility should be available. Additionally, it could be interesting to investigate the accumulation rate of elements in lichen transplants and relate it to the respective emissions from the pulp mill.

Lichens could also be used to monitor the accumulation of other pollutants that may be emitted by paper or pulp mills, as polycyclic aromatic compounds, which are pollutants of concern in what regards human and wildlife exposure, as they are carcinogenic.

Annex I

Mean frequency values of each species found at the reference location, in each direction and site were the diversity studies were undertaken. MSFs (mean sum of frequencies) for each cardinal point and LDVs (lichen diversity values) for each site are also provided

	A				B			
	N	S	E	O	N	S	E	O
Calicium abietinum	0	0.6	0.3	0.8	0	0.3	0	0
C. cf. *denigratum*	0	0.2	0	0	0	0	0	0
C. hyperelloides	0	0	0	0	0	0	0	0
Chrysothrix candelaris	3.8	4.3	4.3	3.8	3.5	4.4	4.5	3.3
C. flavovirens	0	0	0	0	0	0	0	0
Cladonia cryptochlorophaea	0	0	0	0	0	0	0	0
C. homosekikaica	0	0	0	0	0	0	0	0
C. macilenta	0	0	0	0	0	0	0	0
C. merochlorophaea	0	0	0	0	0	0	0	0
cf. *Endohyalina ericina*	0	0	0	0	0	0	0	0
Evernia prunastri	0.4	0.1	0.2	0.3	0.3	0.3	0.1	0.2
Flavoparmelia caperata	1.2	1.5	1.0	1.0	1.5	1.7	1.4	2.2
Hypogymnia physodes	1.9	0.7	1.6	1.2	2.6	0.6	1.3	2.1
H. tubulosa	0.1	0	0	0.1	0.1	0	0	0
Hypotrachyna lividescens	0	0	0	0	0.1	0	0	0
H. pseudosinuosa	0.3	0	0.1	0.1	0.1	0	0	0
H. revoluta	0.1	0	0	0	0	0	0	0
Lecanora expallens	0.3	0.4	0.8	0	0	0	0	0
L. sorediomarginata	0.1	0.2	0.1	0	0	0	0	0
L. strobilina	0	0.3	0.2	0.4	0.2	0.3	0.4	0.4
Lecidea nylanderi	0	0	0	0	0	0	0	0
cf. *Lecidella scraba*	0.2	0	0	0	0.3	0	0	0
Lepraria nylanderiana	0.2	0.1	0.3	0	0	0	0.1	0
Loxospora elatina	0	0	0.2	0	0	0	0	0
Micarea peliocarpa	0	0	0	0	0.1	0	0	0
M. prasina	0	0	0	0	0	0	0	0
M. cf. *adnata*	0	0	0	0	0	0	0	0
Ochrolechia microstictoides	0.4	0	0.2	0	0	0	0	0
Parmelia sulcata	0	0	0.2	0	0	0	0	0
Parmeliopsis hyperopta	0	0	0	0	0	0	0	0
Parmotrema hypoleucinum	0	0	0	0	0.3	0.3	0	0.2
P. perlatum	0.2	0	0.1	0.1	0.8	0	0.1	0.3
P. reticulatum	0.8	0	0.3	0.1	1.3	1.3	1.2	1.3
Pertusaria amara	0	0	0.1	0	0	0	0	0.1
P. cf. *heterochlora*	0.1	0	0	0	0	0	0	0
Punctelia subrudecta	0	0	0	0	0	0	0	0
Pyrrhospora cinnabarina	0.1	0.1	0	0	0	0	0	0
P. quernea	2.5	4.8	3.8	4.7	3.0	4.8	3.6	4.5
Trapeliopsis flexuosa	0	0.2	0	0.2	0	0	0	0
Tuckermanopsis chlorophylla	0	0	0	0	0	0	0	0
Usnea cornuta s. lat.	0.6	0	0.1	0.2	0.4	0	0	0.4
U. mutabilis	0	0.1	0	0	0.2	0.1	0.4	0.3
U. rubicunda	1.3	0.6	0.7	1.3	2.8	1.1	1.4	2.5
U. subscrabosa	0.2	0.2	0.3	0.2	0.6	0.1	0.4	0
MSF	14.5	14.2	14.7	14.1	18.1	15.2	14.8	17.7
LDV		57.5				65.8		

	C				D			
	N	S	E	O	N	S	E	O
Calicium abietinum	0	0	0.1	0	0.1	0	0	0
C. cf. *denigratum*	0	0	0	0	0	0	0.3	0
C. hyperelloides	0	0	0	0	0	0	0	0
Chrysothrix candelaris	3.2	3.6	3.3	2.7	0	0	0.1	0
C. flavovirens	0	0	0	0	3.5	4.5	4.3	4.0
Cladonia cryptochlorophaea	0	0	0	0	0	0	0	0
C. homosekikaica	0	0	0	0	0	0	0	0
C. macilenta	0	0	0	0	0	0	0	0
C. merochlorophaea	0	0	0	0	0	0	0	0
cf. *Endohyalina ericina*	0	0	0	0	0	0	0	0
Evernia prunastri	0.8	0.4	0.9	0.6	0.2	0.3	0.7	0.1
Flavoparmelia caperata	2.3	1.8	1.8	1.8	1.8	0.5	1.4	1.1
Hypogymnia physodes	2.9	0.8	2.2	2.1	3.6	2.2	3.3	2.3
H. tubulosa	0.1	0	0.1	0	0	0	0	0
Hypotrachyna lividescens	0	0	0	0.1	0.1	0	0.1	0
H. pseudosinuosa	0	0.2	0.1	0	0	0	0	0.1
H. revoluta	0	0	0	0	0	0	0	0
Lecanora expallens	0	0	0	0	0	0	0	0
L. sorediomarginata	0.1	0	0	0.1	0	0	0	0
L. strobilina	0.3	0.8	0.8	0.8	0.1	0.2	0	0.3
Lecidea nylanderi	0	0	0	0	0	0	0	0.1
cf. *Lecidella scraba*	0	0	0	0	0.1	0	0	0
Lepraria nylanderiana	0	0	0	0	0	0	0	0
Loxospora elatina	0	0	0	0	0	0	0	0
Micarea peliocarpa	0	0	0	0.1	0	0	0	0
M. prasina	0	0	0	0	0.3	0	0.2	0
M. cf. *adnata*	0	0	0	0	0	0	0	0
Ochrolechia microstictoides	0	0	0	0	0.1	0	0	0
Parmelia sulcata	0	0	0.1	0	0.3	0	0.2	0
Parmeliopsis hyperopta	0	0	0	0	0	0	0	0
Parmotrema hypoleucinum	0.3	0.6	0.3	0.4	0.1	0.1	0.1	0.2
P. perlatum	1.1	0.1	0.5	0.3	0.9	0.4	0.8	0.4
P. reticulatum	1.3	0.2	0.4	0.7	1.8	0.3	1.1	0.5
Pertusaria amara	0	0	0.1	0.1	0	0	0	0
P. cf. *heterochlora*	0	0	0	0	0	0	0	0
Punctelia subrudecta	0	0	0.1	0	0	0	0	0
Pyrrhospora cinnabarina	0	0	0	0	0	0	0	0
P. quernea	3.6	4.9	4.2	4.9	4.3	5.0	4.3	4.8
Trapeliopsis flexuosa	0	0	0.4	0.3	0	0.3	0.6	0
Tuckermanopsis chlorophylla	0	0	0	0	0.2	0	0.1	0
Usnea cornuta s. lat.	1.3	0.1	0.5	0.3	1.3	0.3	0.3	0.2
U. mutabilis	0.2	0	0	0.1	0	0.1	0	0.1
U. rubicunda	2.3	1.2	1.9	1.4	3.8	1.5	2.5	2.0
U. subscrabosa	0.9	0.3	0.7	0.8	0.8	0.1	0.3	0.8
MSF	20.6	14.9	18.4	17.5	23.2	15.6	20.4	16.8
LDV		71.4				76.0		

	E				F			
	N	S	E	O	N	S	E	O
Calicium abietinum	0	0	0.1	0	0	0.5	0	0.3
C. cf. *denigratum*	0	0	0	0	0	0	0	0
C. hyperelloides	0	0	0	0	0	0	0	0
Chrysothrix candelaris	4.9	5.0	5.0	5.0	4.7	4.8	4.8	4.8
C. flavovirens	0.4	0	0	0	0	0	0.1	0
Cladonia cryptochlorophaea	0	0	0	0	0	0	0	0.1
C. homosekikaica	0	0	0	0	0	0	0	0
C. macilenta	0	0	0	0	0	0	0	0.1
C. merochlorophaea	0	0	0	0	0.3	0	0	0.1
cf. *Endohyalina ericina*	0	0	0.1	0	0	0	0	0
Evernia prunastri	0.3	0.3	0	0	0.1	0.3	0.2	0.3
Flavoparmelia caperata	1.2	0.4	0.9	0.3	2.8	1.2	1.2	2.8
Hypogymnia physodes	3.9	0.7	2.1	1.6	4.0	1.4	2.9	2.7
H. tubulosa	0	0	0	0	0	0	0	0.2
Hypotrachyna lividescens	0	0	0	0	0	0	0	0
H. pseudosinuosa	0	0	0	0	0	0	0	0
H. revoluta	0	0	0	0	0.3	0	0	0
Lecanora expallens	0	0	0	0	0	0	0	0
L. sorediomarginata	0	0	0	0	0	0.2	0	0
L. strobilina	0.3	1.0	0.5	1.1	0	0.3	0.1	0
Lecidea nylanderi	0	0	0	0	0	0.3	0.1	1.5
cf. *Lecidella scraba*	0	0	0	0	0.1	0	0	0
Lepraria nylanderiana	0	0	0	0	0.1	0	0	0
Loxospora elatina	0	0	0	0	0	0	0	0
Micarea peliocarpa	0	0	0	0	0	0	0	0
M. prasina	0	0	0	0	0	0	0	0
M. cf. *adnata*	0	0	0	0	0	0	0	0
Ochrolechia microstictoides	0	0	0	0	0.5	0	0	0.3
Parmelia sulcata	0	0	0	0	0.7	0	0	0.3
Parmeliopsis hyperopta	0	0	0	0	0	0	0	0
Parmotrema hypoleucinum	0.3	0.1	0.1	0.2	0	0	0	0
P. perlatum	0.4	0	0.3	0	0.3	0	0	0
P. reticulatum	0.6	0.1	0.5	0	1.9	0.4	1.3	0.9
Pertusaria amara	0.1	0	0	0	0.1	0	0.2	0.3
P. cf. *heterochlora*	0	0	0	0	0	0	0	0
Punctelia subrudecta	0	0	0	0	0	0	0	0
Pyrrhospora cinnabarina	0	0	0	0	0	0	0	0
P. quernea	4.6	5.0	4.4	4.9	1.1	3.7	2.4	3.1
Trapeliopsis flexuosa	0	0	0.2	0	0	0.1	0.2	1.0
Tuckermanopsis chlorophylla	0	0.1	0	0	0.1	0	0.1	0
Usnea cornuta s. lat.	0.3	0	0.1	0	0.8	0	0.1	0.4
U. mutabilis	0	0	0	0	0.2	0	0	0.2
U. rubicunda	1.9	0.3	1.3	0.8	3.3	0.8	1.0	2.5
U. subscrabosa	0.3	0	0	0	3.6	0.5	1.2	2.3
MSF	19.3	12.9	15.7	13.8	24.8	14.3	15.8	24.0
LDV			61.7				78.9	

	G				H			
	N	S	E	O	N	S	E	O
Calicium abietinum	0	0	0.3	0	0.2	0.6	0.3	0.3
C. cf. *denigratum*	0	0	0	0	0	0	0	0
C. hyperelloides	0	0	0.2	0	0	0.5	0.6	0
Chrysothrix candelaris	4.8	4.9	4.8	4.7	4.4	4.7	5.0	4.3
C. flavovirens	0.1	0	0	0	0.5	0	0	0
Cladonia cryptochlorophaea	0	0	0	0	0	0	0	0
C. homosekikaica	0	0	0	0	0.1	0.1	0	0
C. macilenta	0	0	0	0	0.3	0	0.1	0.1
C. merochlorophaea	0	0	0	0	0	0	0	0
cf. *Endohyalina ericina*	0	0	0	0	0	0	0	0
Evernia prunastri	0.5	0.3	0.2	0.5	0.3	0.1	0.1	0.1
Flavoparmelia caperata	1.3	0.7	1.6	0.8	1.5	0.5	0.7	0.8
Hypogymnia physodes	2.0	0.5	1.5	0.7	4.4	0.9	1.8	1.9
H. tubulosa	0	0	0	0	0.2	0	0.2	0.1
Hypotrachyna lividescens	0.3	0	0.1	0	0	0	0	0
H. pseudosinuosa	0	0	0	0	0	0	0	0
H. revoluta	0	0	0	0	0	0	0	0
Lecanora expallens	0	0	0	0.1	0	0.1	0	0
L. sorediomarginata	0	0	0	0	0	0	0	0.1
L. strobilina	0.7	1.2	1.3	1.2	0.1	0.5	0.2	0.5
Lecidea nylanderi	0	0	0	0	0	0	0	0
cf. *Lecidella scraba*	0	0	0	0	0.1	0	0	0
Lepraria nylanderiana	0	0	0	0	0.3	0	0.3	0
Loxospora elatina	0	0	0	0	0	0	0	0
Micarea peliocarpa	0	0	0	0.1	0.1	0	0	0
M. prasina	0	0	0	0	0.3	0.1	0	0
M. cf. adnata	0	0	0	0	0.1	0	0	0
Ochrolechia microstictoides	0	0	0	0	0.5	0	0.2	0
Parmelia sulcata	0	0	0	0	0	0	0	0
Parmeliopsis hyperopta	0	0	0	0	0	0	0	0.1
Parmotrema hypoleucinum	0	0.1	0.2	0.2	0	0	0	0
P. perlatum	0.6	0	0	0.1	0.1	0	0.2	0.2
P. reticulatum	0.4	0.2	0.3	0.3	0.1	0.1	0	0.2
Pertusaria amara	0	0	0	0	0	0	0	0
P. cf. *heterochlora*	0	0	0	0	0	0	0	0
Punctelia subrudecta	0	0	0	0	0	0	0	0
Pyrrhospora cinnabarina	0	0	0	0	0	0	0	0
P. quernea	3.9	4.5	4.5	4.8	3.9	3.7	2.6	3.9
Trapeliopsis flexuosa	0	0.3	0.4	0.3	0.9	0.4	0.5	0.8
Tuckermanopsis chlorophylla	0	0	0	0	0	0	0.1	0.1
Usnea cornuta s. lat.	0.1	0	0	0	0.9	0.5	0.2	0
U. mutabilis	0.1	0	0.1	0.1	0	0	0.1	0
U. rubicunda	1.6	0.4	0.7	0.6	2.9	0.8	0.7	1.4
U. subscrabosa	0.3	0	0.1	0.1	0.5	0	0.3	0
MSF	16.5	13.0	16.0	14.3	22.7	13.6	14.2	14.9
LDV		59.8				65.4		

	I			
	N	S	E	O
Calicium abietinum	0.1	0.5	0	0.2
C. cf. *denigratum*	0	0	0	0.1
C. hyperelloides	0	0	0	0
Chrysothrix candelaris	4.4	4.8	5.0	4.9
C. flavovirens	0.2	0.3	0.5	0.3
Cladonia cryptochlorophaea	0	0	0	0
C. homosekikaica	0	0	0	0
C. macilenta	0	0	0	0
C. merochlorophaea	0	0	0	0
cf. *Endohyalina ericina*	0	0	0	0
Evernia prunastri	0.3	0.2	0.3	0.3
Flavoparmelia caperata	2.8	1.8	2.3	2.0
Hypogymnia physodes	3.8	1.7	3.1	3.0
H. tubulosa	0.1	0.1	0.1	0
Hypotrachyna lividescens	0.1	0	0	0.1
H. pseudosinuosa	0	0.2	0	0.1
H. revoluta	0	0.1	0	0
Lecanora expallens	0	0	0	0
L. sorediomarginata	0.1	0	0.1	0.1
L. strobilina	0	0.6	0.3	0.2
Lecidea nylanderi	0	0	0	0
cf. *Lecidella scraba*	0.1	0	0	0
Lepraria nylanderiana	0	0	0	0
Loxospora elatina	0	0	0	0
Micarea peliocarpa	0.1	0	0	0
M. prasina	0.3	0	0.1	0
M. cf. *adnata*	0	0	0	0
Ochrolechia microstictoides	0.2	0	0.2	0
Parmelia sulcata	0	0	0	0
Parmeliopsis hyperopta	0	0	0	0
Parmotrema hypoleucinum	0.1	0.6	0	0.6
P. perlatum	0.5	0	0	0.2
P. reticulatum	1.9	1.2	1.0	0.7
Pertusaria amara	0.1	0.1	0.1	0
P. cf. *heterochlora*	0	0	0	0
Punctelia subrudecta	0	0	0	0
Pyrrhospora cinnabarina	0	0	0	0
P. quernea	2.9	4.6	2.9	4.0
Trapeliopsis flexuosa	0	0.2	0.3	0.1
Tuckermanopsis chlorophylla	0	0	0	0.1
Usnea cornuta s. lat.	1.0	0.3	0.3	0.7
U. mutabilis	0.2	0	0	0
U. rubicunda	1.9	1.7	1.1	1.9
U. subscrabosa	2.5	0.4	1.0	1.7
MSF	25.1	18.8	19.7	20.7
LDV		84.3		

Notes: Site A, UTM: 29T, 516193E, 4455327N; site B, UTM: 29T, 516022E, 4454747N; site C, UTM: 29T, 514967E, 4454253N; site D, UTM: 29T, 513852E, 4455259N; site E: 29T, 514226E, 4455175N; site F: 29T, 514455E, 4453702N; site G, 29T, 514963E, 4453606 N; site H, 29T, 516961 E, 4455786 N; site I: 29T, 517192E, 4455706 N. Sites A–G: 12 trees studied, sites H–I: 10 trees studied.

Annex II

Checklist of epiphytic lichens found at Dunas de Quiaios
(Figueira da Foz)

Calicium abietinum Pers. *

C. cf. *denigratum* (Vain.) Tibell *

C. hyperelloides Nyl. *

Cetraria crespoae (Barreno & Vázquez) Kärnefelt *

Chrysothrix candelaris (L.) J.R. Laundon *

C. flavovirens Tønsberg * ◊

Cladonia cf. *mediterranea* P.A. Duvign. & Abbayes *

C. cryptochlorophaea Asahina *

C. homosekikaica Nuno *

C. macilenta Hoffm. *

C. merochlorophaea Asahina *

Dimerella pineti (Ach.) Vězda *

cf. *Endohyalina ericina* (Nyl.) Giralt, Van den Boom & Elix *

Evernia prunastri (L.) Ach. *

Flavoparmelia caperata (L.) Hale *

Hypogymnia bryophila McCune *

H. physodes (L.) Nyl. *

H. tubulosa (Schaer.) Hav. *

Hypotrachyna lividescens (Kurok.) Hale * ∫ § £

H. pseudosinuosa (Asahina) Hale * ∫

H. revoluta (Flörke) Hale *

Lecanora albella (Pers.) Ach. *

L. expallens Ach. *

L. sorediomarginata Rodrigues, Terrón & Elix * ◊

L. strobilina (Spreng.) Kieff. *

Lecidea nylanderi (Anzi) Th. Fr. *

cf. *Lecidella scabra* (Taylor) Hertel & Leuckert *

Lepraria elobata Tønsberg *

L. membranacea (Dicks.) Vain. *

L. nylanderiana Kümmerl. & Leuckert *

Loxospora elatina (Ach.) A. Massal. *

Micarea peliocarpa (Anzi) Coppins *

M. prasina Fr. *

M. cf. *adnata* Coppins *

Ochrolechia arborea (Kreyer) Almb. * ◊

O. microstictoides Räsänen *

Parmelia sulcata Taylor *

Parmeliopsis hyperopta (Ach.) Vain. *

Parmelinopsis horrescens (Taylor) Elix & Hale *

Parmotrema hypoleucinum (J. Steiner) Hale [*]

P. perlatum (Huds.) M. Choisy [*]

P. reticulatum (Taylor) M. Choisy [*]

P. robustum (Degel.) Hale [*]

Pertusaria amara (Ach.) Nyl. [*]

P. coccodes (Ach.) Nyl. [*]

P. cf. *heterochroa* (Müll. Arg.) Erichsen [*]

Physcia clementei (Turner) Lynge [†]

Punctelia subrudecta (Nyl.) Krog [*]

Pyrrhospora cinnabarina (Sommerf.) M. Choisy [*]

P. quernea (Dicks.) Körb. [*]

Ramalina farinacea (L.) Ach. [*]

Schismatomma niveum D. Hawksw. & P. James [*]

Trapelia corticola Coppins & P. James [*]

Trapeliopsis flexuosa (Fr.) Coppins & P. James [*]

Tuckermanopsis chlorophylla (Willd.) Hale [*]

Usnea ceratina Ach. [*]

U. cornuta s. lat. Körb. [*]

U. cornuta s. str. Körb. [*]

U. esperantiana P. Clerc [∫]

U. fulvoreagens (Räsänen) Räsänen [∫]

U. glabrescens (Nyl. ex Vain.) Vain. [∫]

U. hirta (L.) Weber ex F.H. Wigg. [∫]

U. mutabilis Stirt. [* ∫]

U. rubicunda Stirt. [* ∫]

U. subscabrosa Nyl. ex Motyka [*]

U. wirthii P. Clerc [∫]

Notes: [†] epiphytic on burnt bark of a shrubby species, [§] epiphytic on *Cistus salvifolius* L., [£] epiphytic on *Cytisus* sp., [∫] epiphytic on *Halimium halimifolium* (L.) Willk., [*] epiphytic on *Pinus pinaster* Aiton, [◊] epiphytic on *P. pinea* L.

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