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Seasonal trends in usnic acid concentrations of Arctic, alpine and Patagonian populations of the lichen *Flavocetraria nivalis*

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Abstract

The widespread secondary metabolite usnic acid, a dibenzofuran derivative, is the principal acetone-soluble compound in the lichen *Flavocetraria nivalis*. Seasonal variation in concentrations were studied in four populations of this lichen, three from Arctic–alpine habitats in the Northern Hemisphere, and one from Patagonian heathland in the Southern Hemisphere. Usnic acid is produced in large amounts, making up between 4% and 8% of thallus dry weight. Large seasonal variation is seen, with a trend towards peak levels in late spring and early summer, and generally low levels during autumn and winter. However, at an Arctic steppe in Central West Greenland, remarkably high levels were also detected during late autumn and early winter. Comparisons with environmental data using model selection procedures show that usnic acid levels of three of the populations are positively correlated with time of season, as measured by the proximity in time to nearest summer solstice, solar radiation levels, and temperature conditions. All these three variables are intercorrelated, thus indicating the same overall trend. For the three driest sites, precipitation rates are included in the models that best explain the variation in usnic acid. However, the explanatory powers of the models are generally low, partly due to high variation between thalli growing together and sampled at the same time. This is the first attempt to compare statistically seasonal variation in usnic acid concentrations and environmental variables, and thus also the first time it is shown that the concentration in various populations of the same lichen species shows different types of correlation with seasonal climatic changes.

Keywords: *Flavocetraria nivalis*; Lichenized ascomycetes; Seasonal variation; Secondary metabolism; Dibenzofurans; Usnic acid; Solar radiation; Ultraviolet radiation; AIC

1. Introduction

Lichens are known for their production of organic compounds from several biosynthetic pathways. Many of these compounds, the so-called lichen products, are only produced by lichens, or are very rarely detected in other organisms (Culberson, 1969; Huneck and Yoshimura, 1996). The occurrence of lichen substances is widely used as taxonomic markers, but lichenologists, ecologists, pharmaceutical chemists and others are becoming increasingly more aware of the potential biological roles of these substances, and their functions have been discussed in several review papers during the last decades, see Rundel (1978) and Huneck (1999) for two reviews well separated in time. Many lichen products are geographically and taxonomically widespread. The best known and most extensively studied product is the dibenzofuran derivative usnic acid, which

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is commonly used as a collective term for (+)-usnic acid, (−)-usnic acid, (+)-isousnic acid and (−)-isousnic acid (Huneck and Yoshimura, 1996; Ingólfsdóttir, 2002, see the latter reference for structures and biosynthetic route).

Usnic acid-producing lichens are particularly common in light-exposed habitats in practically all biomes of the world. In Arctic–alpine regions, most usnic acid-producing species grow on exposed, lichen-rich heaths, ridges, and on exposed boulders (e.g. Bjerke and Dahl, 2002). Usnic acid has been shown to exhibit antiviral, antiprotozoal, antimicrobial, antiproliferative, anti-inflammatory and analgesic activity (Ingólfsdóttir, 2002, and references therein).

As many other aromatic lichen substances with highly conjugated bonds, usnic acid efficiently absorbs ultraviolet radiation (UVR). Because of this property, coupled with its high photochemical and photophysical stability (Quilhot et al., 1994, 1998; Rancan et al., 2002), several authors have postulated the hypothesis that usnic acid acts as a sun screen, shielding the sensitive photobiont layer from the harmful effects of UVR, in particular ultraviolet-B (UV-B, 280–315 nm) radiation (e.g. Galloway, 1993; Quilhot et al., 1994, 1998; Rikkinen, 1995; Solhaug and Gauslaa, 1996). Some experiments using supplementary UVR led to higher levels of usnic acid under enhanced UVR than under ambient solar radiation or photosynthetically active radiation (PAR) without UVR (Buffoni Hall et al., 2002; Bjerke et al., 2002), whereas other experiments and field surveys did not find a relationship between usnic acid and solar radiation (BeGora and Fahselt, 2001; Bjerke et al., 2004a,b; Gauslaa and Solhaug, 2004).

If usnic acid serves as a protectant against harmful UVR levels, one could expect at least some correlation in space and time between usnic acid levels and fluxes of solar radiation, especially if the synthesis is induced by solar radiation (cf. Solhaug et al., 2003). Such trends have been demonstrated in some surveys along temporal and spatial gradients (e.g. Rundel, 1969; Quilhot et al., 1991, 1998; Fernández et al., 1998; Bjerke and Dahl, 2002; Bjerke et al., 2002), whereas other field surveys failed to detect any correlations between usnic acid concentrations and fluxes of solar radiation (Taguchi et al., 1969; Fahselt, 1981, 1984; BeGora and Fahselt, 2001). Despite the many uncertainties regarding the biosynthesis of usnic acid, it has been postulated that UV-B-absorbing lichen products may prove useful as a tool for monitoring spatial or temporal variations in UV-B fluxes. This topic was summarized by Bjerke et al. (2004a) in context with analyses of the spatial variation in usnic acid concentrations of the lichen *Flavocetraria nivalis* along steep, climatic gradients in Svalbard. It was concluded that at this high-Arctic locality, the levels of usnic acid are altered by numerous environmental stimuli, and that it is unlikely that usnic acid levels can be used to monitor UV-B fluxes. The best model included temperature-related variables, all with negative estimates, thus indicating that high usnic acid levels occur at the sites with the lowest summer and winter temperatures.

In order to achieve a better understanding of temporal trends in usnic acid concentrations, the same lichen as used in the spatial survey (*F. nivalis*) was studied at time periods between 12 and 23 months at four widely scattered, highly contrasting sites, three situated in the Northern Hemisphere (West Greenland, North Norway, Svalbard) and one in the Southern Hemisphere (South Chile). The objectives were to assess how usnic acid levels vary naturally over long time spans and evaluate whether variation in usnic acid levels are correlated with environmental variables (temperature, humidity, and solar radiation).

2. Results

The mean concentrations of usnic acid from the four sites are 5.30% (Kangerlussuaq, West Greenland), 5.28% (Tromsø, North Norway), 5.24% (Punta Arenas, South Chile), and 5.11% (Ny-Ålesund, Svalbard), resulting in an overall mean value of 5.25%. Despite these very similar mean values, considerable variation in concentrations was observed between individual thalli, collection dates and sites (Fig. 1). In general, there is a clear trend towards high levels during late spring or summer. For instance, in Tromsø, the highest levels were detected in samples collected in May, June and July with the lowest levels in samples collected between November and February (Fig. 1(a)). In Ny-Ålesund, high mean levels were reached in July (Fig. 1(b)). Similar distinctive differences between the cold and warm seasons were found in Punta Arenas (Fig. 1(d), note that summer solstice here is in December). However, at Kangerlussuaq, remarkably high levels were detected also during late autumn and early winter (Fig. 1(c)). Thus, the correlation between usnic acid levels and season (as measured by proximity in number of days to nearest summer solstice) differ considerably between sites. Significant correlations were found in Punta Arenas ($R^2 = 0.58$, $P = 0.005$), Tromsø ($R^2 = 0.56$, $P < 0.001$), and Ny-Ålesund ($R^2 = 0.37$, $P = 0.027$), but not at Kangerlussuaq ($P = 0.809$). The sampled correlation coefficients are heterogeneous ($X^2 = 8.08$; $\chi^2_{0.05[3]} = 7.81$) due to the deviating value from Kangerlussuaq. The coefficients of the three populations with significant correlations are homogeneous ($X^2 = 1.79$; $\chi^2_{0.05[3]} = 5.99$). The overall correlation for these three populations with the estimated regression line is shown in Fig. 2 ($R^2 = 0.53$, $P < 0.001$). Because proximity to nearest summer solstice is closely and pos-

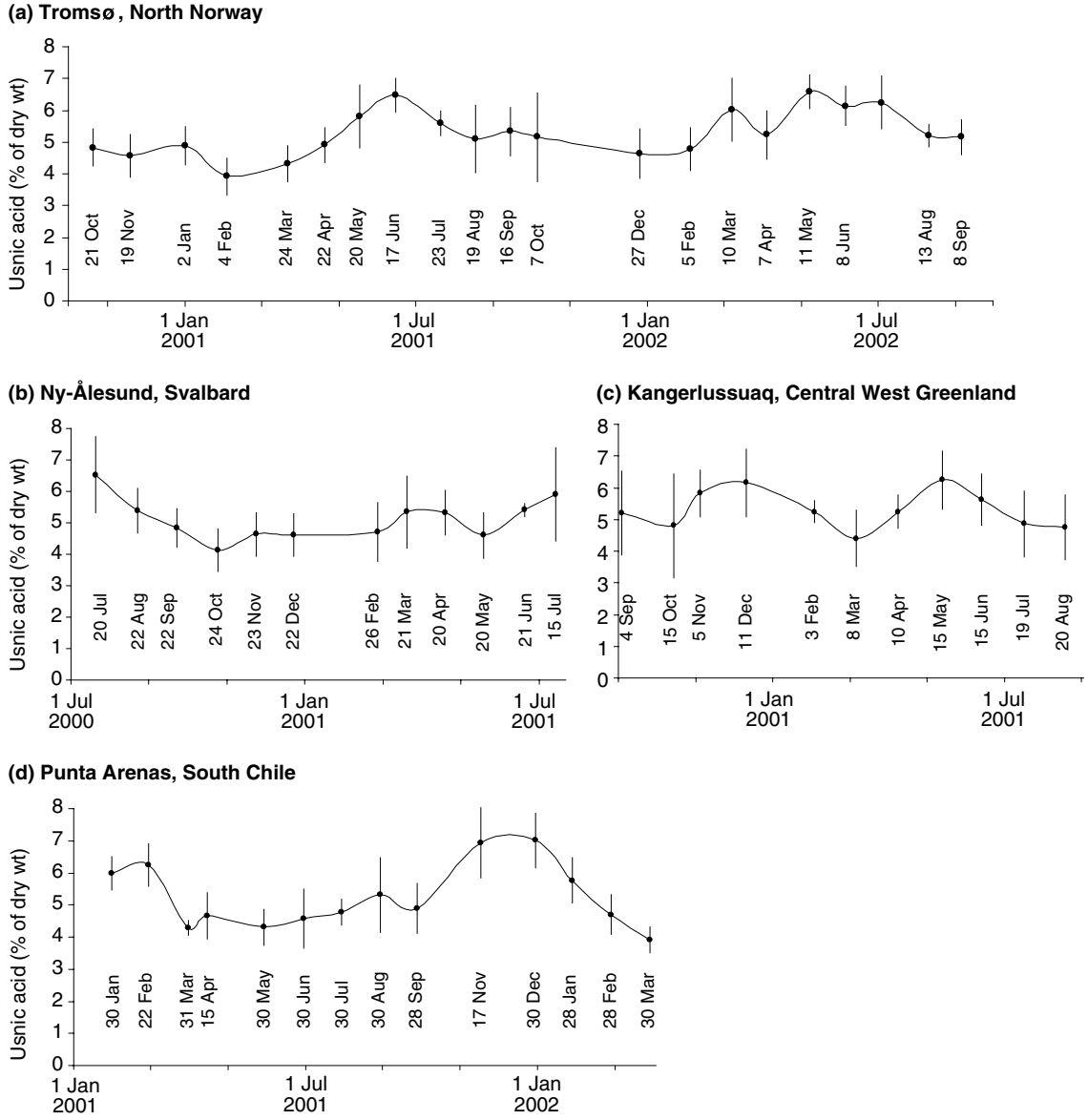

Fig. 1. Temporal trends in usnic acid concentrations of four populations of *Flavocetraria nivalis*: (a) the mountain Fløya, Tromsø, North Norway; (b) Ny-Ålesund, northwestern Spitsbergen, Svalbard; (c) Kangerlussuaq, Central West Greenland; (d) Punta Arenas, southernmost Chile. Values are means of 5–6 thallus samples, ±SD.

itively correlated with mean levels of erythemally weighted solar radiation (CIE) during the 30-d period before sampling ($R^2 = 0.54$), usnic acid levels also show a modest, positive correlation with CIE levels. Since PAR is closely correlated with UVR at these sites ($R^2 > 0.97$), the correlation between CIE values and usnic acid concentration is practically identical to that between PAR and usnic acid concentration. In the model selection analyses, we therefore included CIE values and not PAR values, as a representation of solar radiation in general.

Model selection procedures show that for three of the sites and for the overall data set, the best models for explaining the variability in usnic acid levels include either proximity to nearest summer solstice or CIE_{30} or both variables (Table 1). The best model for the overall data set also includes temperature and altitude, the latter variable a numerical representation of geographical location, thus indicating that there are noteworthy differences between sites. Interestingly, precipitation rates prior to sampling are regarded as important variables at all sites but Tromsø. For the Kangerlussuaq population, precipitation rates during the 30 and 15-d periods prior to sampling are the only variables in the best model, indicating a strong dependence upon humidity at this Arctic, strongly continental (as estimated by continentality indices; Bjerke and Dahl, 2002) steppe site. Precipitation rates for the 30, 15, 7 and 2-d period

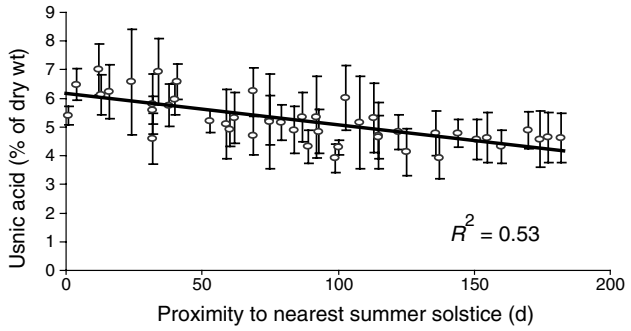

Fig. 2. Scatter plot of usnic acid concentrations including data from the populations Tromsø, Ny-Ålesund and Punta Arenas (vertical axis) vs. the proximity in number of days to nearest summer solstice (21st June in the Northern Hemisphere, 21st December in the Southern Hemisphere). Values are means, ±95% C.I. The linear regression between the two variables are shown together with the correlation coefficient. Data from Kangerlussuaq is not included, because the correlation coefficient of this population deviates significantly from the other three correlation coefficients.

prior to sampling are not closely correlated with each other at any of the sites ($R^2 < 0.46$), except for in one occasion (Punta Arenas, R^2 [$Prec_{30}/Prec_{15}$] = 0.68), thus all these parameters were included in the model selection procedures.

The likelihood of the best models, as shown by Akaike weights (Table 1), is generally low, to a large extent due to high interthalline variation in usnic acid concentration within samplings, which is evidenced graphically by the long error bars in Fig. 2. The data from Punta Arenas are strongly supported by the best model ($w_i = 0.636$), whereas there is less support for the best models of the other sites. This is also indicated by the number of supported models, viz. models with Δ_i less than 2. Thus, for these sites, there exist alternative, plausible models than those presented. Inclusion of second-term interactions in the model selection analyses generally resulted in selection of very complex models with even lower explanatory power. These models are therefore not shown here.

3. Discussion

This study differs from other studies on temporal variability in usnic acid concentration by including long sampling series from multiple sites, and by comparing biological data with environmental data using model selection procedures and likelihood theory. This is therefore, the first time that temporal relationships between usnic acid levels and combinations of extrinsic factors are verified by statistical analyses, and consequently also the first time that it is shown that infraspecific, seasonal trends differ between sites. The longest time series (Quilhot et al., 1991) for usnic acid concentration includes 30 samplings dispersed over three years of the Antarctic lichen *Usnea aurantiaco-ater* (Jacq.) Bory. Each year, they found an abrupt decrease in usnic acid concentrations between May and June, which coincided with the establishment of a permanent snow or ice cover. Concentrations again increased from around November and remained high until May. Legaz et al. (1986) also found very high levels of usnic acid in Spanish specimens of *Evernia prunastri* (L.) Ach. collected from May to July. Hence, our results on *F. nivalis* partly correspond with those on *U. aurantiaco-ater* and *E. prunastri*, as the highest levels of usnic acid generally were detected near summer solstice in all three species.

However, other temporal surveys revealed slightly different seasonal trends. Using various lichen species from northern temperate sites, Taguchi et al. (1969), Ravinskaya and Vainshtein (1975) and BeGora and Fahselt (2001) all found the lowest levels of usnic acid during summer, with the highest levels in late winter or spring. These studies included between 4 and 10 samplings dispersed over an entire year. The high levels of usnic acid in these temperate lichen populations partly coincide with periods of high metabolic activity, as temperate lichens often are inactive for long periods during summer due to drought and heat stress. Boreal and Arctic lichens also often experience drought and heat stress in midsummer (e.g. Sonesson, 2001), and the slight de-

Table 1
AIC_C differences (Δ_i), Akaike weights (w_i) and evidence ratios (w_{min}/w_j) for the two models that best explain the variation in usnic acid concentration at each site and for all sites together

Site	Model	Δ_i	w_i	w_{min}/w_i
Tromsø	Proximity to nearest summer solstice + CIE_{30}	–	0.178	–
(5)	CIE_{30} only	0.4	0.145	1.23
Ny-Ålesund	Proximity to nearest summer solstice + $PREC_{30}$ + $PREC_2$	–	0.094	–
(11)	Proximity to nearest summer solstice + $PREC_{30}$ + $PREC_7$	<0.1	0.093	<1.01
Kangerlussuaq	$PREC_{15}$ + $PREC_{30}$	–	0.155	–
(5)	$PREC_{30}$ only	0.9	0.102	1.53
Punta Arenas	Proximity to nearest summer solstice + $PREC_7$ + $PREC_2$ + $TEMP_{30}$	–	0.636	–
(2)	As above + $PREC_{30}$	1.8	0.264	2.41
All sites	$TEMP_{30}$ + altitude + proximity to nearest summer solstice + CIE_{30}	–	0.085	–
(8)	As above + $PREC_7$	0.1	0.082	1.05

Values in brackets indicate number of models (including the best model) having Δ_i less than two. See text for further information.

cline in usnic acid concentrations from late spring and early summer to late summer in *F. nivalis* from North Norway, Greenland and South Chile may partly be associated with drought stress.

Secondary metabolism in lichens certainly depends upon the availability of liquid water, as shown by Solhaug et al. (2003) and Bjerke et al. (2004b). Thus, it is not surprising that the model selection procedures indicate a positive relationship between usnic acid concentrations at the three driest sites (Ny-Ålesund, Kangerlussuaq and Punta Arenas) and precipitation rates during the 2–30-d period prior to sampling. Nevertheless, the unexpected increase in usnic acid concentrations in Kangerlussuaq during November and December is not related to increased precipitation rates, since July and August received similar amounts of precipitation as did the autumn months ($PREC_{30}$ = 20–22 mm). However, decreasing temperatures led to lower evaporation rates and thus increased availability of water during autumn, and this may be the extrinsic factor that stimulated this late autumn and early winter accumulation of usnic acid, which also was evident in two species of *Rhizoplaca* from the same site (Dahl, 2004).

Precipitation rates are the variables that are least correlated with other environmental variables. Temperature conditions, proximity in time to nearest summer solstice and CIE values are all positively intercorrelated. Thus, inclusion of several of these variables in the best models may merely be regarded as multiple indications of a trend towards having the highest concentrations close to summer solstice, and not that all these variables are regulatory per se.

The high variability is evidenced by large confidence intervals (Fig. 2), and it generally results in low Akaike weights of the best models. The models selected to explain the variability of usnic acid concentrations along spatial gradients also showed low explanatory power (Bjerke et al., 2004a). This was largely explained by high interthalline, intrasite variability in usnic acid concentrations, and is certainly also the case along the temporal gradients. Reasons for high variability between adjacent thalli are not well understood. It has been detected that high genetic variation is present in some lichens and their photobionts, even at small spatial scales (Romeike et al., 2002; Walser et al., 2003). Thus, both inherent genetic factors, biotic factors and abiotic factors may in various ways contribute to high variability (Fahselt, 1984; Bjerke et al., 2004a).

To conclude, we have shown that usnic acid concentrations in widely dispersed populations of *F. nivalis* is correlated with seasonal changes in climatic factors, but that there also are some similarities between the populations, resulting in inclusion of some of the same parameters within the models that best explain the data. Time of the year is a relevant parameter. The most humid site (Tromsø) is the only site where precipitation rates are not included in the best models. High interthalline variability results in low model weights. Since the correlations between usnic acid concentrations and fluxes of solar radiation in some sites are modest only and not evident at all in other sites, the hypothesis that the content of UV-absorbing compounds in lichens can be used to monitor changes in UV-B radiation (see Bjerke et al., 2004a, for a discussion) is rejected.

4. Experimental

4.1. Study sites

The fruticose usnic acid-producing lichen *F. nivalis* (L.) Kärnefelt and Thell is a widespread species in cold areas in the Northern Hemisphere, with a few known localities in the Southern Hemisphere (Bjerke and Elvebakk, 2004). Four easily accessible sites were selected for this study:

1. The mountain Fløya, Tromsø, North Norway (69°37′N, 19°03′E, 560 m a.s.l.). Twenty-one samplings from 21st October 2000 to 8th September 2002.
2. Ny-Ålesund, NW Spitsbergen, Svalbard (78°56′N, 11°56′E, 20 m a.s.l.). Twelve samplings from 20th July 2000 to 15th July 2001.
3. Twelve kilometers SW of Kangerlussuaq, Central West Greenland (66°59′N, 50°59′W, 40 m a.s.l.). Eleven samplings from 4th September 2000 to 20th August 2001.
4. Fifteen kilometers N of Punta Arenas, XII Región de Magallanes, Chile (52°58′S, 70°45′W, 40 m a.s.l.). From a site in Pali-Aike National Park (52°07′S, 68°42′W, 150 m a.s.l.), it was collected and transplanted to an *Empetrum*-heath close to Punta Arenas, which is more easily accessible than Pali-Aike. It was sampled from the transplant site at 14 different occasions between 30th January 2001 and 30th March 2002.

4.2. Quantitative analyses of usnic acid

Lichen samples were air-dried and kept at room temperature in a dry and dark place until quantitative analyses were undertaken. Six samples were analysed from each sampling. Only thallus apices, defined as the uppermost 0.5 cm of the tips, were analysed, as these are the thallus parts containing the highest concentrations of usnic acid (Bjerke et al., 2002). Complete extraction of usnic acid was achieved by immersing the samples three times for 24 h in solutes, first in acetone, second in a 1:1 mixture of acetone and methanol, and finally in pure methanol. The combined extracts were mixed, filtered and diluted to 25 ml. Extracts were analysed

quantitatively by reversed-phased high performance liquid chromatography (RP-HPLC) using a standard column (Nova-Pak C18, 60 Å, 4 μm, 3.9 × 150 mm, Waters Co., Milford, USA), a photodiode array detector, and a mobile phase consisting of methanol and 1% orthophosphoric acid in ultra-pure water, as described in detail by Bjerke et al. (2002). Major amounts of (+)-usnic acid were present in all specimens. Very small peaks of (−)-usnic acid, partly overlapping with (+)-usnic acid, were frequently detected. The peak area ratio between the (−)-usnic acid and (+)-usnic acid was generally less than 1:100. Thus, the small area of (−)-usnic acid was incorporated into the total amount of usnic acid. Voucher specimens from the four localities are deposited in TROM.

4.3. Environmental variables

The intention was to establish plots close to near-ground solar radiation monitors and meteorological stations. Radiation data were available from Kangerlussuaq from a Brewer instrument run by the Danish Meteorological Institute. A multiband filterradiometer, model GUV, run by Norwegian Institute for Air Research (Johnsen et al., 2002), provided radiation data from Ny-Ålesund. A similar instrument was placed in Tromsø, but was moved to Andøya (69° 17′N, 16° 08′E, c. 120 km SW of Tromsø) immediately before we started the sampling in Tromsø (Johnsen et al., 2002). Thus, we had to use radiation data from Andøya for this study. A Brewer instrument that had been monitoring solar radiation in Punta Arenas continuously for numerous years, stopped working just before the start of our sampling period (C. Casiccia, personal communication). Thus, to have a coarse estimate of radiation levels in Punta Arenas, data measurements from NASA's Total Ozone Mapping Spectrometer (TOMS) were retrieved from the Internet (http://toms.gsfc.nasa.gov/ery_uv/euv.html). Data on daily temperature and precipitation rates were available from meteorological stations close to the respective sites. In situ near-ground temperature and humidity were not measured. The radiation and climate data were used to derive several variables. Precipitation rates (PREC), daily mean temperature (TEMP) and daily shortwave radiation (CIE) were calculated for the 30, 15, 7 and 2-d periods prior to harvesting. The variables are abbreviated $PREC_{30}$, $PREC_{15}$, $PREC_7$, $PREC_2$, $TEMP_{30}$, $TEMP_{15}$, $TEMP_7$, $TEMP_2$, CIE_{30}, CIE_{15}, CIE_7 and CIE_2. Shortwave radiation is identical to the CIE weighted irradiance, which is based on the action spectrum for erythema (McKinlay and Diffey, 1987). Prior to model selection procedures, the correlation between the various variables were calculated, and in cases with strong correlation between two variables ($R^2 \geqslant 0.64$), only one of them was included in further analyses. This was almost always the case when comparing the various CIE and TEMP variables, thus $TEMP_{30}$ and CIE_{30} were included to represent all temperature and radiation variables. On the other hand, PREC variables were only rarely strongly correlated with each other. Proximity to nearest summer solstice in number of days was included as an additional variable, and this variable showed strong correlation with CIE variables at some sites. Proximity was further divided into half years, i.e. whether collection date is closer to the forthcoming or to the previous summer solstice, but this variable did not lead to significantly different statistical results, as compared to analyses without categorization into half years.

4.4. Statistical analyses

Estimations of linear regressions, product-moment correlation coefficients and significance tests in correlation were calculated according to Sokal and Rohlf (1995). To assess whether usnic acid concentrations were related to any environmental variables, linear model selection procedures were used. The second-order variant of Akaike's Information Criterion (AIC_C) was applied to find which models that best fit the variability in the response variable. Since it is the relative values, and not the absolute size of the AIC_C values that are of importance (Burnham and Anderson, 2002), only the AIC_C differences (Δ_i) are presented. The relative likelihood of the best models were interpreted by means of model weights termed Akaike weights (w_i), which are expressed on a scale from 0 to 1 where 1 indicates "full evidence" in favour of model i. Moreover, evidence ratios (w_{min}/w_j) are provided for the second best model (model j) as compared to the best model, thus providing the relative likelihood of the model pair. Low ratios ($<\sim 1.5$) indicate that the explanatory power of the two models are almost equal. Model selection procedures, likelihood estimations and interpretations are carried out in accordance with Burnham and Anderson (2002). All analyses were done by using the statistical package S-PLUS 6.1 (Insightful, Seattle, USA) and the library MASS (Venables and Ripley, 2002).

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