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Intermittent low temperatures constrain spring recovery of photosynthesis in boreal Scots pine forests

INGO ENSMINGER*, DMITRY SVESHNIKOV†, DOUGLAS A. CAMPBELL‡, CHRISTIANE FUNK§, STEFAN JANSSON†, JON LLOYD*, OLGA SHIBISTOVA¶ and GUNNAR ÖQUIST†

**Max-Planck-Institut für Biogeochemie, Postfach 100164, 07701 Jena, Germany, †Department of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden, ‡Mount Allison University, Sackville, New Brunswick, E4L 1G7, Canada, §Department of Biochemistry, University of Umeå, S-901 87 Umeå, Sweden, ¶Institute of Forest, Siberian Branch, Russian Academy of Sciences, Akademgorodok, 660036 Krasnoyarsk, Russia*

Abstract

During winter and early spring, evergreen boreal conifers are severely stressed because light energy cannot be used when photosynthesis is pre-empted by low ambient temperatures. To study photosynthetic performance dynamics in a severe boreal climate, seasonal changes in photosynthetic pigments, chloroplast proteins and photochemical efficiency were studied in a Scots pine forest near Zotino, Central Siberia. In winter, downregulation of photosynthesis involved loss of chlorophylls, a twofold increase in xanthophyll cycle pigments and sustained high levels of the light stress-induced zeaxanthin pigment. The highest levels of xanthophylls and zeaxanthin did not occur during the coldest winter period, but rather in April when light was increasing, indicating an increased capacity for thermal dissipation of excitation energy at that time. Concomitantly, in early spring the D1 protein of the photosystem II (PSII) reaction centre and the light-harvesting complex of PSII dropped to their lowest annual levels. In April and May, recovery of PSII activity, chloroplast protein synthesis and rearrangements of pigments were observed as air temperatures increased above 0 °C. Nevertheless, severe intermittent low-temperature episodes during this period not only halted but actually reversed the physiological recovery. During these spring low-temperature episodes, protective processes involved a complementary function of the PsbS and early light-induced protein thylakoid proteins. Full recovery of photosynthesis did not occur until the end of May. Our results show that even after winter cold hardening, photosynthetic activity in evergreens responds opportunistically to environmental change throughout the cold season. Therefore, climate change effects potentially improve the sink capacity of boreal forests for atmospheric carbon. However, earlier photosynthesis in spring in response to warmer temperatures is strongly constrained by environmental variation, counteracting the positive effects of an early recovery process.

Nomenclature

A = antheraxanthin
Car = carotenoid pigments
Chl = chlorophyll
CP29 = protein representing the monomeric minor light harvesting complexes of photosystem II
D1 = reaction centre protein of photosystem II
DEPS = de-epoxidation status of the xanthophyll cycle pigments
Elip = early light-induced protein
F_0 = instantaneous fluorescence at open PSII centres
F_0' = minimal fluorescence at open PSII centres immediately after illumination

Correspondence: Ingo Ensminger, fax + 49 36 41 57 71 00,
e-mail: ingo.ensminger@bgc-jena.mpg.de

F_m' = maximal fluorescence at closed PSII centres under actinic light
F_m = maximal fluorescence at closed PSII centres
F_t = transient fluorescence at partly closed PSII centres under actinic light
$F_v/F_m = (F_m - F_0)/F_m$, the efficiency of open PSII units
I_{mean} = 5-day running mean of the incoming photosynthetically active radiation (mol photons m^{-2} day^{-1})
LHC = light harvesting complex
LHCI-730 = protein of the light harvesting complex of photosystem I
LHCII = protein of the major light harvesting complex of photosystem II
NPQ = $(F_m - F'_m)/F'_m$, nonphotochemical quenching
NPQ = F_m/F_mrec-1) reversible
$\Delta F/F_m' = (F'_m - F_t)/F'_m$, the efficiency of partly closed PSII units
PPFD = photosynthetic photon flux density (μmol photons m^{-2} s^{-1})
PSI or PSII = photosystem I or photosystem II, respectively
PsbS = protein involved in nonphotochemical quenching
RC = reaction centre of the photosystems
V = violaxanthin
Z = zeaxanthin

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Introduction

Significant changes in the climate of boreal regions over the last 100 years are well documented, for Siberia in particular (Serreze *et al.*, 2000). These changes include air temperatures and the duration and extent of snow cover (Groisman *et al.*, 1994; Brown, 2000). It has recently been argued that the increase in temperatures over the last 20 years resulted in large-scale changes in the high northern latitude terrestrial CO_2 exchange, where the phasing of the seasonal cycle of atmospheric CO_2 correlates with large year-to-year variations in both spring and autumn temperatures (Randerson *et al.*, 1999). In particular, warmer air temperatures are hypothesized to be causing an earlier onset of photosynthetic activity. Nevertheless, the factors determining the timing and rate of the spring photosynthetic recovery in boreal conifers are not completely understood in terms of the underlying physiology, nor in terms of the associated environmental triggers (Öquist *et al.*, 2001; Suni *et al.*, 2003).

Scots pine (*Pinus sylvestris*) is one of the most important evergreen conifer species that dominate the boreal forests, and covers about 14% of the Siberian forested area (Shvidenko & Nilsson, 1994; Richardson & Rundel, 1998). To survive severe winter stress in the northern zone, reduced daylength in autumn triggers a cold hardening process involving long-term changes in the metabolism of the tree, followed by dehardening and recovery in spring (Vogg *et al.*, 1998; Öquist *et al.*, 2001). In *Pinus sylvestris*, this cycle includes a down-regulation of photosynthetic capacity in autumn with almost complete suppression during the winter season (Strand & Öquist, 1988; Ottander *et al.*, 1995; Vogg *et al.*, 1998). In autumn, the excitation pressure on the photosystems increases, because photochemical generation of electrons exceeds the consumption of electrons by metabolic processes as metabolism is slowed by low temperatures (Huner *et al.*, 1996). The protective mechanisms against photo-oxidative damage from this increased excitation pressure during winter involve increased nonphotochemical quenching (NPQ) of excitation energy via xanthophylls (e.g. Adams & Deming Adams, 1994; Ottander *et al.*, 1995; Verhoeven *et al.*, 1999a; Gilmore & Ball, 2000; Adams *et al.*, 2002), reduced absorbance and activity of photosystem II (PSII) due to partial breakdown of antenna chlorophylls, inactivation of PSII reaction centres (Öquist *et al.*, 1992; Lee *et al.*, 2001) and increased cyclic flow of electrons around PSI (Ivanov *et al.*, 2001). Dehardening of Scots pine and recovery of photosynthesis with concomitant reorganization of the photosynthetic apparatus does not start until spring (Ottander *et al.*, 1995; Vogg *et al.*, 1998).

Considerable data describe seasonal patterns of photosynthetic adjustment. However, little information is available about rapidly changing environmental conditions during the crucial spring transition period on conifers from harsh climatic environments (but see Bergh & Linder, 1999; Monson *et al.*, 2002). In this work, we thus combine physiological and biochemical data obtained from the field with micrometeorological

measurements in an analysis of photosynthesis/light/temperature relations in *P. sylvestris*. We particularly emphasized the dynamics of recovery during spring. With huge seasonal changes in temperature, light and intermittent low-temperature episodes, this period might account for much of the observed interannual variability in carbon acquisition (Randerson *et al*., 1999).

The purpose of this study was thus to determine (i) seasonal changes in photosynthetic capacity for a central Siberian Scots pine forest; (ii) the timing of the recovery process in spring, and the factors that trigger the onset of photosynthesis; and (iii) effects of intermittent low-temperature episodes on the spring recovery of photosynthesis. The overall aim was to infer possible consequences for spring recovery of photosynthesis in the context of the anticipated future change of the global climate.

Materials and methods

Study site and plant material

Fieldwork was conducted from June 2000 until the end of May 2001 at a pristine forest with an open canopy (leaf area index (LAI) of 1.5 (projected area)) and a mean canopy height of 16 m. The *P. sylvestris* stand is located 30 km west of the Yenisei river (60°45′N, 89°23′E, elevation 90 m), at the eastern edge of the west Siberian lowland, about 600 km north of the city of Krasnoyarsk. For further details of the forests of the region, refer to Wirth *et al.* (1999). The stand, also the subject of a long-term study of ecosystem carbon, water vapour and energy fluxes were located on alluvial sands (classified as Inceptisols) with no underlying permafrost (Lloyd *et al*., 2002).

Trees for the needle sampling were selected randomly within an area of about 1 ha and only second year needles were used for all measurements. During summer, autumn and winter, samples were taken at least once per month. During early and late spring, samples were taken at least twice per week. A shotgun was used to remove sun-exposed branches (8–12 m height) from three different trees per sampling day and all sampling was undertaken at mid-day. For the analysis of photosynthetic pigments and the estimation of thylakoid proteins, samples were placed immediately in liquid nitrogen and stored until required. In the laboratory, needles were homogenized with liquid nitrogen in a mortar, lyophilized and kept in the dark under vacuum until further processing. For measurements of chlorophyll fluorescence and needle gas exchange, twigs of about 20 cm length were kept in sealed plastic bags, transported to the laboratory and stored for 2 h in darkness at either 4 °C (winter samples) or at ambient temperature (spring and summer samples).

Photosynthetic pigments

From the freeze-dried samples, pigments were extracted for 2 h at 4 °C in the dark in acetone (100%) buffered with $NaHCO_3$. A reversed-phase C-18 column (Knaur, Berlin, Germany) was used to separate the pigments by high-performance liquid chromatography (HPLC) according to Ensminger *et al.* (2001). Total chlorophyll and total carotenoids were estimated spectrophotometrically using the equations of Lichtenthaler (1987).

Chlorophyll fluorescence and photosynthetic oxygen evolution

For measurements of photosynthetic capacity, needles were arranged carefully on sticky paper tape and a 2 cm diameter disc was cut. The disc was then exposed within an LD2/3 leaf chamber for simultaneous recording of oxygen evolution (Clark-type electrode, Hansatech, Norfolk, UK) and pulse-modulated chlorophyll fluorescence of PSII (PAM-2000, Walz, Effeltrich, Germany). All experiments were performed at 20 °C in a CO_2-enriched atmosphere (5%, Walker, 1990).

PSII activity was monitored according to Schreiber *et al*. (1994) with the optimum quantum yield measured as the ratio of variable to maximum chlorophyll fluorescence ($F_v/F_m = (F_m - F_0)/F_m$) of dark adapted samples. The efficiency of open PSII reaction centres in light was estimated as the ratio of $\Delta F/F_m' = (F_m' - F_t)/F_m'$ during illumination with actinic light of photosynthetic photon flux densities (PPFDs) of 370 or 1300 $\mu mol\, m^{-2}\, s^{-1}$.

NPQ, taken here as a measure of radiation-less dissipation of absorbed light energy, was calculated according to Bilger & Bjorkman (1990) as $NPQ = (F_m - F_m')/F_m'$. The calculation of winter levels of NPQ is a critical point, as the fully relaxed F_m is required for calculations (Adams *et al*., 1995a, b). During deep sustained quenching of excitation energy by a photosystem primed for winter conditions, F_m measured after brief dark adaptation (e.g. a few hours) does not represent the fully relaxed value. To overcome the problem of an inadequate F_m during winter, we extrapolated relaxed (or summer) values assuming that F_m at its optimum will exceed F_0 by a factor of 5 (Schreiber *et al*., 1994). We then used this calculated value to estimate NPQ. This exerted reasonable values of F_m, as we obtained similar F_m values during summer from direct measurements and for the calculated F_m.

The reversible component of NPQ (NPQ_{rev}) was determined according to Demmig Adams (1998) by subtracting the sustained component of $NPQ = F_m/F_m rec - 1$ (where $F_m rec$ is the partially recovered F_m after 10 min of recovery under dim light) from the maximum NPQ during high light.

Protein extraction, SDS-PAGE and immunoblotting

Proteins were extracted from freeze-dried needles in a buffer containing 0.06 M Tris-HCl (pH 6.8), 2% SDS and 15% sucrose by sonication (three cycles, 20 s each) followed by incubation at 75 °C for 5 min. For SDS-PAGE, 0.02 M DTT was added. The basis upon which the gels are loaded (such as chlorophyll levels or protein levels) can influence the nature of the inferred response. Because we observed only about 10% variability in total protein levels between samples from different seasons, the samples were loaded on an equal protein basis (20 μg protein per slot). Proteins were separated in a Mini-Gel System (CBS Scientific, Del Mar, CA, USA) using the buffer system of Laemmli (1970) with a 12% (w/v) stacking gel (17.5% 4 M urea w/v for the separation of the PsbS) and a 4% (w/v) resolving gel.

After separation, polypeptides were transferred to a PVDF membrane (0.2 μm pore size, Biorad, Hercules, CA, USA) and probed with specific antibodies against LHCI-730, D1 (Global D1, Agrisera, www.agrisera.se, Sweden), LHCII, CP29, PsbS and early light-induced protein (Elip). Goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham, Little Chalfont, UK) was used as a secondary antibody. A chemoluminescent detection system (ECL detection kit, Amersham) was used to visualize the complexes. One immunoblot, representative of at least three separate blots from different protein extractions and gel runs was used to demonstrate dynamic changes in polypeptides over the seasons.

Net ecosystem carbon flux and climate data

Canopy-level fluxes of carbon dioxide were measured continuously with an eddy covariance system throughout the study period. Measurements were made at a height of 27 m, 5 m above the mean tree height (Lloyd *et al.*, 2002). An associated weather station also provided PPFD (LI-190SA, LiCor Inc., Lincoln, NB, USA) and air temperature (HMP35D, Vaisala, Helsinki, Finland), both being measured at the top of the tower. Soil temperature was measured with Pt100 elements at 5 cm depth. All meteorological data were collected every 10 s with 10 min average values stored on a datalogger (Dl 3000, DeltaT, Burwell, UK) and subsequently converted to 30 min means off-line. These derived 30 min means have been used here to calculate daily climatologies for the experimental period, including minimum and maximum temperatures, average daily temperatures, maximum and daily integrated PPFDs and daily average soil temperatures. In all cases, daily averages have been calculated from midnight to midnight local time.

Statistics

The software package SPSS release 11.0 (SPSS, Chicago, IL, USA) was used to perform statistical tests and procedures.

Results

Seasonal patterns of climate and photosynthetic pigments

Figure 1 shows the seasonal variation in the mean daily air temperatures and PPFDs as well as their 5-day running means for 2001/2002. It illustrates the likely climatic constraints for plant growth over much of the year. The average daily air temperatures were below 0 °C from October 2000 until mid-April 2001. The first night-time frost occurred on 23 September 2000 with the last frost in spring recorded on 22 May 2001. In September and October, both air temperature and PPFD declined rapidly at this site. In contrast, during late March and early April, I_{mean} was as high as typically observed during August and September, but accompanied by subzero temperatures. This situation in spring is typical for central Siberia and is attributable to a strong high-pressure atmospheric cell, the 'Siberian High', typically centred over the Eurasian continent from mid-January till at least late March. The Siberian High tends to produce cloudless conditions and associated

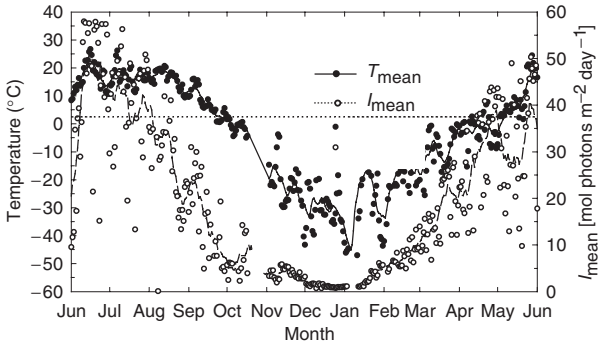

Fig. 1 Annual climate at Zotino field site. Daily mean air temperatures as well as the 5-day running mean (T_{mean}) are given. Incoming photosynthetically active radiation (I_{mean}) is also given as daily values and as a 5-day running mean (mol photons m^{-2} day^{-1}). Closed circles denote daily mean temperature, and open circles denote daily average of incoming photosynthetically active radiation.

low air temperatures over much of the middle-to-high Eurasian latitudes during this time (Gong & Ho, 2002). Further data on diurnal variations in PPFD and air temperature typical for this site in April/May can be found in Figure 1 of Lloyd *et al*. (2002).

Chlorophyll (Chl) levels were significantly higher during summer, compared with other times of the year (Fig. 2a). Although there were no statistical differences between samples during the cold season, Chl levels varied considerably relative to summer levels. In autumn after cold hardening, Chl content decreased to 77% of the summer average, with a further decline during winter to only 61%. In early spring, Chl increased to about 86% of the summer levels, but then subsequently dropped to 75% in late spring. Total carotenoids (Car) per Chl stayed constant throughout the seasons, except for slight decreases during the transition periods in autumn and late spring. Only the difference between early spring and late spring is statistically significant (Fig. 2b). Violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) represent the pigments of the photoprotective xanthophyll cycle (V + A + Z). They increased from autumn through early spring, until the fraction of the total carotenoid pool present as V + A + Z was over double that observed during summer (Fig. 2c).

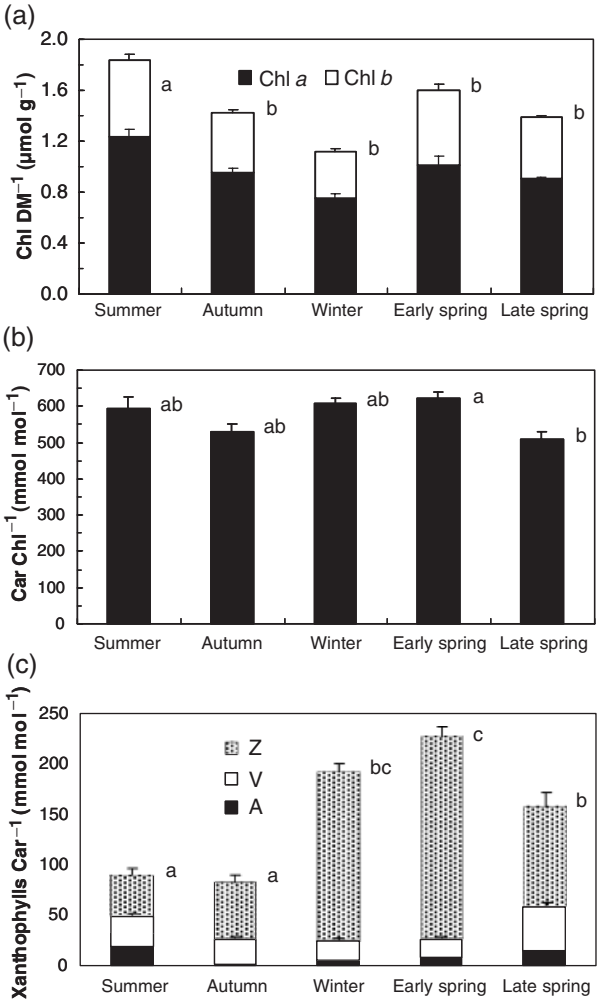

Fig. 2 Annual pattern of the composition of photosynthetic pigments of Siberian Scots pine. (a) Total chlorophyll per dry mass [(Chl a + Chl b) DM^{-1}]; (b) total carotenoids per total Chl ($Car\ Chl^{-1}$); (c) xanthophyll cycle pigments per total carotenoids ($(V + A + Z)\ Car^{-1}$). Summer = 11 June–06 September ($n = 11 \pm$ SE); autumn = 07 September–10 December ($n = 22 \pm$ SE); winter = 11 December –15 March ($n = 12 \pm$ SE); early spring = 16 March –30 April ($n = 60 \pm$ SE); late spring = 01 May –01 June ($n = 53 \pm$ SE). Letters are used to indicate significantly differing groups ($P < 0.05$, Tukey's *post hoc* test). V, violaxanthin; Z, zeaxanthin; and A, antheraxanthin.

Seasonal patterns of thylakoid proteins

The highest levels of D1, a PSII reaction centre protein, occurred in summer, compared with lower levels in September, reflecting downregulation due to the cold hardening process (Fig. 3). Winter levels of D1 were somewhat lower and remained stable until March,

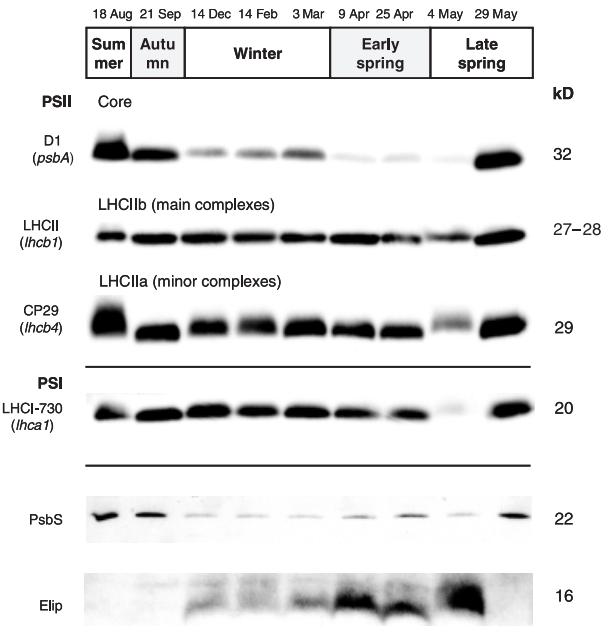

Fig. 3 Western blots of SDS-PAGE reflecting changes in chlorophyll binding and reaction centre proteins during the seasons. Samples were probed with antibodies raised against the reaction centre D1 protein of photosystem II (PSII), the light-harvesting chlorophyll (Chl) binding proteins LHCII, CP29, LHCI-730 and the PsbS and early light-induced protein (Elip) protein (corresponding gene in brackets). Samples were collected on indicated dates at solar noon. Lanes were loaded on equal protein basis.

followed again by a last drop in spring. D1 content finally recovered in late May. By contrast, LHCII (the major light harvesting complex of PSII), CP29 (representing one of the monomeric minor LHCII complexes) and LHCI-730 of PSI remained fairly high throughout winter. These proteins did, however, show a transient but drastic decrease during the spring recovery period between 25 April and 04 May, with full recovery only in late May.

Levels of the PsbS protein, which is involved in NPQ, roughly paralleled the changes in D1. Highest levels occurred from late spring through autumn, followed by lower levels throughout winter, subsequently increasing in spring with the exception of one low value measured in mid-to-late spring. A complementary pattern of changes was observed in levels of the Elip (Fig. 3).

Dynamics of the spring recovery

Environmental variability during spring. Maximum PPFD (from the 30 min mean values) was already high during spring, with maximum values around 1300 µmol $m^{-2} s^{-1}$ in the beginning of April and 1500 µmol $m^{-2} s^{-1}$ in May (Fig. 4a). I_{mean} doubled, increasing from 20 to 40 mol $m^{-2} day^{-1}$ in April, but was again lower, around 30 mol $m^{-2} day^{-1}$, due to increased cloudiness for most of May (Fig. 4a). Although PPFD was already high in early spring, air temperature was typically below 0 °C, with daily minima typically between −12 and −6 °C and reaching as low as −20 °C in April (Fig. 4b). On some occasions, however, maximum air temperatures went as high as 10 °C during the day (e.g. 18 April), conditions that if sustained would be favourable for photosynthesis. In late spring, minimum air temperatures only occasionally dropped below 0 °C, and then only during clear cold nights or early in the morning, when there was little to no irradiance coming into the forest.

Soil temperature, on the contrary, was uncoupled from short time changes in air temperature. Because of the buffering snow cover of the ground, soil temperature at 5 cm depth did not vary for most of the spring period and remained between 0 °C and −1 °C until 23 May (Fig. 4b).

Photosynthetic activity at the canopy level during spring. Net ecosystem CO_2 exchange (NEE) was measured with an eddy flux system and is represented by the daily mean NEE (Fig. 5). Net respiration is indicated by positive values with release of CO_2 to the atmosphere, whereas net photosynthesis is indicated by negative fluxes, with uptake of atmospheric CO_2. During early

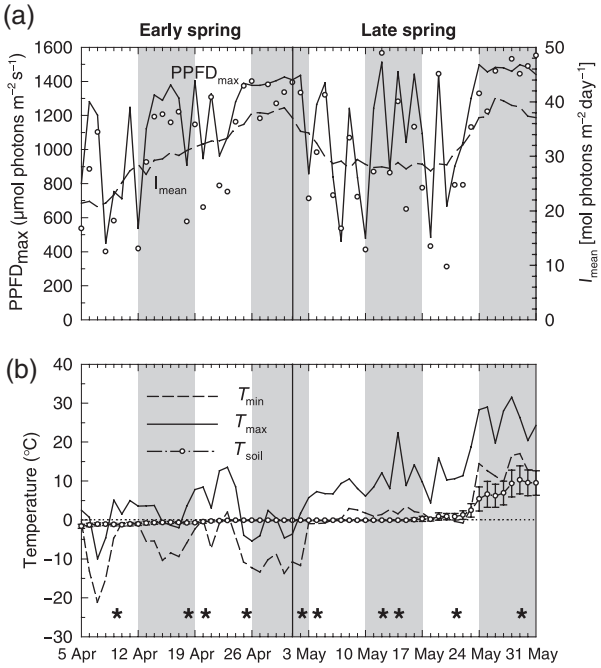

Fig. 4 Spring climate at Zotino field site. (a) Maximum photosynthetic photon flux density during the day ($PPFD_{max}$, solid line), 5-day running mean of the incoming photosynthetically active radiation (I_{mean}, in mol photons $m^{-2} day^{-1}$, dashed line), open circles denote daily mean values; (b) Air temperature given as daily minimum (T_{min}), daily maximum (T_{max}) and daily mean soil temperature (T_{soil}) measured at 5 cm depth as the average value of $n = 4$ measurements ± standard deviation. Asterices indicate dates on which chloroplast proteins were detected by SDS-PAGE and immunoblotting (see Figs 3 and 8).

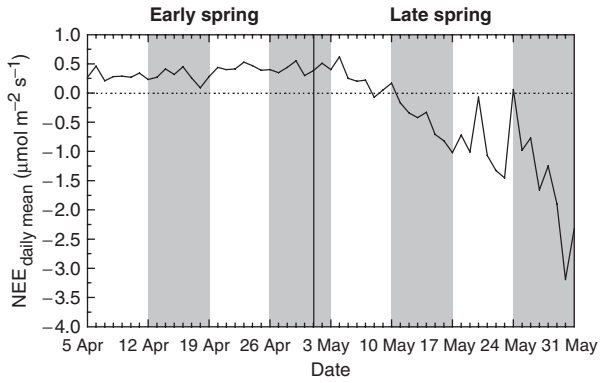

Fig. 5 Time course of the net ecosystem CO_2 exchange rate (NEE) as measured with the eddy covariance system above the canopy ($NEE_{daily\ mean}$). Positive values indicate net respiratory release of CO_2 to the atmosphere, and negative values indicate net photosynthetic uptake.

spring, only low respiration rates with mean fluxes between 0.25 and 0.5 µmol $m^{-2} s^{-1}$ CO_2 occurred in the field. The onset of detectable canopy-level photosynthesis was on 4 May when NEE started to

decrease, coinciding with the rise of mean air temperature above 0 °C (Fig. 4b). The first day showing net photosynthesis was on 8 May (Fig. 5), when minimum temperatures were finally established well above 0 °C (Fig. 4b). In addition, in May the potential for photoinhibition was lower than in late April, since PPFDs were lower and temperatures were higher (Fig. 4a). During the May recovery of NEE, two major disturbances of the trend in NEE were observed. On 18 May, NEE increased from −1.0 back to −0.7, while on 21 May NEE increased from −1.0 to near zero (Fig. 5) because of extensive cloud cover (Fig. 4a). But for 24 May, the sharp increase in NEE coincided with sharp temperature increases and the sudden rise of soil temperature above 0 °C (Figs 4b and 5), which presumably resulted in a large increase of CO_2 flux from soil respiration.

Photosynthetic activity at the needle level during spring. For samples taken from the field and assayed in the laboratory at 20 °C, Figure 6 shows the changes in the rate of photosynthetic oxygen evolution, optimum and apparent quantum yield of PSII (F_v/F_m and yield, respectively) as well as in NPQ throughout the spring period. Freezing temperatures and night frosts on 21 April, 24 April until 2 May, 21 May and 22 May coincided with drops in rates of net photosynthetic oxygen exchange and a lower quantum yield, suggesting a detrimental effect on the recovery processes. In particular, F_v/F_m proved to be a strong indicator of these temperature-induced disturbances. By contrast, dark respiration did not show any coincidence with frost duration or intensity.

23 April was the first day of the year without freezing temperatures, on which maximum PPFD reached around 900 μmol photons $m^{-2} s^{-1}$. On 23 April, increases in oxygen evolution and quantum yield were already observed under favourable laboratory conditions. The following day was, however, again characterized by freezing temperatures, and oxygen evolution and quantum yield decreased immediately. Air temperature then remained below 0 °C until 2 May, while maximum PPFD and integrated incoming photosynthetically active radiation stayed fairly high. But then, with air temperatures above 0 °C and decreased irradiance, photosynthetic parameters and dark respiration (−5.3 μmol O_2 $m^{-2} s^{-1}$) increased again.

Onset of photosynthesis and consistent changes at the canopy and at the needle level. From the fluxes observed at the canopy level as well from data obtained with needles in the laboratory, 8 May was an important date. Following on from a couple of relatively warm days of only moderate I_{mean}, on this warm day dark respiration rates

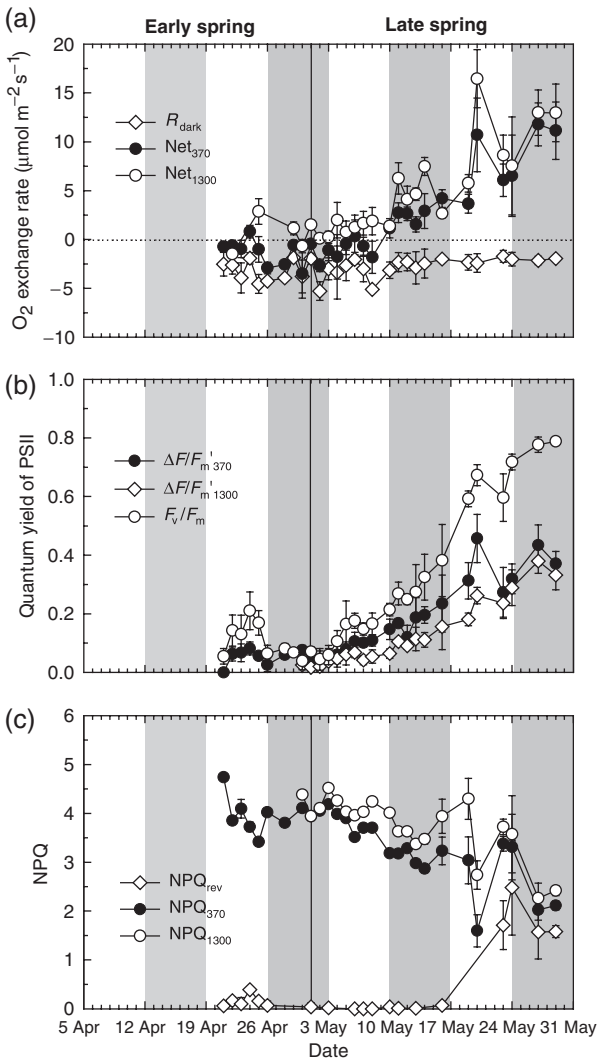

Fig. 6 Time course of spring recovery of photosynthesis in *Pinus sylvestris*. (a) Dark respiration (R_{dark}) and photosynthetic net O_2 evolution (Net_{370} and Net_{1300}); (b) photosystem II (PSII) quantum yield (F_v/F_m), and effective yield of open centres under illumination ($\Delta F/F_m'$); (c) Nonphotochemical quenching (NPQ) and the reversible component of NPQ (NPQ_{rev}). Respiration and F_v/F_m were obtained from samples after 20 min adaptation in the dark. Closed and open circles indicate data obtained from samples measured at PPFDs of 370 and of 1300 μmol $m^{-2} s^{-1}$, respectively. Each data point represents the mean of $n = 1$–3 measurements (± SD).

increased to −5.1 μmol O_2 $m^{-2} s^{-1}$, whereas F_v/F_m still expressed low photochemical efficiency of PSII (Fig. 6a, b). At the canopy level, photosynthesis matched respiration as CO_2 flux indicated the first day of net carbon uptake by the stand in 2001.

NPQ generally decreased during spring (Fig. 6c) but transiently increased (e.g. 16 and 18 May) on days with high $PPFD_{max}$. In contrast to NPQ measured under illumination at 370 and 1300 μmol $m^{-2} s^{-1}$, the reversible

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component of NPQ (NPQ_{rev}) measured after 10 min of dark acclimation remained low for most of the spring period. Therefore, much of the substantial observed NPQ in early spring reflected an only limited dynamically regulated dissipation of excess absorbed light energy. The difference between NPQ and NPQ_{rev} thus represented sustained winter-type excitation dissipation with constitutively increased levels of NPQ, because dissipation via photochemistry was mostly impaired during the cold season. Until 23 May, partial relaxation of photosynthesis from sustained winter quenching was only observed during the first warm period from 21 April until 24 April, when NPQ_{rev} also showed an increased contribution of dynamic NPQ. This was not, however, observed during the early phases of the second recovery phase that occurred after about 3 May.

Photosynthetic pigments during spring. Chl concentrations were variable during early spring and were lowest after the cold spell at the end of April (Fig. 7a). With increasing temperatures and moderate light conditions from early to mid-May, the amount of Chl a and b increased again, while the amount of carotenoids remained fairly constant throughout the spring (Fig. 7a). The carotenoid composition, however, varied greatly. The pool of the xanthophyll cycle pigments (V + A + Z) expressed on a total carotenoid basis decreased significantly after 15 May compared with the first half of May (paired sampled *t*-test, Fig. 7b). Lutein increased, once the overall warming had commenced and in early May, levels were significantly lower than those observed after 15 May (paired samples *t*-test, Fig. 7b).

The de-epoxidation state of the xanthophyll cycle pigments (DEPS), expressed as (0.5A + Z)/(V + A + Z), indicates the chemical conversion of xanthophylls into the de-epoxidized form, with zeaxanthin being completely de-epoxidized and antheraxanthin by only 50%. DEPS was above 0.9 early on in spring, indicating sustained conversion of the V + A + Z pool into zeaxanthin. The first dip in DEPS appeared on 23 April. This coincided with the first warm days and the fairly low maximum PPFD on this day, and the rise in quantum yield and oxygen evolution mentioned above. On the following days, temperatures dropped again to below freezing and maximum PPFD increased DEPS again. The relaxation of sustained high levels of DEPS from winter stress was clearly evident only after 8 May and thus started after the last heavy frost events had occurred after 3 May (Fig. 4b and Fig. 7). Therefore, the pattern of declining DEPS in May was consistent with the transition in NEE from net respiration to net photosynthesis.

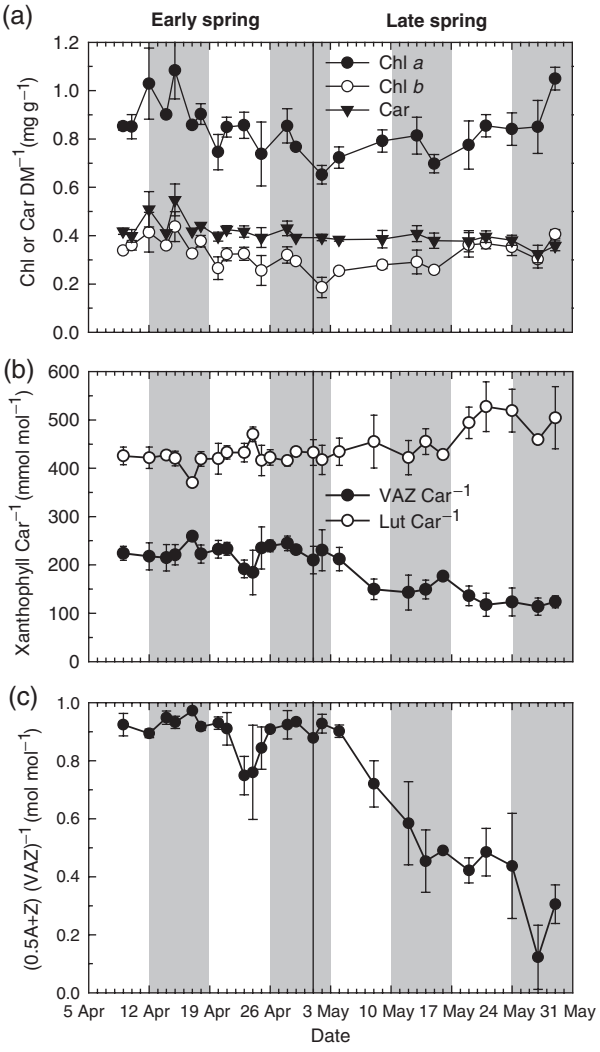

Fig. 7 Time course of spring recovery of photosynthesis in second-year needles of *Pinus sylvestris*. (a) Chlorophyll a and b (Chl a and Chl b) and carotenoids (Car) on a needle dry mass (DM) basis; (b) xanthophyll cycle pigments per total carotenoids ((V + A + Z) Car^{-1}) and lutein per total carotenoids ($Lut\,Car^{-1}$); (c) de-epoxidation status of the xanthophyll cycle pigments [(0.5A + Z) $(V + A + Z)^{-1}$]. Each data point represents the mean of $n = 1$–3 samples (± SD). V, violaxanthin; Z, zeaxanthin; A, antheraxanthin.

Spring recovery and proteins of the chloroplast

Levels of the reaction centre protein D1 (Fig. 8) and fluorescence parameters (Fig. 6) indicated the presence of low levels of functional PSII carried over from the winter acclimated state during the dehardening in spring. In addition, a second band occurring above the 32 kD region reflected increasing amounts of D1 precursor and hence potentially increasing turnover of D1 under light stress conditions. This was, however, impaired by freezing temperatures and increased PPFD

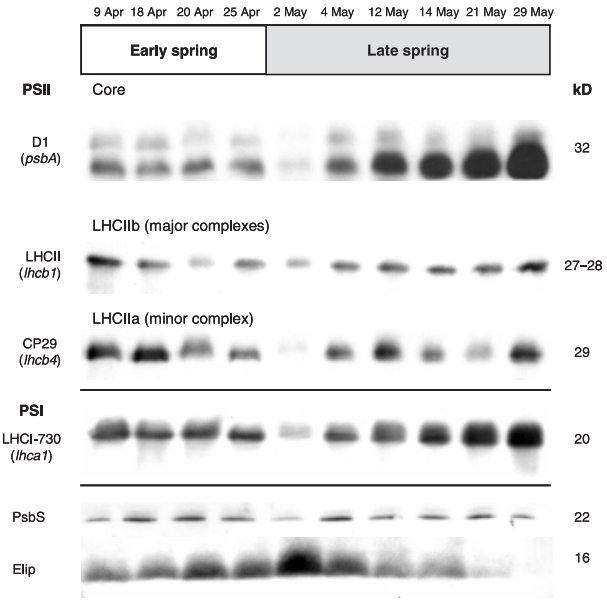

Fig. 8 Western blots of SDS-PAGE reflecting changes in chlorophyll binding proteins during the spring recovery of photosynthesis. Samples were probed with antibodies raised against the reaction centre D1 protein of photosystem II (PSII), the light-harvesting Chl binding proteins LHCII, CP29, LHCI-730 and the PsbS and early light-induced protein (Elip) proteins (corresponding gene in brackets). Samples were collected on indicated dates at solar noon. Lanes were loaded on equal protein bases.

after 24 April, resulting in the loss of D1 protein, decreased F_v/F_m (Fig. 6) and loss in Chl (Fig. 7). Winter levels of LHCII decreased slowly with increasing PPFD during early spring and did not recover until end of May. CP29 and LHC-730 decreased after the cold spell after 24 April. The subsequent recovery observed upon the more sustained rise in air temperature after 5 May revealed different patterns amongst the different proteins. D1, LHCII and LHCI-730 increased constantly until 29 May, whereas CP29 levels decreased after the frost of 21 May.

PsbS decreased during loss of D1 as well, but increased immediately upon exposure to warmer temperatures and moderate light conditions later on in May. Elip protein, on the other hand, showed a complementary increase (Fig. 8) during the freezing at the end of April. In the absence of strong photoinhibitory conditions after May 2, the expression of D1 and PsbS rapidly increased again, whereas Elip decreased.

Parameters controlling spring recovery of photosynthesis

Measurements of stand NEE, chlorophyll fluorescence and oxygen evolution provided data on spring recovery of photosynthesis in Scots pine. We used the general linear model (GLM) procedure with a forward selection method within a univariate analysis of variance to test for statistically significant effects of the environmental factors shown in Fig. 4 on the photosynthetic parameters during the spring period. Relevant factors and DEPS were then used for further linear regression analysis. The changes in photosynthetic parameters were best explained by their significant relation with either the 5-day running mean of air temperature or by DEPS (Table 1), DEPS itself being closely correlated with the combined effects of maximum photon flux density and minimum air temperature (Table 1). Using the linear regression models (Table 1), we calculated the threshold values for positive net photosynthesis as affected by DEPS. We found very similar DEPS levels to be required for positive photosynthetic rates during spring onset of photosynthesis, being 0.75 for the whole canopy gas exchange (NEE, Fig. 9a) and 0.78 on the needle level (O_2Net_{370}, Fig. 9b). Stem (data not shown) and soil temperature did not reveal statistical relationships to the parameters describing photosynthesis.

Discussion

Downregulation of photosynthesis during cold hardening and winter stress

Acclimation during autumn included loss of chlorophylls to lower light absorption (see also Ottander *et al*., 1995; Vogg *et al*., 1998; Gilmore & Ball, 2000), along with an increase in xanthophylls, which could increase the potential for photoprotection via the xanthophyll cycle in the LHC complexes (Figs 2c and 7b, c; Demmig Adams & Adams, 1996a, b; Horton *et al*., 1996). Interestingly, although autumn hardening involved some loss of LHC, the antenna proteins LHCII, CP29 and LHCI-730 stayed relatively high during the winter period. The major LHCII complex binds about 60% of the Chl and is a major binding site for lutein, whereas proteins of the minor antenna complexes like CP29 bind only about 5% of the Chl and are binding sites for violaxanthin and zeaxanthin (Horton *et al*., 1996; Bassi & Caffarri, 2000). Perhaps even more important with respect to NPQ is the involvement of PsbS in the xanthophyll cycle involved in the generation of reversible NPQ (Li *et al*., 2000). Although zeaxanthin stayed high, PsbS levels declined during winter in parallel with loss of D1, but Elip (that also bind xanthophylls) transiently increased. This is consistent with the assumption that Elip, interacting with xanthophylls, may have a role in photoprotection, but more data are needed to verify this hypothesis. In winter, the rapidly reversible NPQ_{rev} component of thermal dissipation was low, in parallel with the low levels of PsbS

Table 1 Factors affecting spring recovery and onset of photosynthesis of field-grown *Pinus sylvestris* from the Central Siberian stand at Zotino

Indicator	Variable	Adjusted R^2	Significance	Regression model
F_v/F_m	DEPS	0.874	$P<0.0001$	$y=-0.972x+0.941$
	T_{mean}	0.856	$P<0.0001$	$y=0.035x+0.142$
O_2 Net_{370} ($\mu mol\,m^{-2}\,s^{-1}$)	DEPS	0.867	$P<0.0001$	$y=-16.511x+12.824$
	T_{mean}	0.811	$P<0.0001$	$y=-0.625x+0.600$
NEE ($\mu mol\,m^{-2}\,s^{-1}$)	DEPS	0.783	$P<0.0001$	$y=-1.924x+2.574$
	T_{mean}	0.743	$P<0.0001$	$y=0.106x+0.168$
DEPS [$(0.5V+Z)\,(V+A+Z^{-1})$]	$PPFD_{max}$ (x_1), T_{min} (x_2)	0.706	$P<0.003$	$y=-0.0003x_1-0.0243x_2+1.025$

DEPS, de-epoxidation state of the xanthophyll cycle pigments; NEE, net ecosystem CO_2 exchange; PPFD, photosynthetic photon flux densities; V, violaxanthin; Z, zeaxanthin; and A, antheraxanthin.

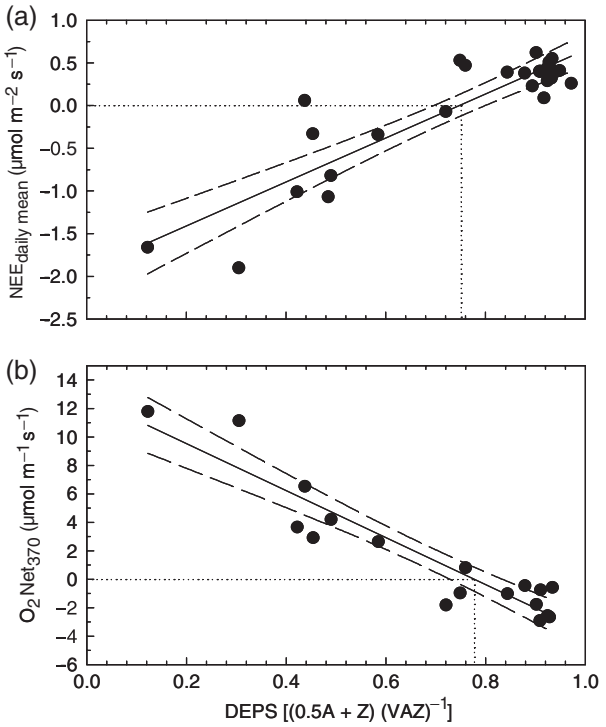

Fig. 9 Relationship between de-epoxidation status of the xanthophyll cycle pigments [$(0.5A+Z)\,(V+A+Z)^{-1}$] and net ecosystem CO_2 exchange rate (NEE) as measured with the eddy covariance system above the canopy ($NEE_{daily\ mead}$) (a), and with needle-level photosynthetic net O_2 evolution (O_2Net_{370}) (b). Dashed lines indicate 95% confidence limits of the regression line. Intercepts with the *x*-axis are 0.75 in (a) and 0.78 in (b). Details of the linear regression model are given in Table 1. V, violaxanthin; Z, zeaxanthin; A, antheraxanthin.

but in marked contrast to the winter accumulation of zeaxanthin. This pattern supports a different localization and function for zeaxanthin as a scavenger of oxidative radicals during the prolonged winter down-regulation, rather than simply as an element of the mechanism for inducible NPQ (Havaux & Niyogi, 1999; Verhoeven *et al.*, 1999b), which is largely suppressed during winter stress in Scots pine in favour of more sustained quenching mechanisms.

The observed loss of PsbS in parallel with that of D1 during autumn and winter (Fig. 3) contradicts earlier data showing increased contents of PsbS in pine under winter and artificial frost-hardening conditions when the content of D1 drops (Ottander *et al.*, 1995; Savitch *et al.*, 2002). Furthermore, Ottander *et al.* (1995) report a stronger autumn induced drop of the light harvesting antenna in Scots pine than we observed here. At present, we do not understand these different results but we tentatively ascribe them to different climatic conditions in the more maritime winter climate of Scandinavia (Ottander *et al.*, 1995), compared with the more continental climate of Central Siberia in our study; PsbS may play an important role in dynamic NPQ but not in the strong sustained winter quenching of Siberian Scots pine as suggested earlier (Öquist & Huner, 2003).

Cold hardening, and particularly the subsequent winter stress conditions, also involved drops in the levels of the PSII reaction centre protein D1. (Figs 2 and 3). Net degradation of D1 under excessive light is well established (Sundby *et al.*, 1993; Anderson *et al.*, 1997) and a major loss in winter in *P. sylvestris* confirms earlier findings (Ottander *et al.*, 1995). Similarly, degradation of the major LHC is a regulated proteolytic process during the acclimation to high light (Yang *et al.*, 1998). Under cold temperature and illumination, down-regulation of functional PSII and LHC and the consequent drop in PSII photochemistry are important protective mechanisms.

Spring recovery and dehardening. Spring recovery of photosynthesis in Central Siberian Scots pine is a complex process of assembly, degradation and changes in properties of the chloroplast. The patterns

observed here demonstrate the importance of spring light and temperature episodes in the boreal zone (Table 1, Figs 4 and 8). In late March/April, sunlight increases quickly because of the rapidly increasing solar angle. The ground is usually still snow covered so a large fraction of the radiant energy is reflected through the albedo effect. As a consequence, air temperatures remain low during the early phase, while the needles of Scots pine are exposed to rapidly increasing PPFD. This high light/low-temperature environment in early spring suppresses photosynthesis, as shown in our data by low photochemical energy conversion yields for PSII both in the light and after brief dark acclimation (Fig. 6b), increased NPQ (Fig. 6b) and low fluxes in gas exchange both at the stand and at the needle level (Figs 5 and 6a). The early spring between mid-March and end of April therefore places high demands on photoprotection with highest amounts in xanthophyll cycle pigments (mainly Z) in early spring (e.g. 12 until 17 April), rather than during the colder but darker winter season (Figs 1 and 2c). During the period 2 May until the end of May (referred here as late spring), the fraction of xanthophylls decreased, emphasizing the importance of V + A + Z during sustained winter stress (Fig. 2c).

When warm temperatures and moderate light occurred during spring, as on 23 April, DEPS considerably decreased (Fig. 7c), but was immediately recovered upon re-exposure to cold temperatures and excess light. Such conditions should acidify the chloroplast thylakoid lumen and keep the luminal violaxanthin de-epoxidase enzyme activated (Eskling *et al*., 2001), thus favouring the almost complete conversion of V into Z not only in winter but especially under early spring conditions.

In late spring (from 15 May), lutein increased, perhaps reflecting a requirement for increased triplet quenching capacity in functional chloroplasts through lutein (which may account for more than 90% of Chl* quenching as suggested by Bassi & Caffarri, 2000). The preferred binding sites of lutein are the LHC proteins encoded by the genes *lhca1*, *lhcb1* and *lhcb4*, which increased in parallel with lutein. (The *lhcb5* and *lhcb6* are also preferred binding sites, but these were not studied here.) Together, the concomitant increase in lutein and LHC protein clearly indicates the developmental changes during late spring onset of photosynthesis in Scots pine.

With increases in mean temperatures from 1 May, (V + A + Z)/Car and DEPS decreased in late spring in the dehardening process (Strand & Öquist, 1988; Beck *et al*., 1995), due to the effect of $PPFD_{max}$ and T_{min} on DEPS as revealed by our statistical analysis (Table 1). The calculated threshold values predict positive rates of photosynthesis for DEPS below 0.75 (for needles) and 0.78 (for the whole canopy), which are within the range of DEPS measured on 8 May, the first day in 2001 expressing net carbon uptake of the Scots pine stand (Fig. 5). In addition, the values also correspond with DEPS measured on 23 April. During a moderately warm period, on this day we observed increased photosynthetic activity (Fig. 6), obvious changes in xanthophyll cycle pigments (Fig. 7b, c) and increased levels of chlorophyll binding proteins (20 April). Nevertheless, no detectable increase in carbon uptake of the ecosystem was measured in the field (Fig. 5). These findings show that photosynthetic reactivation in winter-stressed needles correlates with a sustained drop in DEPS below about 0.8. However, these findings do not rule out the possibility that the onset of photosynthesis is an indirect effect due to warmer soil temperatures as suggested for a high-elevation subalpine forest in Colorado with harsh winter growth conditions (Monson *et al*., 2002). In this high-elevation forest, reactivation of photosynthesis in spring was tied to soil temperature and the availability of liquid water after the snow melt. Studies across a range of Eurasian boreal forests have shown that once snow melt has commenced, soil temperatures typically rise immediately to close to 0 °C, remaining almost invariant at this 'zero curtain' level until snow melt is completed. During this time, there is almost certainly sufficient liquid water available to support any air temperature-controlled photosynthetic recovery (Suni *et al*., 2003).

By mid-May, sustained winter quenching had relaxed and sustained inhibition of photosynthesis had ended (Figs 6c and 7c). Some recovery had already taken place considerably earlier, but was constrained by the previous frost period around 25 April. The effect of this frost was visible on the decreased protein levels of D1, LHCII, CP29 and LHCI-730 in samples from 2 May, suggesting that intermittent frost can reverse the reorganization of the photosynthetic apparatus and thereby delay the full photosynthetic exploitation of otherwise favourable conditions. On the level of thylakoid bound proteins, protective processes involve complementary functions for PsbS and Elips. PsbS is constitutively present in functional PSII complexes (Funk, 2001). Loss of these complexes involved a concomitant decrease of PsbS at the end of April/early May under low-temperature/high light conditions (Fig. 8). Elip protein, which is proposed to accumulate in nonappressed regions of thylakoid membranes where photodamaged PSII complexes migrate (Adamska, 2001), accumulated during low-temperature conditions. This supports a protective function for Elip protein during light stress and low temperature, with a transient binding of free

chlorophyll molecules in a pigment-carrier function to prevent the formation of free radicals (Noren *et al.*, 2003).

Seasonal changes in photosynthesis in a Central Siberian Scots pine. The dynamic changes in the photosynthetic machinery during the winter season in Siberian Scots pine suggest that after cold hardening, physiological activity does not completely cease during winter. Instead, there is photometabolic activity, indicated by the variable amounts of reaction centre protein D1 or changes in F_v/F_m, which are linked to the variable climate during the cold season. In this respect, cold hardiness in evergreen conifers allows for a continuum of opportunistic acclimation processes that balance light absorption and light utilization and control the arrangement of the safety systems of the photosynthetic machinery. A major process involved in this winter acclimation is probably linked to the stoichiometry of active PSII vs. PSI that allows the adjustment of photosynthetic electron transport during winter (Ivanov *et al.*, 2001).

Possible consequences in the context of anticipated future change in global climate. Because the net carbon balance of boreal forests is the small residual of two much larger fluxes, photosynthesis and respiration, the carbon status of these forests is extremely sensitive to climate (Goulden *et al.*, 1998). Understanding of seasonal patterns of photosynthesis like the hardening and dehardening processes and their modulation by abiotic factors thus becomes an important issue in predicting tree responses to anticipated future climate changes (Saxe *et al.*, 2001). Here we report a continuing adjustment of photosynthetic metabolism to the variable winter climate. In contrast to the initial autumn cold hardening process in Scots pine, triggered by a rapidly declining photoperiod (Strand & Öquist, 1988), our data suggest that the onset of photosynthesis in spring is driven by the physiological state of the chloroplast, including the excitation pressure on PSII and PSI as a result of the light and temperature regime, imposed by the surrounding aerial environment. In principle, this suggests that earlier and increased spring photosynthesis should have occurred for the boreal conifer forests in response to generally warmer temperatures over the last few decades.

However, we observed that intermittent frost constrains the recovery of photosynthesis and delays the spring transition from positive to negative NEE. This, together with year-to-year variations in spring temperatures, might have a considerable impact on the future carbon balances of these forests and presumably account for much of the observed interannual variability in carbon acquisition of boreal forests (Randerson *et al.*, 1999). Moreover, the damage to the photosynthetic machinery involves not just high respiratory costs for repair. Once early dehardening has occurred, the costs for maintaining the cellular systems in the observed state primed for recovery will increase, due to increased maintenance respiration and concomitant loss of carbohydrate reserves (Ögren, 1997).

In terms of the carbon budget of the forest, we cannot yet quantify from our data to what extent the positive response of photosynthesis to generally warmer spring temperatures will be counteracted by the costs of repair and increased maintenance respiration for subsequent frost and cold temperature episodes. But a promising tool to estimate such effects on the ecosystem level is the de-epoxidation status (DEPS) of the xanthophyll cycle pigments. Here we have demonstrated that the spring pattern of NEE correlates with specific environmental, molecular, pigment and physiological changes. These changes, well predicted by DEPS, could be detected by remote sensing (e.g. Gamon *et al.*, 1990; Nichol *et al.*, 2000, 2002) and used to modify NEE estimates to account for transient chilling episodes during the spring reactivation phase (Rahman *et al.*, 2001).

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