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Norwegian University of
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Too Much of a Good Thing

Photoprotection in Boreal Conifers During Winter

Rannveig Bø Fløystad

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Supervisor: Richard Strimbeck, IBI

Norwegian University of Science and Technology
Department of Biology

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Rannveig Bø Fløystad

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Abstract

Photochemical utilization of light energy is inhibited in boreal conifers during winter and spring because the carbon fixation reactions slow down due to low temperature. Incoming light can be very strong due to reflection from the snow cover. This constitutes a considerable physiological stress to the plant and excess energy must be dissipated as heat through non-photochemical quenching (NPQ) to avoid damage. The effects of light, temperature and ecological adaptions on NPQ that remains active day and night, termed sustained NPQ, were investigated in this study. Current-year shoots from four species of *Picea* were exposed in growth chambers to combinations of high and low light (200/80 μmol m^{-2} s^{-1}) and high and low temperature (10/0 °C) in a 2×2×4 factorial experiment. Maximum photochemical efficiency of photosystem II (F_v/F_m) was measured pre-dawn using chlorophyll fluorimetry. A mixed effects model was fitted, which showed that high light and low temperature caused strong reduction in F_v/F_m. This indicated sustained non-photochemical quenching. The effect of light was stronger at low temperature. *P. sitchensis* showed the most flexible response to changes in light and temperature. This is probably a beneficial adaption in an oceanic climate, where mild periods allow photosynthesis during winter. A similar recovery experiment where the shoots were brought to room temperature before measurement confirmed that it was principally ΔpH-independent sustained NPQ that was observed in the main experiment.

It was hypothesized that light stress in combination with low temperature is the cause of the chlorotic foliage observed in conifers in spring. Color analyses were done by pixel count in images of the samples. No sign of winter chlorosis was found. Violaxanthin-levels, measured using HPLC, were lower for high-stress treatments, indicating participation of the xanthophyll cycle in sustained NPQ. No differences in expression of the protein PsbS between the treatments were found. This is supportive for the hypothesis that even if PsbS is involved in rapidly reversible NPQ, it is not involved in the sustained form of NPQ.

Sammendrag

Fotokjemisk utnyttelse av absorbert lysenergi blir forhindret i boreale bartrær om vinteren og våren fordi lav temperatur senker takten på karbonfikseringsreaksjonene. Samtidig kan lysintensiteten være svært høy på grunn av refleksjon fra snødekket. Dette utgjør et betydelig fysiologisk stress for planten, og overskytende energi må avgis som varme via ikke-fotokjemisk energiavgivelse (IFE) for å forhindre skade. I denne studien ble effekten av lysintensitet, temperatur og økologiske tilpasninger på ikke-fotokjemisk energiavgivelse som opprettholdes hele døgnet, såkalt vedvarende IFE, undersøkt. Fire arter i slekten *Picea* ble eksponert for kombinasjoner av lys (200/80 μmol m^{-2} s^{-1}) og temperatur (0/10 °C) i et 2×2×4 faktorialt eksperiment. Maksimal fotokjemisk effektivitet av fotosystem II F_v/F_m ble bestemt ved hjelp av klorofyll fluorimetri. En lineær modell som ble tilpasset dataene viste at sterkt lys og lav temperatur ga en betydelig nedgang i $F_v/F_{m,}$, hvilket indikerer vedvarende IFE. Effekten av lys var sterkere ved lav temperatur. *P. sitchensis* viste en mer fleksibel respons enn de andre artene. Dette er trolig en gunstig tilpasning til et kald-temperert klima der varmeperioder muliggjør fotosyntese deler av vinteren. Et oppfølgningseksperiment der skudd ble oppbevart i romtemperatur i 30 minutter før fluorescens-målingene ble gjort bekreftet at det i all hovedsak var ΔpH-uavhengig vedvarende IFE som ble observert i hovedeksperimentet.

Det ble foreslått at den overnevnte stressituasjonen forårsaker klorose i bartrær om våren. Fargeanalyse av skuddene ble gjort med bildepunkt-telling i fotografier av prøvene, men ingen tegn til klorose ble funnet for noen av behandlingene. Violaxanthin-innholdet i skuddene bestemt ved hjelp av HPLC. Det var lavere violaxanthin-innhold i skudd som ble utsatt for både sterkt lys og lav temperatur, hvilket indikerer at xanthofyll-syklusen er involvert i vedvarende ikke-fotokjemisk energiavgivelse. Det ble ikke funnet noen forskjell i uttrykk av proteinet PsbS mellom behandlingene. Dette tyder på at PsbS ikke er involvert i vedvarende IFE, selv om det er involvert i rask reversibel IFE.

Abbreviations

A	Antheraxanthin
AIC	Akaike information criterion
ATP	Adenosine triphosphate
BME	β-mercaptoethanol
Chl	Chlorophyll
D1	PSII reaction center polypeptide
DEPS	De-epoxidation state
ELIP	Early light-induced protein
EPS	Epoxidation state
ETC	Electron transport chain
F_0	Minimal chlorophyll fluorescence from dark-adapted leaves
F_m	Maximal chlorophyll fluorescence from dark-adapted leaves
F_v	Variable chlorophyll fluorescence in dark adapted leaves
F_v/F_m	Maximum quantum efficiency of PSII photochemistry in dark-adapted leaves
HL	High light treatment
HPLC	High performance liquid chromatography
HT	High temperature treatment
kDa	kilodalton
LL	Low light treatment
LT	Low temperature treatment
LT_{50}	Median lethal temperature
LUT	Lookup tables
MCMC	Markov chain Monte Carlo
ML	Maximum likelihood
NPQ	Non-photochemical quenching
P_{680}	Central reaction center chlorophyll molecule
PAM	Pulse amplitude modulation
Pheo	Pheophytin
PPFD	Photosynthetic photon flux density
PQ	Plastoquinone
PsbS	PSII subunit and gene product of *PsbS*
PSII	Photosystem II
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene difluoride
Q_A	Plastoquinone molecule at site A in PSII reaction centers
Q_B	Plastoquinone molecule at site B in PSII reaction centers
REML	Restricted maximum likelihood
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
V	Violaxanthin
VDE	Violaxanthin de-epoxidase
Z	Zeaxanthin
ZE	Zeaxanthin epoxidase

1. Introduction

1.1. Winter ecophysiology of boreal conifers

Some places on earth are more inhospitable than others. The temperature in the continental climates of interior Canada and Siberia can stay extremely low for months during winter. For example, the monthly mean minimum temperature of Yakutsk in Siberia stays below -10 °C for half of the year, and reaches as low as -44 °C in January (Hyderometeorological Centre of Russia, 2013). Still, conifers like spruces (*Picea*) and firs (*Abies*) inhabit these and other extreme climates, and these trees even retain their foliage throughout the winter (Eckenwalder, 2009). In contrast to the ground vegetation, snow cover cannot offer the crown of the trees any insulation. Conifers thus require physiological adaptions to avoid tissue death. Frost tolerance, including accumulation of sugars, dehydration and possibly vitrification of cytoplasm (Strimbeck and Schaberg, 2009), is an essential feature. Additionally, there is need for a protection mechanism against too much light in cold conditions during winter and spring (Oquist and Huner, 2003).

1.2. The problem of excess light in a seasonally cold environment

Although plants rely on light for photosynthesis, they are frequently exposed to more light energy than they can utilize. For plants in most environments, fluctuations in incoming light can be strong in short time spans due to passing clouds, shading from other leaves, or solar intensity variations throughout the day (Larcher, 2003). However, conifers in boreal climates experience too much light energy for extended periods. During winter and spring, low temperature causes the enzymatic carbon fixation reactions in the Calvin cycle to slow down (Oquist and Huner, 2003). There is also a reduced rate of energy consumption since growth ceases in winter (Huner et al., 1993). However, light harvesting by photosynthetic pigments in the evergreen needles is unaffected by temperature (Blankenship, 2002) and incoming light can, especially in spring, be very strong due to reflection from snow cover. This increases the excitation rate of the chlorophyll molecules. The imbalance between energy harvest and consumption leads to an energy imbalance in the leaves during winter (Huner et al., 1998).

The primary photochemical event in photosynthesis takes place in the reaction center of photosystem II, embedded in the thylakoid membrane (Figure 1.1), where light energy is

converted to chemical energy (Blankenship, 2002). As a chlorophyll molecule in the antenna absorbs a photon of light, one of the chlorophyll's electrons is placed in a higher excitation state (Nobel, 2009). Antenna chlorophylls transfer the electronic excitation to the chlorophyll molecule of the reaction center (P_{680}). The excited electron of P_{680}* is then donated to an acceptor molecule, pheophytin. Subsequent energy transfer to Q_A^- and other downstream components in the electron transport chain happens via redox reactions. The rate-limiting step in a cold environment is the oxidation of reduced quinone A (Q_A^-), because downstream carbon fixation processes cannot oxidize the Q_A at the same rate as it is reduced by P_{680}* (Huner et al., 1998). In plants experiencing low temperatures and strong light, a higher share of the total Q_A-pool is thus reduced. As long as Q_A is reduced, the electron transport chain is blocked and the reaction centers cannot process incoming excitations from the antenna chlorophylls. The reaction center is therefore said to be closed and plants with closed reaction centers experience an increased *excitation pressure* (Huner et al., 1998). The ratio $[Q_A^-]/([Q_A^-] + [Q_A])$ gives an estimate of the relative PSII excitation pressure to which the organism is exposed. High light and low temperatures will increase the excitation pressure.

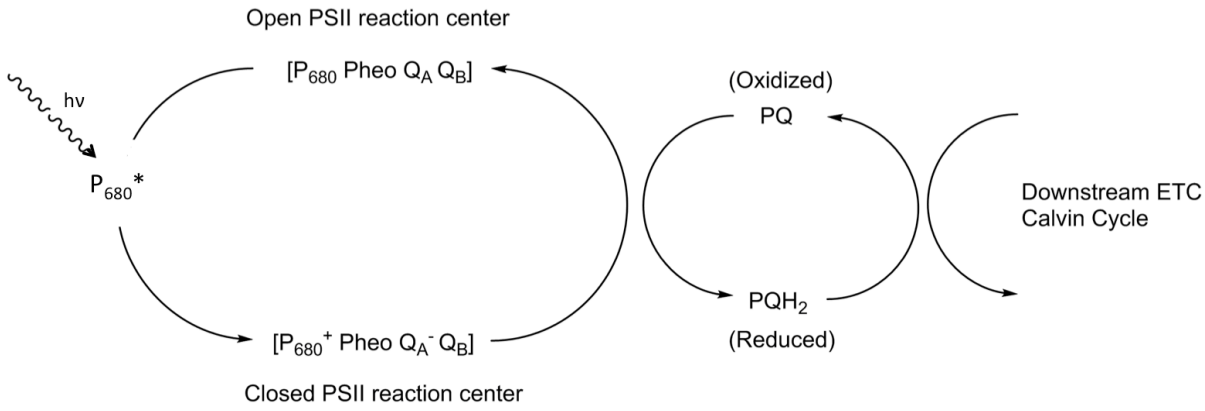

Figure 1.1: Schematic representation of the photochemical event in the reaction center of PSII. The excited chlorophyll molecule (P_{680}*) produces the radical pair P_{680}^+ and Q_A^-, and the system is considered closed until downstream carbon fixation processes oxidize Q_A^- via Q_B, the PQ-pool and other components of the electron transport chain. After Huner et al (1998).

If the excitation energy in excess is not dealt with in an alternative way, reactive oxygen species (ROS) can be formed as energy absorbed by chlorophyll molecules in the antenna or in the reaction center is transferred to O_2 instead of being used in photosynthesis (Noguchi, 2002; Adams et al., 2004). Singlet excited oxygen ($^1O_2$*) and superoxide (O_2^-) cause

oxidative damage to lipids, pigments, nucleic acids and proteins, and can therefore cause damage to or death of cells (Wise, 1995; Triantaphylides et al., 2008). Excess energy thus represents a considerable stress to plants and photoinhibition of photosynthesis is readily observed in overwintering evergreens (Oquist and Huner, 2003). One possible consequence of high excitation pressure is photobleaching of needles, which is commonly observed in spring, when light intensities are strong (Figure 1.2) (Baronius et al., 1991). This phenomenon, termed winter chlorosis, shows reversibility in summer (Strimbeck, pers. comm.) in contrast to the necrosis caused by freezing (Strimbeck et al., 2007). We therefore hypothesized that the color change is caused by winter light stress.

Figure 1.2: Winter chlorosis in *Picea abies* in April in Trøndelag, Norway. The needles appear more yellow than during the growing season and early winter.

Damage induced during winter can reduce the potential for growth the following summer both because repair takes energy and because the photosynthetic apparatus might be damaged (Close et al., 1999; Adir et al., 2003). Evolution of a protection mechanism therefore seems beneficial. It might also be a prerequisite for the evergreen habit (Demmig-Adams and Adams, 2006). If the leaves are severely damaged, it would not be advantageous to keep them through winter.

1.3. Non-photochemical quenching of excess energy

To avoid reactive oxygen formation, energy can be dissipated as heat. This process is called non-photochemical quenching (NPQ), as opposed to utilization of energy by the photochemical route for subsequent reduction of carbon (Muller et al., 2001) .

Non-photochemical quenching occurs in at least two main forms: *rapidly reversible NPQ* and *sustained NPQ* (Demmig-Adams and Adams, 2006; Demmig-Adams et al., 2006b). The rapidly reversible form is turned on and off within minutes, allowing adjustments to rapid fluctuations in the present light conditions (Li et al., 2000). It is thought to be an important feature of all land plants and almost all other photosynthetic eukaryotes that require protection from temporary increased excitation pressure caused by high light (Muller et al., 2001). Other terms relating to the same phenomenon are qE, feedback de-excitation and flexible- or ΔpH-dependent NPQ (Demmig-Adams and Adams, 2006).

The sustained form of NPQ is found in plants experiencing a prolonged stress, in addition to light stress, that causes a decline in the photosynthetic capacity (Demmig-Adams and Adams, 2006). This includes coniferous evergreens (Ottander et al., 1995; Lamontagne et al., 2000; Savitch et al., 2002; Verhoeven et al., 2009), which demand protection from increased excitation pressure during winter when low temperature poses an additional stress (Huner et al., 1998). The sustained form is slowly relaxed (Muller et al., 2001; Demmig-Adams and Adams, 2006; Demmig-Adams et al., 2006b), requiring in conifers up to six days under optimal conditions to disengage (Verhoeven, 2013). Rapidly reversible and sustained NPQ seem to work in an additive fashion (Porcar-Castell, 2011). That is, the plants can utilize both modes at the same time. Sustained non-photochemical quenching will be in focus in this thesis.

There is strong evidence that non-photochemical quenching takes place in the antenna systems of PSII (Adams and Demmig-Adams, 1992; Adams et al., 1995; Muller et al., 2001). Besides antenna quenching, some evidence suggests a role for the reaction center of PSII in the quenching of excess energy, also in evergreen conifers (Ivanov et al., 2002; Ivanov et al., 2008; Krieger-Liszkay et al., 2008). The two processes are not mutually exclusive (Bukhov et al., 2001; Sveshnikov et al., 2006). Reaction center quenching will only be considered briefly in this thesis.

1.4. Molecular mechanisms of non-photochemical quenching

Rapidly reversible NPQ has been more thoroughly studied than sustained NPQ. Some of the molecular mechanisms seem to be similar in both modes (Demmig-Adams and Adams, 2006). The review of the mechanisms will therefore start with knowledge obtained from the rapidly reversible mode, before mechanisms of the sustained mode are discussed.

The xanthophyll cycle

The rapidly reversible mechanism relies on the build-up of the proton gradient across the thylakoid membrane as the PQ-pool gets reduced at a high rate and ATP consumption in the dark reactions can not keep pace (Muller et al., 2001). This leads to protonation of the 22 kDa photosystem II protein PsbS (considered below) and activates *the xanthophyll cycle*. The xanthophyll cycle involves conversion between the xanthophylls zeaxanthin, antheraxanthin and violaxanthin depending on the light conditions (Muller et al., 2001). A high proportion of zeaxanthin is associated with heat dissipation of energy in the antenna systems and protection of chloroplasts under high light (Demmig-Adams, 1990; Horton et al., 1996; Bassi and Caffarri, 2000). Energy quenching by zeaxanthin is thus thought to be an important feature of non-photochemical quenching. The conversion from violaxanthin to zeaxanthin is a de-epoxidation reaction with antheraxanthin as an intermediate (Blankenship, 2002). This conversion is mediated by violaxanthin de-epoxidase (VDE), which is activated by high concentration of H^+ in the thylakoid lumen in high light situations. (Eskling et al., 1997; Bugos et al., 1998). In low light, pH increases, and VDE is no longer active. A second enzyme called zeaxanthin epoxidase (ZE) has a higher pH-optimum. It epoxidizes zeaxanthin, giving antheraxanthin and finally violaxanthin (Muller et al., 2001). The proportion of zeaxanthin to the total xanthophyll pool is thus determined by the activity of VDE and ZE, which in turn depends on lumenal pH and energy balance of PSII. This implies that in the morning, after hours of dark adaption, the xanthophyll pool is dominated by violaxanthin, and the system is not primed for heat dissipation.

Sustained NPQ

In contrast, plants exhibiting sustained NPQ show a high proportion of zeaxanthin pre-dawn (Adams and Demmig-Adams, 1994; Ottander et al., 1995; Savitch et al., 2002; Adams et al., 2006). This means that the system is in a heat dissipative state even in the morning, and therefore has a more persistent protection against high excitation pressure. Energy dissipation via zeaxanthin is thus expected to be a part of this mode as well (Ensminger et al., 2004; Demmig-Adams et al., 2006b). It is important to realize that two separate mechanisms seem

to be responsible for the pre-dawn heat dissipative state (Demmig-Adams and Adams, 2006). Low temperature can inhibit enzymatic relaxation of the pH-gradient overnight such that the rapidly reversible form is still in operation, leading to retained zeaxanthin pre-dawn. However, if brought to higher temperature, zeaxanthin retention due to this process ceases, and the heat dissipation of energy decreases rapidly (Verhoeven et al., 1998; Verhoeven, 2013). This is considered the *ΔpH-dependent* version of sustained non-photochemical quenching (Demmig-Adams and Adams, 2006). In other cases, zeaxanthin levels will not increase and heat dissipation will not relax even if the plants are transferred to higher temperature. This form of NPQ is consequently *independent of a pH-gradient* (Verhoeven et al., 1998) and this mode will be in focus in this thesis. How zeaxanthin can be retained without the pH-gradient is not known, but a role for the protein PsbS is proposed.

The PsbS protein – is it involved in sustained non-photochemical quenching?

The PsbS protein is a 22 kDa photosystem II subunit, belonging to the LHC protein superfamily (Jansson, 1999). It is expected to be located between the light harvesting complex and the reaction center of PSII (Niyogi et al., 2005). In 2000, evidence that PsbS plays an important role in rapidly reversible quenching of excess energy was found (Li et al., 2000) and the following hypothesis was proposed: when pH decreases in the thylakoid lumen, amino acid residues on PsbS get protonated. The protonation brings about a conformational change, which facilitates the binding of the energy quencher zeaxanthin. Even though the exact mechanism is still discussed, there is considerable evidence for a role of PsbS in rapidly reversible NPQ (Muller et al., 2001; Niyogi et al., 2005).

In 2003, Öquist and Huner suggested a role for PsbS also in sustained quenching. A conformational change within the antenna was proposed as a mechanism for keeping the xanthophylls in an energy-dissipating state in the absence of a pH-gradient. PsbS was considered a key protein in the formation of the quenching protein-xanthophyll aggregate. Induction of the conformational change was hypothesized to rely on means other than lumenal acidification, like cold-induced minor polypeptides, protein phosphorylation or frost desiccation. The key role for PsbS was suggested in the light of studies showing increasing levels of PsbS during winter (Ottander et al., 1995; Savitch et al., 2002).

On the other hand, Zarter and colleagues (2006b) found little or no increases in PsbS in Subalpine fir (*Abies lasiocarpa*) and Lodgepole pine (*Pinus ponderosa*), respectively, during

winter at high altitude (3022m) in Colorado, USA. Also, Demmig-Adams and colleagues (2006a) did not find increasing PsbS levels in leaves of a tropical perennial evergreen (*Monstera deliciosa*) that showed sustained NPQ after transfer from low light to high light. Actually, the leaves that showed the greatest increase in sustained NPQ exhibited greatest *reduction* in PsbS levels. (The tropical plant employs sustained NPQ because of the additional stress caused by low nutrient and water ability.) On that basis, they suggest that PsbS does not have a role in sustained NPQ. Similar results have been found in other evergreens experiencing winter conditions (Ensminger et al., 2004; Zarter et al., 2006a; Busch et al., 2007).

1.5. Chlorophyll fluorimetry

As described above, energy from a photon absorbed by a chlorophyll molecule in the reaction center can be 1) used in photochemical reactions and thus drive photosynthesis or 2) it can be dissipated as heat through non-photochemical quenching. Additionally, there is a third pathway for the energy – it can be re-emitted as chlorophyll fluorescence, which is radiation of slightly longer wavelength than of the light absorbed (Nobel, 2009) As the three processes are competitive, a change in the rate of either photochemistry or non-photochemical quenching will change the yield of chlorophyll fluorescence (Butler, 1978). Measuring the fluorescence from PSII reaction center chlorophyll molecules is therefore a non-invasive method that can give insight into the energy partitioning taking place in the leaf (Maxwell and Johnson, 2000).

A healthy, non-stressed leaf kept in darkness is not experiencing any excitation pressure and therefore no protective NPQ is operating. Additionally, all the reaction centers are open and ready to process incoming excitations photochemically. Nevertheless, also in such a leaf, some energy is emitted as fluorescence when illuminated with weak, non-actinic measuring light (Baker, 2008). This is called the minimal, or background, fluorescence F_0. It arises because there is always a probability for the chlorophyll molecules to fluoresce when illuminated, and the plant cannot use this share of the absorbed energy. A short, intense flash of light, a saturation pulse, can be given that leads to closure of all the reaction centers (Baker, 2008). Following such a closure, all the energy absorbed has to be released as fluorescence, since the photochemical route is closed and no NPQ is present in the dark-adapted leaf. This is called the maximum level of fluorescence F_m and represents the total energy absorbed (Baker, 2008). Subtracting F_0 from F_m yields the variable fluorescence

$F_v = F_m - F_0$ (Baker, 2008). F_v is thus proportionally equal to the maximum amount of absorbed energy that can be used for photosynthesis. The ratio F_v/F_m can be shown to equal the maximum quantum yield of PSII photochemistry (Kitajima and Butler, 1975), which is also known as maximum photochemical efficiency of PSII. In healthy, non-stressed leaves, F_v/F_m is remarkably constant at around 0.83 (Bjorkman and Demmig, 1987).

If the leaf is exposed to light that will drive photosynthesis (actinic light), NPQ is activated. If a saturation pulse is applied, the maximal fluorescence recorded F'_m will be lower than for the dark-adapted leaf (Baker, 2008). The difference in maximal fluorescence from a dark-adapted and light-adapted leaf is proportional to the amount of energy dissipated as heat through non-photochemical quenching. The Stern-Vollmer expression for F_m-quenching $[(F_m - F'_m) - 1]$ (Bilger and Bjorkman, 1990) can be used for estimating NPQ.

Observations of sustained NPQ using chlorophyll fluorimetry

In continuously stressed plants, like conifers that experience high excitation pressures over an extended period during winter, lower maximum quantum yield of PSII photochemistry (F_v/F_m) is observed (Ottander et al., 1995; Strand, 1995; Lundmark et al., 1998; Demmig-Adams et al., 2006b). This can be the result of sustained non-photochemical quenching which lowers F_m also in the dark-adapted leaf, or due to damage in the reaction center that gives lower rates of photochemical quenching and concomitantly increasing F_0 (Demmig-Adams et al., 2012). Hence, decline in F_v/F_m is clearly a sign of stress, but it can be attributed to two different processes. F_v/F_m is nevertheless the recommended fluorescence parameter for detection of sustained non-photochemical quenching (Adams and Demmig-Adams, 2004). In overwintering evergreens, sustained NPQ is moreover found to be the main reason for declining F_v/F_m (Adams et al., 2004; Ensminger et al., 2004; Porcar-Castell, 2011). Also, by careful examination of F_0 and F_m the mechanisms behind the reduced maximum quantum yield of PSII might be revealed (Maxwell and Johnson, 2000).

The Stern-Vollmer quenching of F_m normally used for estimating rapidly reversible NPQ is not applicable to plants under prolonged stress because the true F_m cannot be measured due to sustained quenching of F_m-fluorescence in the dark. This would underestimate the amount of NPQ taking place (Adams et al., 1995). F_v/F_m, a ratio of two measured fluorescence metrics, is more robust (Adams et al., 1990; Demmig-Adams et al., 2012).

1.6. Aim of study

As described above, non-photochemical quenching can be sustained when there is an increased excitation pressure over a long time period, giving evergreen conifers crucial protection against too much light energy during winter. Nevertheless, the phenomenon is not thoroughly characterized. Several studies have presented detailed recordings of F_v/F_m, de-epoxidation state of the xanthophyll cycle and related parameters in the field (Adams et al., 1995; Ottander et al., 1995; Ensminger et al., 2004; Porcar-Castell, 2011), but few controlled experiments have been performed. There is also lack of knowledge about species-specific ecological adaptions to long lasting high excitation pressure. Questions remain regarding how non-photochemical quenching, probably involving the xanthophyll cycle, can continue to be in a heat dissipative state throughout the night and for several days. A role for PsbS is suggested, but there is conflicting evidence as reviewed above.

The aim of this study was to describe the separate and combined effects of high light and low temperature stress on photoprotection occurring in winter in boreal evergreen conifers. The experimental design was applied to four species with different geographical distribution and cold tolerance to reveal ecological adaptions of photoprotection. The hypothesis was that species originating from areas with severe winter cold would show a continuous high level of sustained NPQ, while species growing in areas where warm spells and therefore photosynthesis is likely to occur should have a more flexible response. Chlorophyll fluorescence measurement of F_v/F_m was used to quantify sustained NPQ. Color changes of the needles were monitored to investigate light stress in combination with low temperature as the cause of winter chlorosis. To validate that the quenching observed was actually ΔpH-independent sustained NPQ, a follow up experiment monitoring recovery was performed.

Analyses of the expression of the photosystem II-protein PsbS and pigment composition were performed for an investigation of the underlying mechanisms of sustained photoprotection. Levels of fluorescence quenching for F_0 and F_m were monitored to reveal any signs of damage to PSII, and to possibly gain insight into whether reaction center quenching was involved or not.

2. Materials and Methods

2.1. Main experiment

Materials and Sample Collection

Shoots from four boreal species of *Picea (Picea abies* L. Karst.; *P. obovata* Ledeb.; *P. rubens* Sarg. and *P. sitchensis* (Bongard) Carr.) originating from temperate continental and temperate oceanic environments were harvested February 28, 2012 from stands at Ringve Botanical Garden (63°26'56''N, 10°27'12''E) in Trondheim, Norway. Ringve Botanical Garden is situated a few hundred meters from Trondheimsfjord. The climate is classified as southern boreal and moderately oceanic (Strimbeck and Schaberg, 2009). The temperature at Trondheim Airport, Værnes, the nearest weather station similarly situated near the fjord, was 4.8 °C on the day of sampling. Shoots of previous summer's growth were used. The samples were brought back to the lab in an insulated container with ice.

Experimental design

A 2×2×4 factorial experiment was performed in growth chambers. The fixed factors investigated and the levels used are shown in Table 2.1.

Table 2.1: Fixed factors and levels in main growth chamber experiment

Factor	Levels
Light	High light (HL) = 200 µmol $m^{-2}s^{-1}$
	Low light (LL) = 80 µmol $m^{-2}s^{-1}$
Temperature	High temperature (HT) = 10.0 °C
	Low temperature (LT) = 0.0 °C
Species	*Picea abies* (AB)
	Picea obovata (OB)
	Picea rubens (RU)
	Picea sitchensis (SI)

Light and temperature treatments were replicated across three sample shoots from three individual trees of each species. At least 17 needles from each shoot were measured individually in the fluorescence measurements. The experiment as a whole was not replicated due to limitations in growth chambers so Temperature and Light effects may be confounded

with other possible chamber effects. Individual trees were still treated as independent for the purpose of analysis, as growth chamber conditions were closely controlled and monitored and no significant differences from the programmed light and temperature was observed.

Set-up

The two side shoots of a three-parted shoot of tree number 1 of *Picea abies* were recut under water (to avoid embolisms) and placed side by side in a floral foam strip (Oasis® Floral Foam Maxlife). Shoots from the number 1 trees of *Picea obovata* and *Picea sitchensis* were assembled in the strip in the same way. For *Picea rubens*, whole three-parted shoots were used because of the shoots' small size. See Figure 2.1 for illustration. The number 2 and 3 trees were identically arranged in floral foam strips. 12 trays with one strip of each tree number were made and three trays were placed under each of the four light/temperature-regimes.

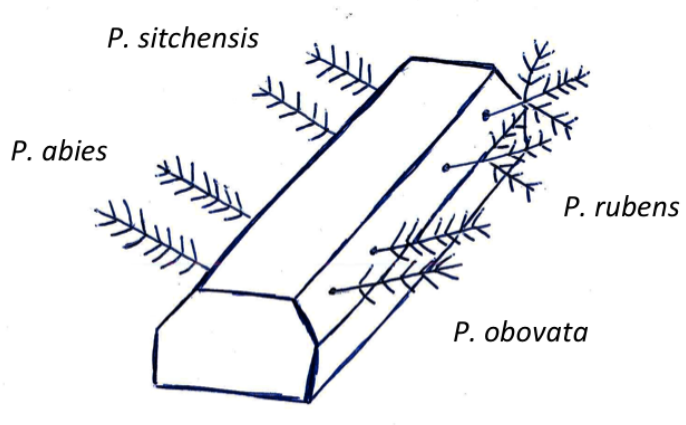

Figure 2.1: Assembly of conifer shoots in floral foam strips. Care was taken to achieve a similar angle to the light for each shoot. Two shoots were used for each species to get enough material for all analyses.

Low temperature growth chambers (Percival model LT-36VL, CLF Climatics, Percival Scientific Inc, USA) were used with Philips Alto II fluorescent lamps. One chamber was used for HL and LL treatments at low temperature and a second chamber was used for HL and LL treatments at high temperature. A 16-hour day/8-hour night photoperiod was used with non-ramping onset and offset of light. The temperature settings were constant throughout the twenty-four hour cycle. The shoots were sufficiently watered in the high temperature chamber. For the low temperature chamber, the water was frozen. Two individual thermometers were used for temperature records. Light intensity was monitored daily (LI-1000 Datalogger and Quantum Sensor, LI-COR Inc., USA) in the middle of the shelves,

and on a weekly basis in 9 positions (middle, four corners, four sides) to observe spatial heterogeneity in light intensity.

Sampling and chlorophyll fluorescence measurements were done after 7, 14 and 21 days. Shoots from one tray were harvested pre-dawn after 8 hours dark adaption at each sampling date, including the 3 individual trees of each species. The samples were kept cool in the dark until chlorophyll fluorescence measurements and image analysis were done. For protein and pigment analyses, 5mm needle segments were placed in 1.5 mL microcentrifuge tubes after cutting and immediately immersed in liquid nitrogen. The samples were stored at -80 °C until further analyses were done. The shoots showed signs of desiccation after 21 days, which may have affected the results. Further investigations (pigment and protein analyses) and statistical analyses of the fluorescence data were therefore focused on the samples from day 14. Day 14 is preferred over day 7 due to the longer exposure to the different light/temperature regimes.

2.2. Recovery experiment

Experimental design

A 2^4 factorial experiment was performed to show the recovery kinetics of two species, *P. obovata* and *P. sitchensis*. The fixed factors are shown in Table 2.2.

Table 2.2: Fixed factors and factor levels in the recovery experiment

Factor	Levels
Light	High light (HL) = 200 µmol $m^{-2}s^{-1}$
	Low light (LL) = 80 µmol $m^{-2}s^{-1}$
Temperature	High temperature (HT) = 10.0 °C
	Low temperature (LT) = 0.0 °C
Species	*Picea obovata* (OB)
	Picea sitchensis (SI)
Recovery	Control, no recovery
	30 min. recovery in room temperature

The time for recovery was set to no more than 30 minutes to be sure that the relaxed component of NPQ was the ΔpH-dependent mechanism reported from earlier studies

(Verhoeven, 2013). The replication structure of the recovery experiment was the same as for the main experiment. Temperature and light intensity were monitored as in the main experiment, but with a different light-measuring device (PAR Quantum Sensor, Skye Instruments Ltd, UK).

Sample collection and set-up

Shoots were harvested from 3 individual trees of each species February 20, 2013. Harvesting procedures and tree stands used were the same as in the main experiment. The temperature at Trondheim Airport, Værnes, was -6.1 °C at the day of sampling. The shoots were assembled in floral foam strips and trays, and one tray was placed under each of the four different light and temperature conditions.

Chlorophyll fluorescence measurements were done pre-dawn after at least 6.5 hours of dark adaption. For investigation of recovery kinetics, shoots were brought to room temperature for 30 minutes before chlorophyll fluorescence measurements were done. Control shoots were kept cool in the same way as in the main experiment until fluorescence measurements were done.

To avoid different recovery times, measurements were completed for one tree one species at a time for the room temperature treated shoots. For the control shoots, measurements were completed for one tree for both species at a time. The samples were randomized for both room temperature and control measurements.

2.3. Chlorophyll fluorescence measurements

A pulse amplitude modulated fluorometer (PAM 210, Heinz Walz GmbH) was equipped with a modified head: The light diode was masked with tape to form a slit that accommodated the needles. This was done to make sure that the area of measurement was equal between all samples, independent of the size of the needle. In this way, also measurements of F_0 and F_m could be compared between samples. The following settings of the fluorometer were used: Measuring light intensity 0.08 µmol $m^{-2}s^{-1}$; measuring light emission peak: 650 nm; saturation pulse intensity: 2800 µmol $m^{-2}s^{-1}$; saturation pulse length: 0.4 s; saturation pulse emission peak: 665 nm. Light intensities are given according to the instruction manual of the PAM-210 (Heinz Walz GmbH).

F_0 and F_m, the minimal and maximal fluorescence from a dark-adapted needle, were measured. F_0 corresponds to the level of fluorescence when Q_A is fully oxidized and F_m is the level of fluorescence when Q_A is fully reduced after a saturation pulse (Baker, 2008). Maximum quantum yield of PSII photochemistry $F_v/F_m = (F_m - F_0)/F_m$ was thereafter calculated by the in-built software (DA-TEACH Data Acquisition Software, v1.01, Heinz Walz GmbH).

In the main experiment, samples were grouped according to their tree-number, and handled in order 1-3. Within these groups, the samples were randomized. Zero offset determination on the fluorometer was performed before measuring each group of shoots.

2.4. Color analysis

The purpose of the analyses was to separately count the pixels with orange, yellow and green hue, and subsequently calculate the percentage needle area of each color (Strimbeck et al., 2007). Needles were cut into 5mm segments. Needles from each sample were put in an individual well in a 12-well clear plastic culture dish. The plastic container was scanned with a flatbed image scanner (Xerox Workcentre 5755, Stamford, CT, USA) using standard settings, 300 dpi resolution and black background. The pictures were saved as high quality JPEG-files. Images were analyzed using ImageJ public domain software (U.S. National Institutes of Health, Bethesda, MD, USA). First, the original image (Figure A1, A in Appendix A) was separated into three image slices with grayscale values for the parameters hue, saturation and brightness. The hue slice was converted back to colors using the Spectrum look-up table (LUT) (Figure A1, B). To count only the pixels that were part of the needles a mask for the background was made, using the brightness slice with a threshold value of 100 on the grayscale LUT. The needle area remained white, while the background was masked in black (Figure A1, C). The mask was added to the hue-image (Figure A1, D). Threshold Spectrum LUT values of 15-35, 36-50 and 51-90 were used for separately selecting and counting orange, yellow and green pixels respectively (Figure A1, E, F, G). By drawing a circular region of interest around each well, each sample was analyzed separately. Percentage green needle area for each sample was calculated from the following equation:

$$\frac{Area_{\text{Green}}}{Area_{\text{Green}} + Area_{\text{Yellow}} Area_{\text{Orange}}} * 100\% \qquad (2.1)$$

The same calculation was completed for yellow and orange areas for each sample.

2.5. Pigment analysis

Pigment extraction

Chilled 100 % acetone (HPLC-grade) was used for extraction of pigments from the samples. The needle pieces (approx. 0.1g) were ground in a mortar kept on ice with acetone (approx. 3mL). The extract and the remaining needle pieces were transferred to a glass vial and set for extraction in a -20 °C freezer overnight. Each sample extract was filtered into a 2mL HPLC glass vial through a 3mL syringe with a 0.2µm PTFE filter. All procedures were completed under subdued light.

HPLC analysis

A Hewlett-Packard 1100 HPLC system (Hewlett-Packard Company, Palo Alto, CA, USA) was used for the analyses, with quaternary pump system, auto sampler and injector and a UV-Vis diode array (using the range 350-700 nm, <1nm spectral resolution). The solid phase consisted of a Waters Symmetry C8-column (4,6 mm x 150 mm, 3,5 µm particle size, Waters Corporation). For the mobile phase, a solvent system based on two solvents A and B was used. (A: Methanol: Acetonitrile : Hydrous pyridine (50 : 25 : 25); B: Acetonitrile: Acetone (80 : 20)). Methanol was used for washing the system. All reagents were HPLC-grade and made the same day as the analyses were done. 77 µL of the sample was drawn from each vial by the auto sampler. 23 µL of water was added and mixed automatically before injection. The temperature in the column was 25 °C and in the auto sampler 0 °C. Flow rate was 1.000 mL/min.

Peaks were quantified using Agilent ChemStation software (Agilent Technologies, Santa Clara, CA, USA). To identify the peaks, the absorption spectrum and retention times for each peak were verified with literature (Jeffrey et al., 1997) showing each pigment's spectrum and retention time in the same solvent. Absorption at 440 nm was used for determining the concentration of the pigments. Concentration of each pigment in each sample extract was found by using the following equation:

$$Concentration = \frac{Area_{440nm} * Response\ factor_{440nm}}{Injected\ volume\ of\ sample} \quad (2.2)$$

where $Area_{440}$ is the definite integral of the absorption curve at 440 nm for the pigment. The specific extinction coefficients for calculation of the response factors were provided by

Jeffrey et al. (1997). HPLC calibration was performed using prepared and purchased standards (Sigma-Aldrich Co, US). No zeaxanthin was found, and therefore, violaxanthin levels were presented instead of the intended epoxidation status EPS = (V+0,5A)/(Z+A+V) where Z, A and V are zeaxanthin, antheraxanthin and violaxanthin, respectively (Thayer and Bjorkman, 1990). Since the extraction was not done quantitatively, no absolute amount of violaxanthin (V) per sample weight was calculated. Instead, the amount of violaxanthin was expressed on a chlorophyll a+b basis by dividing the concentration of violaxanthin by the concentration of Chl a+b for each sample:

$$[V]/[Chl\ a+b] = V/Chl\ a+b \tag{2.3}$$

2.6. Protein analysis

Protein extraction

Proteins were extracted from needles by grinding frozen tissue in LN_2 in a mortar. Approximately 80 μg of ground tissue (exact weight recorded for each sample) was added to a test-tube containing chilled 180 μL extraction solution (33 % sucrose, 0.5 % SDS (w/v), 12 % β-mercaptoethanol (BME) (v/v)). 500 μL phenol was immediately added, and the sample was vortexed and centrifuged for 4 min at 14000 rpm (4 °C). The phenol phase was drawn off. Based on the recorded mass of powder added to each test tube, the volume of phenol extract that contained proteins from 10 mg or 5 mg for each sample was added to new test tubes. For the samples from tree number 1 from each species, 10 mg equivalents were used. Because precipitates from this volume were difficult to dissolve in SDS lysis buffer, a volume corresponding to 5 mg was chosen for the remaining samples (number 2 and 3-trees). When running gels, different loading volumes were used to correct for these differences.

Adding 0.4 mL ice-cold 80 % methanol with 0.1 M ammonium acetate to the test tubes and leaving them in a freezer for 40 minutes precipitated the proteins. After centrifuging for 30 min at 14000 rpm (4 °C), the supernatant was discarded. The protein pellet was washed with 0.4 mL ice-cold 100 % methanol, centrifuged for 4 min at 14000 rpm (4 °C) and the supernatant was discarded. A second washing step using 80 % acetone was performed. The pellet was air dried briefly. The pellet was dissolved in 50 μL 1X SDS lysis buffer with BME (2 % SDS, 10 % glycerol, 0.01 % bromophenol blue (w/v), 62.5 mM Tris pH 6.8, 5 % BME

(v/v)), by heating at 85 °C for about 10 minutes (30 minutes for samples with proteins from 10 mg tissue).

SDS-PAGE

The 22 kDa protein PsbS was detected using Western blotting. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed for separation of proteins in the sample according to the proteins' molecular weight. Proteins in the extracts were separated on a 13 % acrylamide resolving gel (Laemmli 1970) at 30 mA constant current for 60 minutes in running buffer (Bio-Rad, 2012). Mini-Protean®3 Cell equipment (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used. Visualization of separated protein bands was done by using Coomassie® G-250 staining according to the manufacturer's instructions (SimplyBlue™ Safe Stain, Life Technologies Ltd, Paisley, UK).

Samples were loaded on an equal tissue basis (proteins from 1mg needles per lane). Each gel was loaded with all treatments (HT-LL, HT-HL, LT-LL, LT-HL) from one replication (one tree). Since there were 8 lanes available on each gel, *P. rubens* was loaded on the same gels as *P. abies*, and *P. sitchensis* was loaded on the same gels as *P. obovata*.

Western blotting

Equilibration of the gel for Western blotting was done in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol (v/v), pH 8.3) for 15 min. Electroblotting for transfer of proteins from the gel to the PVDF membrane (Bio-Rad, 162-0177) was carried out for 60 minutes at 100 V constant voltage in transfer buffer. Mini Protean Trans-Blot® equipment (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used. The membrane was subsequently blocked for 45 minutes in blocking solution (3 % gelatin, 0.02 % sodium azide (w/w) in Tris Buffered Saline (TBS)).

The following antibodies were used for detection: Primary antibody anti-PsbS from rabbit (AS09 533, Agrisera AB, Vännäs, Sweden) and secondary antibody for immunodetection by alkaline phosphatase-conjugate (Goat anti-rabbit IGg (H+L) AP conjugate, Bio-Rad). Both primary and secondary antibody were diluted 1:1000 in 1 % gelatin TBS (1 % gelatin, 0.0067 % sodium azide (w/v) in TBS).

The membrane was incubated with primary antibody for 2 hours. A brief rinse in TBS and

3 x 7 min washes in TTBS (0.05 % Tween 20 (v/v) in TBS) was completed before incubation with secondary antibody. After 45 min of incubation with secondary antibody, a second rinse using TBS and TTBS as described above was completed. Alkaline phosphatase-conjugate substrate (Bio-Rad 170-6432) was used for colorimetric detection. Development time was 12 minutes.

Gel quantification

The blots were scanned at 600 dpi using an UMAX Astra 6400 flatbed color scanner (UMAX Technologies, Inc, USA) with default settings except the following: Shadow = 40; mid-tone value (gamma) = 1.5. Relative optical densities of signals were quantified using built-in gel quantification functions in ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Background was subtracted (rolling ball radius = 40) and the intensity of the bands was analyzed in a horizontal fashion.

2.7. Statistical analyses

Main experiment and recovery experiment

For chlorophyll fluorescence data from the main experiment, a subset consisting of data from the samples harvested after 14 days was analyzed. 15 outliers out of a total of 1057 observations were removed.

A linear mixed effects model was fitted to the F_v/F_m data using restricted maximum likelihood (REML). The function `lmer` in package *lme4* (Bates et al., 2013) in the statistical environment R (R Development Core Team, 2012) was used. The factors Temperature, Light and Species were treated as fixed effects, while Tree was treated as a random effect. The Akaike Information Criterion (AIC) was used to decide between competing models. In the model selection procedures, maximum likelihood (ML), not REML, was used to fit the models to estimate the correct AIC values. The model with the lowest AIC has most support. ΔAIC is defined as $AIC_{\text{best model}} - AIC_{\text{competing model}}$. Competing models that have $|\Delta AIC| \leq 2$ also have substantial support and should be considered. Those in which $4 \leq |\Delta AIC| \leq 7$ have substantially less support, and models having $|\Delta AIC| > 10$ do not have any support (Burnham and Anderson, 2004). The assumption of normality and homogeneity was checked visually using Q-Q-plot, plots of residuals vs fitted values and plots showing distribution of residuals within each group (Tree). MCMC-estimated p-values (pMCMC) were found using the function `pvals.fnc` from the package *languageR* (Baayen, 2011).

The same approach was used for analyses of F_v/F_m data from the recovery experiment. However, separate linear mixed models were fitted for the two species. The fixed factors included in the analyses were "Treatment" and "Recovery". The factor Treatment had four levels: HT-EHL; HT-LL; LT-HL and LT-LL. Treatment was used instead of Light and Temperature as two separate factors because the light level at HL in the HT-chamber was observed to be higher than the light level at HL in the LT-chamber (see Table 3.2). Tree were used as random factor. One outlier was removed from a total of 450 observations for *P. sitchensis*. There were 467 observations for *P. obovata* and no outliers were removed.

Pigment, protein and color analyses

For the pigment, protein and color analyses data, the linear mixed model was not used. The reason for this is that the assumption of normal distribution of the residuals was violated when the data was analyzed with `lmer`. Instead, the Kruskal Wallis test for different means was used (Function `kruskal.test` in package *stats* in R (R Development Core Team, 2012)). Means of the four groups HT-HL, HT-LL, LT-HL, LT-LL were compared. Multiple comparison analyses (`kruskalmc` in package *pgirmess* (Giraudoux, 2011)) were performed where differences were significant ($p<0.05$).

Graphics were prepared using R (R Development Core Team, 2012) including the package *ggplot* (Wickham, 2009) and package *scales* (Wickham, 2012).

3. Results

3.1. Climatic data and growth chamber conditions

The temperature recordings from the weather station at Værnes Airport in the winter months preceding the main experiment are shown in Table 3.1. The samples were collected February 28, 2012.

Table 3.1: Temperature recordings from the weather station at Værnes Airport in the winter season of 2011-2012.

Month	T_{min} (°C)	T_{max} (°C)	T_{mean} (°C)
November	-4.5	14.5	4.8
December	-9.0	9.8	0.4
January	-13.4	6.3	-1.9
February	-13.7	8.1	-0.4

Growth chamber conditions were monitored as explained in chapter 2 and are summarized in Table 3.2. The two individual thermometers showed nearly identical values. The built-in thermometer was chosen as reference for the reported temperatures shown in Table 3.2.

Table 3.2: Growth chamber conditions in the two experiments. Mean value and highest measured deviation from the mean due to temporal heterogeneity is shown. Variation in light intensity due to spatial heterogeneity is shown in parentheses.

Chamber	Temperature (°C)	Rel. humidity (%)	Light intensity (µmol $m^{-2}s^{-1}$)	
			HL	LL
Main experiment (n=10)				
HT	10.0±0.3	63±4	206±20 (±21)	80±3 (±8)
LT	0.3±0	48±4	203±36 (±25)	80±5 (±8)
Recovery experiment (n=10)				
HT	9.9±0.1	57±1	347±13 (±25)	81±2 (±5)
LT	0.3±0	50±1	205±4 (±7)	82±4 (±4)

For the main experiment, one day of 164 µmol $m^{-2}s^{-1}$ accounts for the pronounced variation for the LT-LL treatment. When this day is excluded, the second highest deviation was 15 µmol $m^{-2}s^{-1}$ from the mean.

Due to technical problems, the light intensity in the recovery experiment was 347 µmol m^{-2} s^{-1} instead of the intended 200 µmol m^{-2} s^{-1} for the high light treatment in the high temperature chamber throughout the whole experiment period.

3.2. Changes in maximum photochemical efficiency of photosystem II

After 14 days of exposure in the growth chambers, the shoots showed different levels of maximum photosynthetic efficiency of PSII (F_v/F_m) depending on the light and temperature conditions. There were also species-specific responses. The results are shown in Figure 3.1.

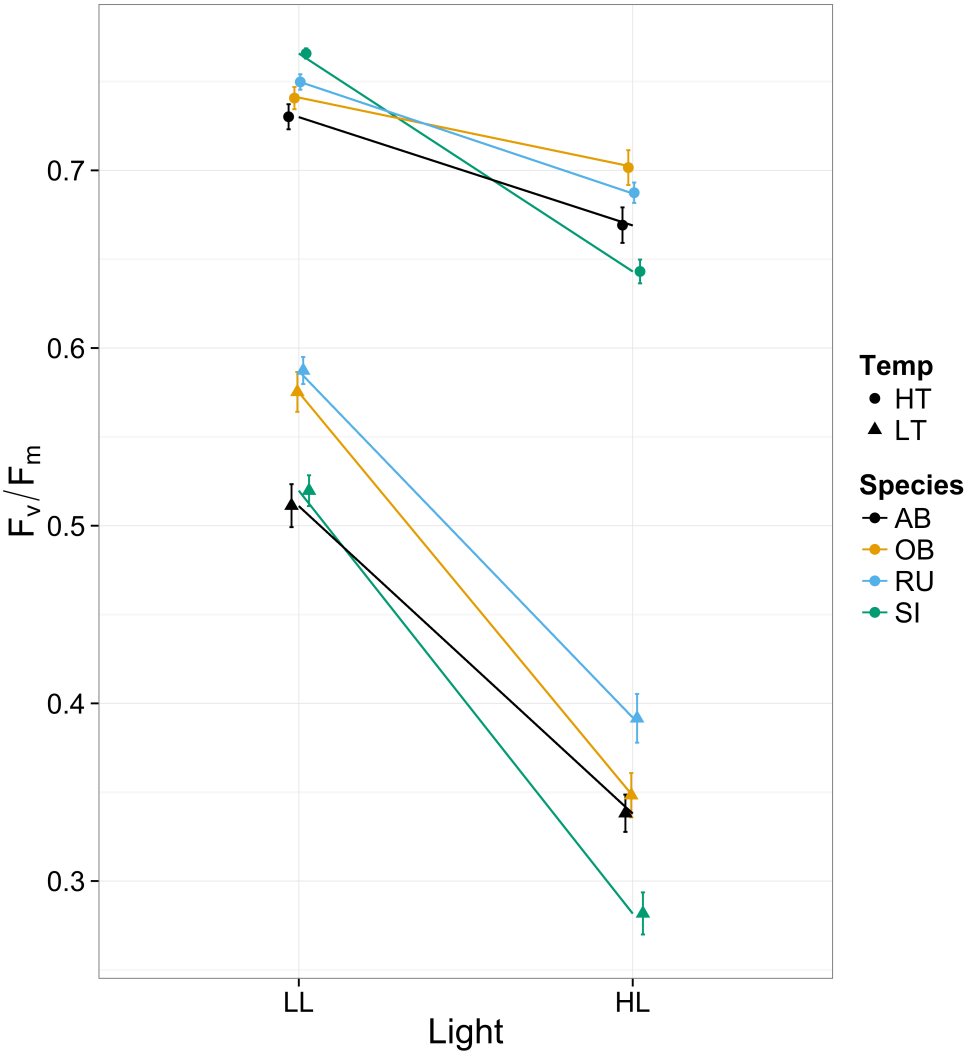

Figure 3.1 Maximum photochemical efficiency of PS II (F_v/F_m) for *Picea abies* (AB), *P. obovata* (OB), *P. rubens* (RU) and *P. sitchensis* (SI) grown under combinations of high and low temperature (10/0 °C) and high and low light (200/80 µmol m^{-2} s^{-1}) for 14 days. F_v/F_m was measured after 8 hours of dark-adaption. Shoots from 3 individual trees per species constituted replication; minimum 17 needles were measured per shoot. Error bars ±SE.

In order to quantify the effects of light, temperature and ecological adaption on sustained NPQ a linear mixed model was fitted to the F_v/F_m data. Light, Temperature and Species were treated as fixed effects, and Tree was treated as a random effect. The variance due to Tree constituted 28 % of the total variance ($Var_{Tree}/(Var_{Tree} + Var_{Residual}) = 0.28$). Using the Akaike Information Criterion (AIC) for discrimination between candidate models, the full model was found to explain the data best (Table B1, Appendix B). The absolute difference in AIC to the next best model was 8. The estimated effects, t-values and MCMC-estimated p-values for the chosen model are given in Table 3.3. The intercept levels for the fixed factors were high light, high temperature and *Picea abies*. The main fixed effects estimate the increase in F_v/F_m from the intercept-value when changing one factor's level while the two others remain at intercept-levels. The two-way interaction between factor 1 and factor 2 is the difference in response to factor 1 when factor 2 is at different levels (factor 3 is at the intercept level). The three-way interaction between factor 1, 2 and 3 is the difference in response to factor 1 when levels of factor 2 and 3 are changed.

Table 3.3: Random and fixed effects in a linear mixed model fitted by restricted maximum likelihood. For fixed effects, the levels for the intercept are high temperature (10 °C), high light (200 µmol m^{-2} s^{-1}) and species *Picea abies* (AB). The other levels are low temperature (0 °C), low light (80 µmol m^{-2} s^{-1}) and species *P. obovata* (OB), *P. rubens* (RU) and *P. sitchensis* (SI).

Random effects:			
	Variance	Std.dev	
Tree	0.0018	0.042	
Residual	0.0046	0.068	
Fixed effects:			
	Estimate	t-value	pMCMC
(Intercept, HL-HT-AB)	0.6668	25	<0.001
Light LL	0.0604	4.8	<0.001
Temp LT	-0.3230	-26	<0.001
Species OB	0.0355	0.97	0.262
Species RU	0.0200	0.54	0.530
Species SI	-0.0243	-0.66	0.443
Light LL × Temp LT	0.1068	6.2	<0.001
Light LL × Species OB	-0.0218	-1.3	0.219
Light LL × Species RU	0.0025	0.15	0.885
Light LL × Species SI	0.0623	3.6	<0.001
Temp LT × Species OB	-0.0250	-1.5	0.145
Temp LT × Species RU	0.0277	1.7	0.101
Temp LT × Species SI	-0.0378	-2.2	0.028
Light LL × Temp LT × Species OB	0.0786	3.3	<0.001
Light LL × Temp LT × Species RU	0.0258	1.1	0.278
Light LL × Temp LT × Species SI	0.0080	0.3	0.745

Effect of species

There was no main effect of species on F_v/F_m at high temperature and high light which are the model intercept levels shown Table 3.3. This means that there were no difference in F_v/F_m due to species for this treatment. The greatest range of responses between the species occured at low temperature and high light (LT-HL). Here, *P. sitchensis* showed the lowest maximum photochemical efficiency observed in the experiment. Running a model with changed intercept level for temperature showed a negative significant effect of *P. sitchensis* at LT-HL (Table B2). That is, *P. sitchensis* had a lower F_v/F_m than the other species for this treatment. For the combinations HT-LL and LT-LL, no effect of species was found (models not shown). This means that according to the models there is no significant main effect of species, except for *P. sitchensis* in LT-HL conditions.

Effect of temperature and interaction with species

While F_v/F_m at high temperature was slightly lower than the F_v/F_m -value for non-stressed tissue (about 0.83 (Bjorkman and Demmig, 1987)), low temperature caused strong decreases in F_v/F_m, see Figure 3.1. The effect of temperature in the fitted model (Temp LT = -0.3230) was strong and significant for the intercept species *P. abies* (Table 3.3) and similar for *P. rubens* and *P. obovata*. For *P. sitchensis*, the response to temperature was even stronger, indicated by the significant interaction between temperature and species SI (Temp LT × Species SI).

Effect of light and interaction with species

Shoots exposed to high light showed a more pronounced decrease in F_v/F_m than shoots exposed to low light. Grand means are 0.51 and 0.64, respectively. In the model, this can be seen as the significant effect of light (Light LL = 0.0604), which means that different levels of light explain much of the variation in the data. The effect was the same for *P. rubens* and *P. obovata*, while *P. stichensis* showed a much stronger response (Light LL × Species SI = 0.0623, implying that going from HL to LL for *P. sitchensis* causes an additional increment in F_v/F_m of 0.06 compared to *P. abies*). *Picea sitchensis* was thus more responsive to light than the other species at 10 °C, showing the highest F_v/F_m in low light and the lowest F_v/F_m in high light (Figure 3.3). Under low temperature conditions, *P. sitchensis* and *P. obovata* showed a stronger response to light than *P. abies* and *P. rubens* (Table B2).

Interaction between light and temperature

There was a strong interaction between light and temperature (Light LL × Temp LT = 0.1068). This means that going from HL to LL gives an increase in F_v/F_m that is 0.1068 larger at low temperature than at high temperature. At high temperature, the increase is 0.0604, as mentioned above. The interaction can also be seen from figure 3.1, as the response to light is steeper at LT than HT. In other words, there is a stronger response to light at low temperature.

Three-way interactions

The significant three-way interaction indicates a different response to light by the different species dependent on temperature. The significant three-way interaction is attributed to the fact that *P. obovata* showed a stronger response to light than *P. abies* at low temperature (steeper line for *P. obovata* than *P. sitchensis*), while there was a weaker response to light at high temperature (less steep line). *P. sitchensis* did not show a significant three-way interaction even though its response was distinctive as seen from the two-way interactions. This is because *P. sitchensis* showed a stronger response than *P. abies* to light at both high and low temperature.

3.3. Relationship between parameters F_0, F_m and F_v/F_m

The parameters for calculating maximum photosynthetic capacity of photosystem II, (F_v/F_m), namely maximal fluorescence (F_m) and minimal fluorescence (F_0) were compared for the four treatments. As shown in Figure 3.2, F_0 was not changed by different treatments. High light and low temperature did reduce F_m. F_v/F_m was reduced in the same manner. A Pearson's product-moment correlation test for F_v/F_m against F_0 and F_m revealed a strong correlation between F_v/F_m and F_m (p-value< 0.001) but no correlation for F_v/F_m and F_0 (p-value = 0.70). This reveals that the changes in F_v/F_m are due to quenching of the maximum fluorescence.

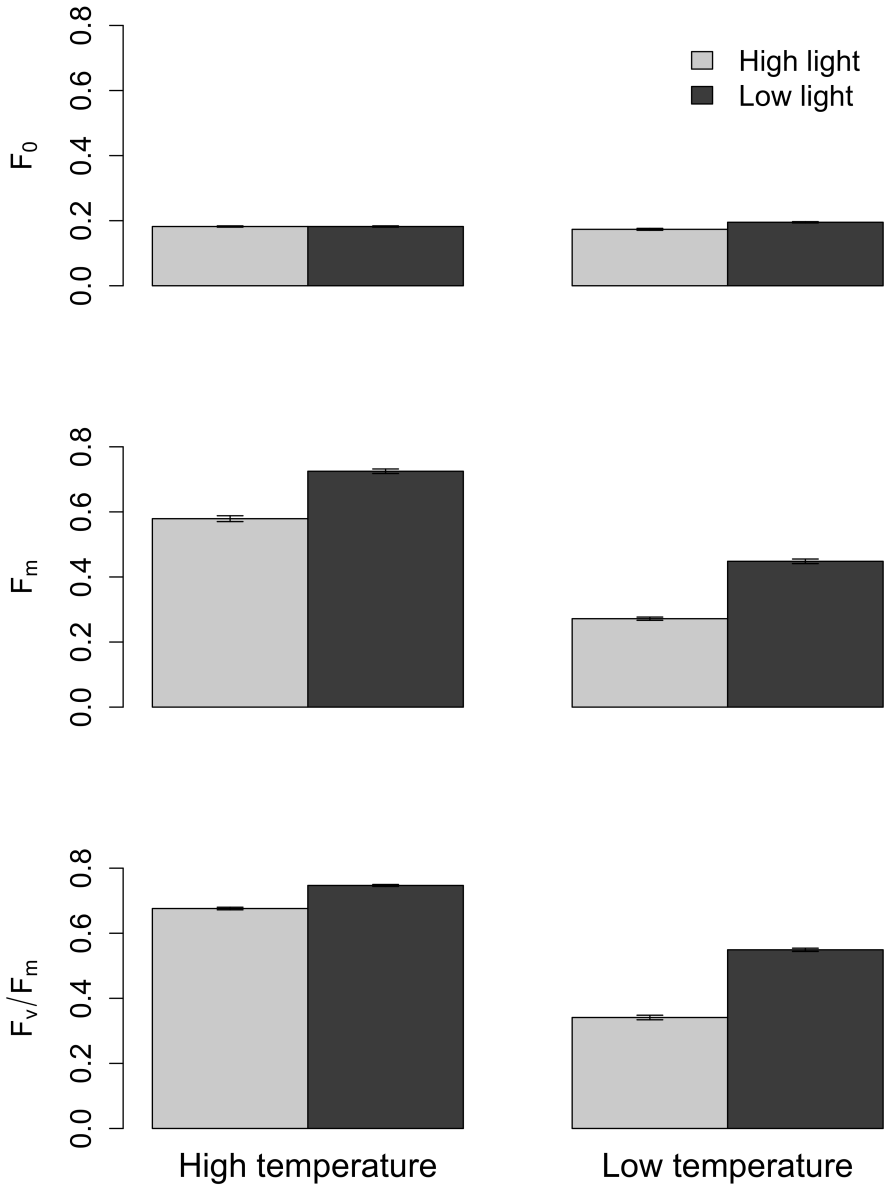

Figure 3.2: Minimal fluorescence (F_0), maximal fluorescence (F_m) and maximum photochemical efficiency of PSII (F_v/F_m) in four species of *Picea* grown under combinations of high and low temperature (10/0 °C) and high and low light (200/80 µmol m^{-2} s^{-1}) for 14 days. Measurements were done pre-dawn after 8 hours of dark-adaption. Shoots from 3 individual trees per species constituted replication; minimum 17 needles were measured per tree. Error bars ±SE.

3.4. The recovery experiment

To confirm that the conifers in this study were actually in a sustained, ΔpH-independent NPQ-state, a nearly identical recovery experiment was performed for *P. obovata* and *P. sitchensis*. Shoot were kept at room temperature in the dark for 30 minutes before chlorophyll fluorescence measurements to allow recovery of ΔpH-dependent sustained non-

photochemical quenching. The control shoots were handled as in the main experiment, that is, no recovery in room temperature before chlorophyll fluorescence measurements. The results are presented in Figure 3.3.

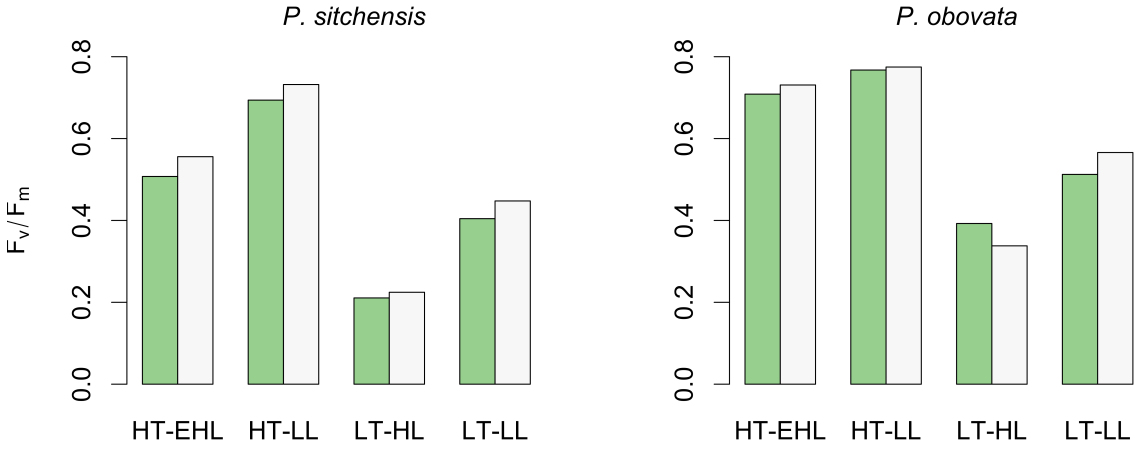

Figure 3.3: Maximum photochemical efficiency (F_v/F_m) for *Picea* spp. grown under combinations of high and low temperature (10/0 °C) and extra high, high and low light (350/200/80 µmol m^{-2} s^{-1}) for 14 days. Shoots were harvested after at least 7 hours of dark-adaption. Green bars show measurements performed directly after harvest. White bars show measurements done after 30 min of recovery in room temperature (20 °C) in darkness.

Linear mixed models considered are shown in table B3, Appendix B. Akaike Information Criterion was used for model selection. Treatment was used as the fixed factor representing the light and temperature levels because the light levels were unintentionally extra high (350 µmol m^{-2} s^{-1}) in the high temperature chamber. For *P.sitchensis,* model 2 (including the two main effects Treatment and Recovery) had the lowest AIC. The full model had |ΔAIC|-value of 2, meaning that there were two candidate models for explaining the data. For *P. obovata* there was only one candidate model (the full model) to explain the variation in the data. The other models had a |ΔAIC|>38. The estimate for the fixed effects including the p-values, and also the variance and standard deviations for the models, are shown in Table 3.4.

Table 3.4: Random and fixed effects for linear mixed models estimating F_v/F_m in the recovery experiment estimated by restricted maximum likelihood. The estimated values of F_v/F_m are shown for each treatment. The four treatments are combinations of high and low temperature (10/0 °C) and extra high, high and low light (350/200/80 µmol m^{-2} s^{-1}). "Recovery Yes" is the level of the factor Recovery where shoots were kept at room temperature for 30 minutes before measurements were done. No interaction effects are included for *P. sitchensis* model 2 since interactions were not included in the model.

Random effects	***P. sitchensis*, model 2**		***P. sitchensis*, full model**		***P. obovata*, full model**	
	Variance	**Std.dev.**	**Variance**	**Std.dev.**	**Variance**	**Std.dev**
Tree	0.0006	0.024	0.0006	0.024	0.0160	0.040
Residual	0.0054	0.074	0.0054	0.074	0.0044	0.066
Fixed effects						
	Estimate	**pMCMC**	**Estimate**	**pMCMC**	**Estimate**	**pMCMC**
Treatment LT-LL	0.408	<0.001	0.404	<0.001	0.512	<0.001
Treatment HT-LL	0.696	<0.001	0.695	<0.001	0.766	<0.001
Treatment LT-HL	0.201	0.008	0.212	0.008	0.392	<0.001
Treatment HT-EHL	0.513	<0.001	0.506	<0.001	0.708	<0.001
Recovery Yes	0.037	<0.001	0.044	<0.001	0.052	<0.001
HT-LL × Yes			-0.006	0.780	-0.044	0.011
LT-HL × Yes			-0.030	0.137	-0.111	<0.001
HT-EHL ×Yes			0.006	0.761	-0.027	0.133

P. sitchensis

By looking at the actual size of the effects in the two models (Table 3.4), Treatment and Recovery show strong and significant effects in both models. Treatments with higher light intensity and/or lower temperature show lower F_v/F_m-values (see also Figure 3.3). The effect of Recovery is small but significant in both model 2 and the full model (0.037 and 0.044, respectively). This imply a small increase in F_v/F_m when the samples were brought to room temperature for 30 minutes before measurement. The interactions Treatment × Recovery are neglible (0.03 and smaller) and not significant in the model where they are included. No interaction effects should therefore be regarded significant and thus there is most evidence for model 2 without the interaction effects.

P. obovata

Treatment had a strong and significant effect, with increasing light intensity and/or lower temperature giving a decline in F_v/F_m as in the main experiment. F_v/F_m was higher for *P. obovata* than for *P. sitchensis* in all treatments The main effect of Recovery was significant with an effect size of 0.052 for the intercept-level of Treatment, which was LT-LL. The effect of Recovery was different for the different treatments, as evident from the

significant interaction terms of Treatment and Recovery. Treatment LT-HL showed the largest difference from the intercept-level LT-LL, with a decrease in F_v/F_m (0.052 + (-0.111) = -0.059) after 30 minutes of recovery, see Figure 3.3. The increase in F_v/F_m was slightly positive for HT-LL and HT-EHL. Thus, Recovery did have a significant effect on F_v/F_m for *P.obovata*. It gave an increase of 0.052 for one treatment, it was minor for two of the treatments and negative for one treatment.

3.5. Color analysis

For all treatments, green needle area constituted more than 85 % of the total needle area (Figure 3.4). There was no effect of light or temperature on percentage green needle area, as analyzed by Kruskal-Wallis test (Kruskal-Wallis chi-squared = 1.95, df = 3, p-value = 0.58). The remaining needle area was mostly yellow. Less than 3.2 % was orange in all treatments.

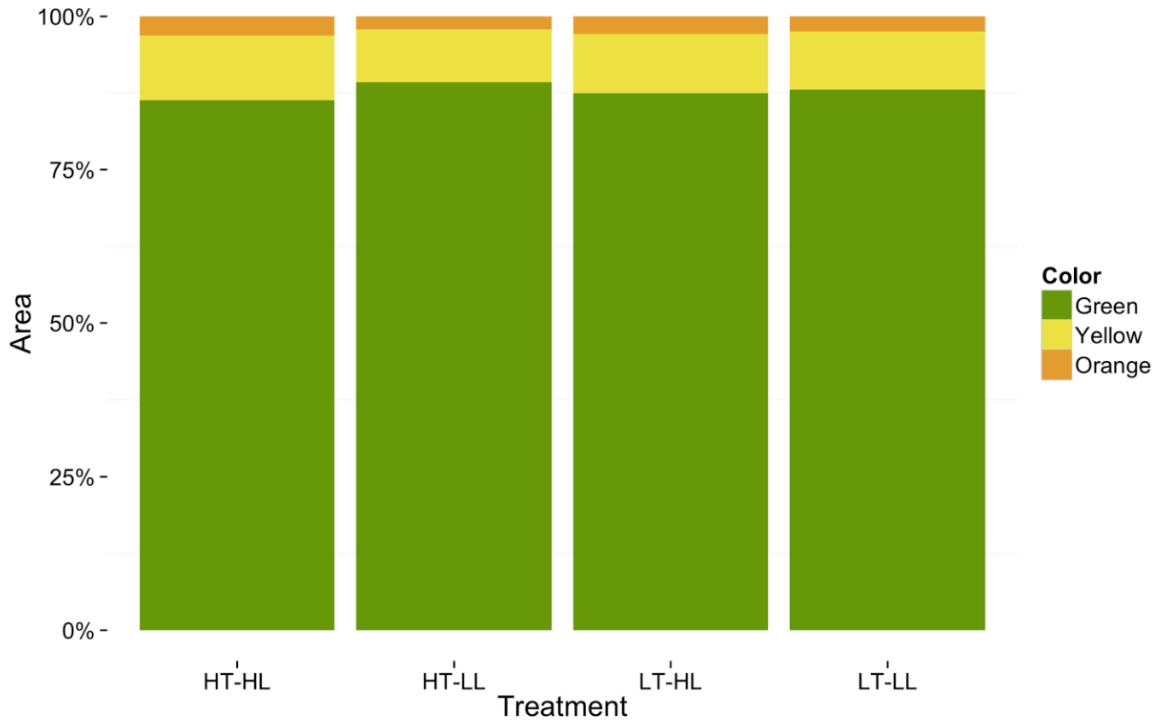

Figure 3.4: Fraction of green, yellow and orange colored needle area of conifer needle sections from shoots of four species of *Picea* grown under combinations of high and low temperature (10/0 °C) and high and low light (200/80 µmol m^{-2} s^{-1}) for 14 days. n=12 for all treatments.

3.6. Pigment analysis

Violaxanthin levels of the needles were analyzed using HPLC (Figure 3.5). Kruskal-Wallis rank sum test for the means of the four treatments HT-HL, HT-LL, LT-HL, LT-LL showed a significant difference between the groups (Kruskal-Wallis chi-squared = 33.40, df = 3, p-value < 0.001). Multiple comparisons revealed differences between treatments at p=0.05 as shown in Figure 3.5. Violaxanthin levels were significantly higher for both the LL and HL treatments at high-temperature than for the HL treatment at low temperature, but the LL treatment at LT could not be distinguished from the HT-HL treament. (At *p*=0.06, these treatments were significally different.) No significant differences were found between light levels at neither high nor low temperature.

No zeaxanthin was detected in the HPLC-analyses of the conifer needles for unknown reasons. (See chapter 4.5 for discussion.)

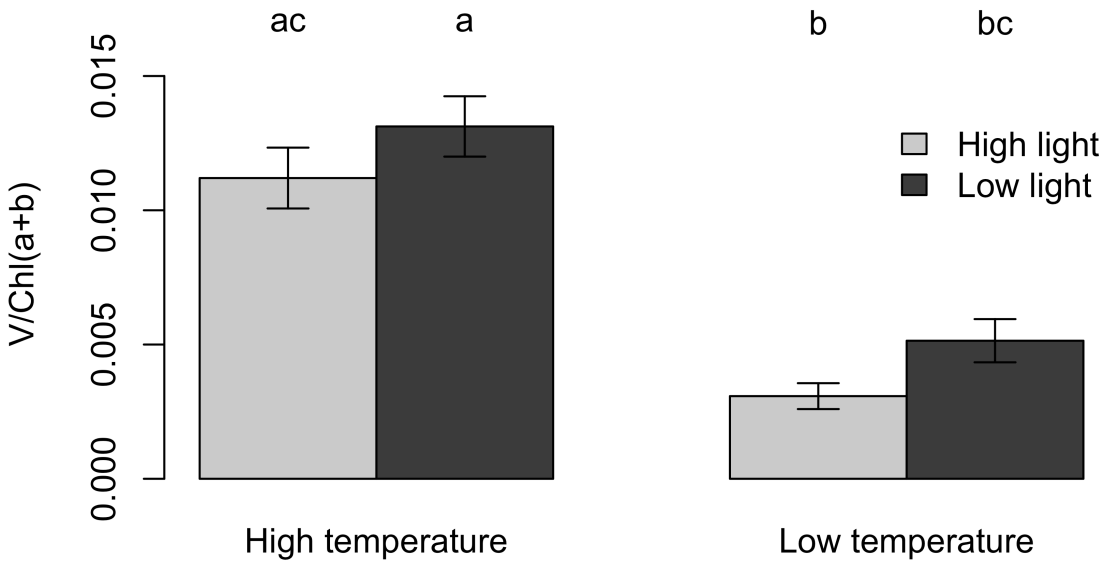

Figure 3.5: Relative levels of violaxanthin to Chlorophyll a+b ([Violaxanthin]/([Chl a] + [Chl b])) in four species of *Picea* grown under combinations of high and low temperature (10/0 °C) and high and low light (200/80 µmol m^{-2} s^{-1}) for 14 days. Samples were harvested after 8 hours of dark-adaption. Means with the same letter (a, b or c) are not significantly different (p=0.05). n= 12 for HT-treatments, n=11 for LT-treatments. Error bars ±SE.

3.7. Protein analysis

Expression of the 22 kDa protein PsbS was analyzed for the four treatments for all species. Relative optical densities from the Western blots are shown in Figure 3.6. The expression levels showed no difference between the four different treatments (Kruskal-Wallis chi-squared =4.25, df=3, p=0.24). In Figure 3.6, a trend of higher PsbS-levels at low temperature was observed. Nevertheless, in separate analyses for the four species this trend was not present (Figure B1, Appendix B).

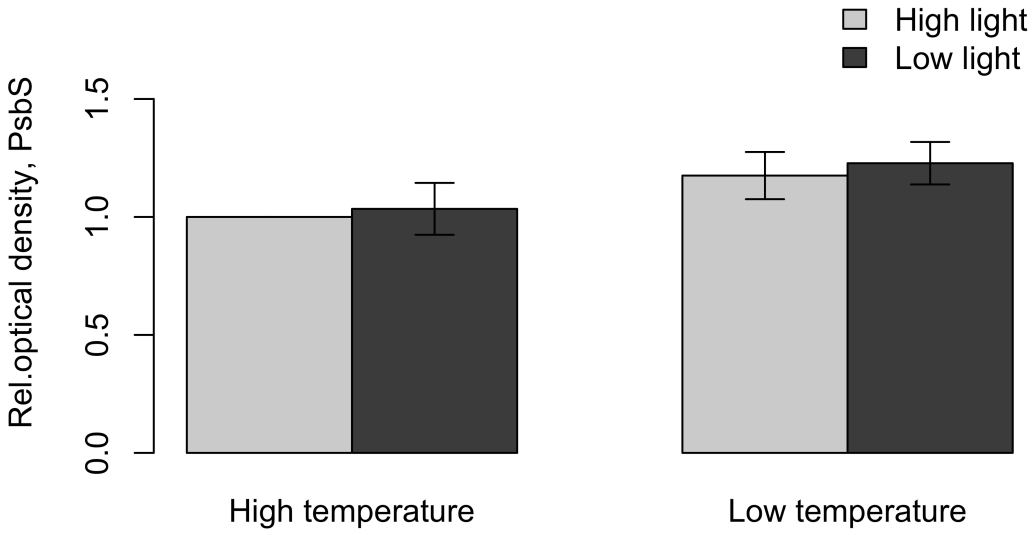

Figure 3.6: Western blot protein expression results for PsbS in shoots of four species of *Picea* grown under combinations of high and low temperature (10/0 °C) and high and low light (200/80 µmol m^{-2} s^{-1}) for 14 days. Relative optical density was calculated as optical density of the sample relative to the optical density of the HT-HL-treatment for each species for each blot. All treatments for each species were run in the same blot. Replicates for the species were run on different blots. For HT-HL n=12, HT-LL n=11, LT-HL n=12, LT-LL n=10. Error bars ±SE.

Pictures of two representative blots are given in Figure 3.7. Blot A is a blot with weakly developed bands and Blot B is a blot with strongly developed bands. In the experiment, 2 out of 6 blots were like blot B, while blot A is representative for the remaining blots.

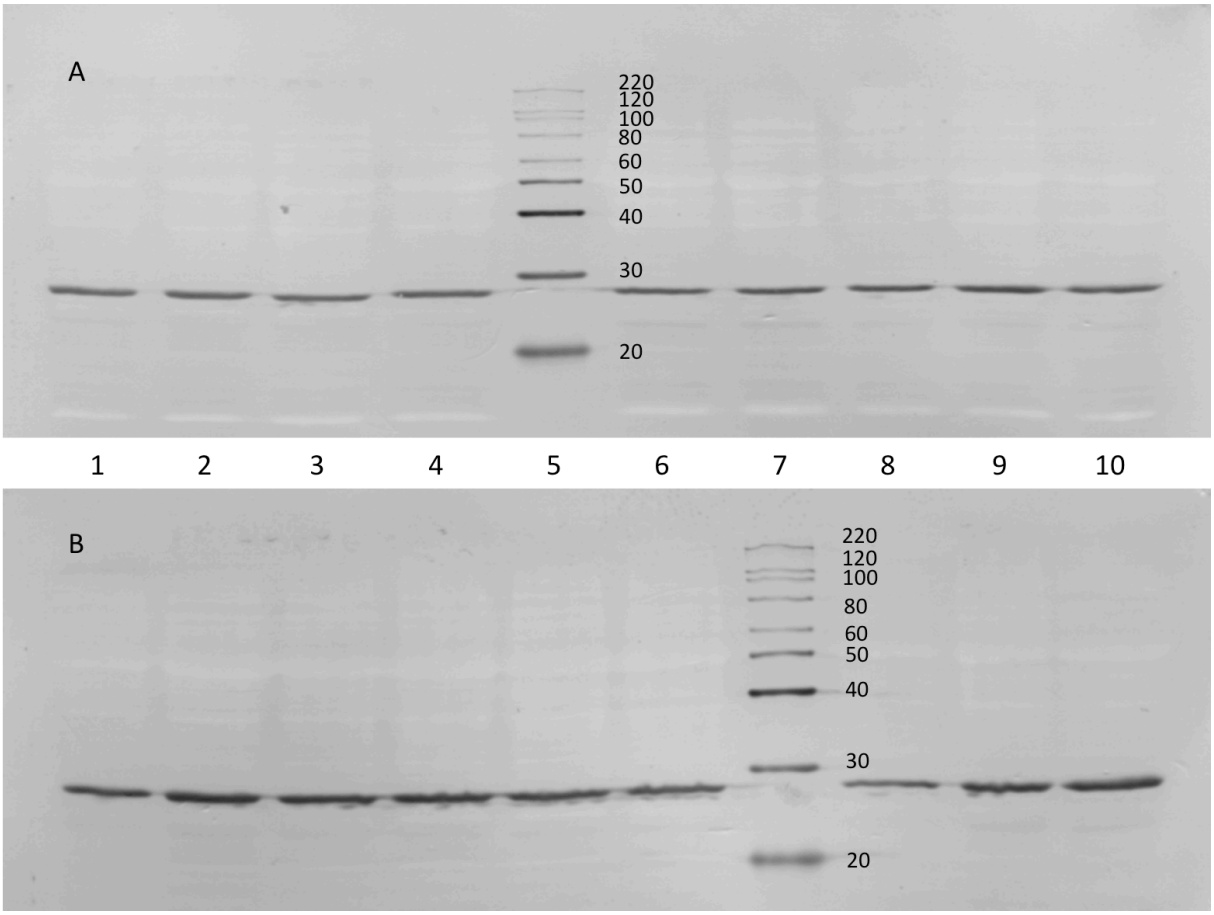

Figure 3.7: 2 representative western blots showing PsbS expression in *P. rubens* and *P. abies* grown under four different light/temperature conditions. Blot A and B show the number 2- and 3-trees, respectively. Application order for the treatments: HT-HL, LT-HL, HT-LL, LT-LL. Blot A) Lane 1-4: *P. rubens*; lane 6-9: *P. abies*; lane 5: Protein standard, molecular weights in kDa (MagicMarker™XP Western Protein Standard 20-220kDa, Invitrogen Corp. USA). Blot B) Lane 1-4: *P. rubens*; lane 5-6,8-9: *P. abies*; lane 7: MagicMarker™XP protein standard.

4. Discussion

The aim of this study was to investigate the separate and interactive effects of light and temperature on sustained non-photochemical quenching in four species of boreal conifers, and test hypotheses regarding the molecular mechanism behind these effects. A factorial experiment applied to four species of *Picea* revealed that temperature had a strong effect on development of sustained non-photochemical quenching while light had a smaller influence. The results also revealed that high light caused a larger reduction in photochemical efficiency of PSII at low temperature (0 °C) than at high temperature (10 °C). The study showed that the species had different responses to the abiotic stresses. No visible chlorosis or necrosis was observed for any of the species in any treatments. In a follow-up experiment on the recovery of maximum photochemical efficiency of PSII, a minor relaxation was found after 30 minutes, indicating some ΔpH-dependent sustained NPQ. However, the quenching observed was mainly ΔpH-independent sustained NPQ,

To ensure that zeaxanthin was retained overnight and is a part of the molecular mechanism in the sustained mode of quenching of excess energy, pigment composition was analyzed for shoots exposed to the different treatments. Violaxanthin and antheraxanthin, but not zeaxanthin, were detected. Violaxanthin concentration was expressed on a chlorophyll basis, and showed trends of decreasing levels at high excitation pressures. The results point toward an involvement of the xanthophyll cycle, even though the full picture is not revealed because of a lack of information on zaexanthin levels.

The hypothesis that PsbS is involved in sustained non-photochemical was tested. By investigating the levels of PsbS expression in the different light and temperature treatments, no evidence for involvement of PsbS was found.

Regarding the localization of the non-photochemical quenching inside the chloroplast, involvement of the xanthophyll cycle points toward antenna quenching. The fluorescence measurements, however, indicate only reaction center quenching, according to a hypothesis by Bukhov et al. (2001). This will be discussed briefly at the end of this chapter.

4.1. Low temperature and high light induce sustained NPQ in boreal conifers

The 2x2x4 factorial experiment showed that both low temperature and high light have a significant negative effect on the maximum photochemical efficiency of PSII (Figure 3.1). This was verified by fitting a linear mixed model using restricted maximum likelihood (Table 3.3). The full model was solely best for explaining the variance in the data. The difference in AIC (|ΔAIC|) to the next best model was 8 (Table B1), which implies that the other models have very little support (Burnham and Anderson, 2004). The effect of temperature was shown to be much stronger than the effect of light (estimated effects -0.32 vs 0.06 respectively (Table 3.3)). This means that by decreasing the temperature from 10 °C to 0 °C (for intercept levels *P. abies* and high light) F_v/F_m is estimated to decrease by 0.32 while changing the light intensity from 200 μmol m^{-2} s^{-1} to 80 μmol m^{-2} s^{-1} (for *P. abies* at high temperature) F_v/F_m will increase by 0.06. The magnitude of the effects found for *P. abies* are representative for the other species as well, except for *P. sitchensis*, which showed stronger responses.

Under low temperature and/or high light as experienced by the shoots in this experiment, the need for protection against production of reactive oxygen species is increased. Presence of low values of F_v/F_m implies that the plants experience a stress additional to light to such an extent that the flexible, rapidly reversible NPQ is replaced by a sustained non-photochemical protection mechanism (Demmig-Adams et al., 2006b).

The observations are in accordance with data from numerous studies on conifers and other plants with evergreen habit that experience winter conditions. Field studies under different temperatures have shown lower photochemical efficiency in sun-exposed than shaded leaves, and strongest declines have been found at low temperatures (Ottander et al., 1995; Strand, 1995; Adams et al., 2004; Ensminger et al., 2004; Zarter et al., 2006a; Zarter et al., 2006b; Verhoeven, 2013). For Douglas fir (*Pseudotsuga menziesii*), F_v/F_m -levels of 0.45 and 0.75 were found in sun and shade needles when temperature on the day of measurement was -3.5 °C (Ebbert et al., 2005). Light intensities were 50-60 μmol m^{-2} s^{-1} in the shade and 1800-2000 μmol m^{-2} s^{-1} in the sun on clear days. The results from the present experiment showed somewhat lower photochemical efficiency, but direct comparisons are difficult because of lack of information about the light and temperature conditions prior to sampling in the field experiment. Lundmark et al. (1998) established a correlation between F_v/F_m and

mean daily temperature based on a two-year field study in exposed needles of *Picea abies*. According to their results, mean daily temperatures of 10 °C and 0 °C corresponds to F_v/F_m values of approximately 0.65 and 0.40, respectively. Information on light intensity was not provided. The results are however close to the results in this experiment for *P. abies* at high light (0.67 and 0.34 at high and low temperature, respectively). This is an indication that the conditions in the chambers can be representative for the actual conditions experienced by conifers in nature.

Few similar controlled lab experiments have been performed, but Lamontagne and colleagues (2000) exposed *Picea mariana* to combinations of light and low temperature. Frost (-4°C) was applied 15 days before fluorescence measurements. F_v/F_m clearly showed the same pattern as in the present study. Direct comparisons of the numbers are difficult due to the frost treatment and different temperature regimes. Under day/night temperatures of 15 C°/5°C F_v/F_m was approx. 0.70 at 80 µmol m^{-2} s^{-1} and 0.55 at 240 µmol m^{-2} s^{-1}. At 5 °C/5 °C the values were 0.65 and 0.45 respectively. A similar set-up as in the present experiment but with temperatures of 5 °C and -5 °C, and light intensities of 50 µmol m^{-2} s^{-1} and 350 µmol m^{-2} s^{-1} gave results for F_v/F_m at HT-HL, HT-LL, LT-HL and LT-LL of 0.57, 0.79, 0.04 and 0.31, respectively, for *Pinus sylvestris* (Sveshnikov et al., 2006). With the slight exception of the HT-LL-treatment, F_v/F_m values are consistently lower than in the present study, which is expected because of the lower temperatures. A strong, significant interaction between light and temperature was found in the present experiment. The effect of light was stronger when the temperature was low. This supports previous findings as well (Lamontagne et al., 2000; Porcar-Castell, 2011). It can be explained by less efficient oxidization of Q_A^- at low temperature (see section 1.2), which leads to more closed reaction centers for the same increase in light. The plants are thus more sensitive to increasing light at low temperature.

It should be noted that the stronger effect of temperature than light is only valid for the levels of light and temperature chosen in this experiment. Different levels of the factors would give different effect sizes. That is, if higher light level or lower temperature were chosen, a stronger effect of light and temperature would probably occur. Instantaneous photosynthetic photon flux densities up to 1000 µmol m^{-2} s^{-1} are observed on bright days in spring in conifer crowns (Strimbeck et al., 1993) and even higher photon flux densities are observed at the

alpine timberline (Yamazaki et al., 2003). Therefore, it is reasonable to believe that F_v/F_m will decrease even more in nature than what was reported here, where high light-treatment corresponded to 200 μmol m^{-2} s^{-1}. F_v/F_m as low as 0.2 is reported in field experiments for *Pinus sylvestris* (Ottander et al., 1995; Lundmark et al., 1998), indicating that lower maximum photochemical efficiency is possible to obtain in more extreme conditions. Extremely high irradiances were not possible using the fluorescent lighting in the growth chambers, and it would not be representative for field conditions to keep a much higher irradiance during the whole day. For example, in southern Finland the daily mean PPFD is seldom higher than 300 μmol m^{-2} s^{-1} during winter (Porcar-Castell, 2011). Additionally, higher light levels would probably lead to desiccation of the needles.

The linear mixed model showed a low repeatability expressed as the ratio of variation within Tree to total variation (28 %, see section 3.2). This means that a quite high share of the variance was due to variance between individual trees. Possible reasons include genetic variation or environmental adaptions to the specific site where the shoots were harvested. More likely, there is an effect of the position inside the growth chambers. The shoots from each tree were arranged in separate floral foam strips that subsequently were placed side by side in the growth chambers. The position of the blocks was the same throughout the experiment period. Since the light intensity in the chambers showed spatial variation (Table 3.1), there was different illumination on each of the blocks comprising each set of replicate trees. This could have given different results for the different Tree replicates, and thus a higher variation between individual trees.

There was no true replication for the light and temperature effects due to equipment limitations. The results are in close accordance with other studies, but the exact effect of temperature and light should be interpreted with care until further growth chamber studies have been performed.

4.2. Species differ in their response to high excitation pressure

The four species considered were chosen because of their different natural distributions and corresponding ecological adaptions. A summary of their natural distributions and data on their cold tolerance are summarized in Table 4.1.

Table 4.1: Original range and low-temperature tolerance data for foliage of the *Picea*-species investigated in the experiment

Species	Natural distribution[1]	LT_{50} (°C)[2]	USDA hardiness zone[3]
P. obovata	Northern Europe and Siberia	-[4]	1
P. abies	Northern Europe	-47.9	4
P. rubens	North-east coast of North America, Appalachian mountains	-38.3	3
P. sitchensis	North-west coast of North America	-35.7	7

1 (Eckenwalder, 2009), 2 (Strimbeck et al., 2007), 3(Bannister and Neuner, 2001), 4 No temperature caused death of tissue, therefore true LT_{50} could not be determined

As shown in Figure 3.1 and confirmed through the linear mixed model, *P. sitchensis* is the species with unique response to light and temperature stress. It was more flexible than the other species, with a remarkably strong response to light at high temperature compared to the other species, and showed the strongest depression of maximum photochemical efficiency of PSII recorded in the experiment at low temperature and high light. On the other hand, *P. sitchensis* showed the highest maximum photochemical PSII efficiency recorded in the experiment at high temperature and low light. This flexibility can be an advantage in the original climate of the species, see Table 4.1. In the coastal areas where *P. sitchensis* has its origins, the extreme minimum temperature will rarely be very low (for USDA hardiness zone 7, average annual extreme minimum between -17.8 °C and -12.2 °C) and more commonly milder temperatures characterize the winters. Under these conditions, winter photosynthesis can occur during warm spells (Neilson et al., 1972; Schaberg et al., 1998). For that reason, a flexible protection mechanism could be beneficial for *P. sitchensis*.

In contrast, continental climates are characterized by severe cold that continues for months. Under such conditions, no photosynthesis can take place during the winter. Periods with conditions favorable of photosynthesis are likely to be rare. Consequently, species adapted to such climates could be better off shutting the photosynthetic machinery down with a high degree of photoprotection during the whole winter. This would thus definitely apply to *P. obovata* (Table 4.1). Regarding *P. abies*, the range of origin is mostly characterized by

continental climate, so it is highly cold tolerant too[1]. *P. rubens*, with its coastal origin, would probably be like *P. sitchensis* and benefit from a flexible protection mechanism. It has previously been shown that the highly cold tolerant *Abies balsamea* does not perform photosynthesis during warm spells, in contrast to *P. rubens* which shows recovery (Strimbeck et al., 1995). The results show no difference in the response to light between the three species *P. obovata, P. abies* and *P. rubens* at high temperature. At low temperature, *P. obovata* was as responsive as *P. sitchensis*, while *P. rubens* and *P. abies* were less responsive. The latter is not in agreement with the hypothesis stated above. According to the hypothesis, *P. obovata* should have a more constant level of sustained NPQ, since it experience long periods unfavorable for doing photosynthesis. The hypothesis is therefore not fully supported even though the results for *P. sitchensis* are supportive.

Few other studies have been performed on species-specific responses to photoprotection, so the possibilities for explaining the data in light of other studies are limited. It is generally recognized that shade adapted leaves show higher susceptibility to photoinhibition than sun-adapted leaves within a species (Taiz and Zeiger, 2006). Robakowski (2005b) proposed a hypothesis including a trade-off between the shade tolerance of the species and resistance against low-temperature induced photoinhibition. However, results showed that shade tolerant plants are actually less prone to low-temperature induced photoinhibition, and other studies have found no correlation between shade-tolerance and photoinhibition (Robakowski, 2005b, a; Wyka et al., 2007). At this point, the results are thus unclear about the connection between shade tolerance and photoinhibition induced by low temperature. Also, the species investigated in the present experiment are all shade tolerant (Burns and Honkala, 1990; Gustafson et al., 2011) or intermediate shade tolerant (*P. abies*) (Wyka et al., 2007), so differences due to this trait would probably not be responsible for differences observed. However, little attention was paid to the previous sun exposure of the harvested shoots. This can probably have introduced unwanted variation in the data. The temperature preceding the experiment can also be a factor affecting the results. The lowest temperature recorded was -13.7 °C, and the mean temperature in January was -1.9 °C (Table 3.1). This might contribute to the absence of a constant high level of NPQ the boreal species. However, extreme cold tolerance is achieved in such conditions (Strimbeck et al., 2007). If the two

[1] Since it is so widespread, it can be found to have different degrees of cold tolerance depending on place of origin. This might explain the difference in hardiness zone status designated by Bannister and Neuner (2001), and T_m found by Strimbeck (2007).

mechanisms are dependent on the same environmental cues for onset, like photoperiod and frost, sustained NPQ should have commenced. In conclusion, it seems likely that sustained NPQ is fairly adjustable under the tested conditions both for continental and coastal species, but with *P. sitchensis* showing the most flexible mechanism.

Ultimately, is much sustained NPQ a benefit for the tree? Should the trees exhibiting low F_v/F_m -values be considered well adapted, or incompetent to utilize incoming light? Low F_v/F_m -values can as explained above be indicative of either high NPQ or low photochemistry rates. Depressed F_v/F_m and concomitantly increased F_0 are widely used and accepted as indicators of photodamage, probably due to degradation of the D1-protein (Ottander et al., 1995; Maxwell and Johnson, 2000). In the present experiment, the F_0-values were constant indicating no damage to the photosystems (Figure 3.2). Low F_v/F_m -values in high light and/or low temperature are thus most likely related to higher sustained NPQ-levels. In some species, like winter wheat, high photosynthetic rates can be maintained or increase during winter (Savitch et al., 2002). The need for assimilates is still high because growth and development continue in the cold season. This species does not show increased NPQ during winter. However, if photosynthetic rates decrease in winter, concomitant with termination of growth, less sugar consumption and cold hardiness, a high NPQ is essential to avoid photo-oxidative damage (Demmig-Adams et al., 2006b). This is the case for boreal conifers and increased sustained NPQ should thus be viewed as an adaption to cold environments. This interpretation implies that *P. sitchensis* is best adapted since it shows high NPQ-levels at high excitation pressure. However, if the other species have better requirements for photosynthesis in harsh conditions, they can to a greater extent maintain photochemical quenching and therefore be equally well adapted even with less NPQ. To fully reveal the question on how sustained NPQ relates to different species and their adaptions, studies including measurements on photosynthetic performance, like daily carbon exchange rates, should be performed.

4.3. ΔpH-dependent NPQ or ΔpH-independent NPQ?

The chlorophyll fluorescence measurements in the main experiment were performed pre-dawn, i.e., after 8 hours of dark-adaption at the assigned temperature (0 °C or 10 °C). The lowered F_v/F_m reported should thus reflect sustained levels of non-photochemical quenching. Nevertheless, a ΔpH-dependent, rapidly reversible NPQ can appear sustained over night at low temperatures, as mentioned in section 1.4, and the recovery experiment was performed to

confirm that the ΔpH-independent sustained NPQ consituted the NPQ observed in the main experiment.

In *P. sitchensis*, a slight increase (estimated to 0.037) was observed. There was no difference in recovery-effect between the treatments. This is indicative of a slight share of the non-photochemical quenching observed being ΔpH-dependent NPQ. For *P. obovata*, the results are less consistent. There was an increase in F_v/F_m at LT-LL (estimated 0.052) and smaller increases at HT-EHL and HT-LL. The smaller increases for these two treatments can be due to the already high values of F_v/F_m for these two treatments, possibly making a further increase in F_v/F_m less likely. For the LT-HL treatment, a decline in F_v/F_m was observed after 30 minutes of recovery, which was unexpected. There should be no reason for a decline in maximal photochemical efficiency when brought to higher temperature. The model shows a high variation between individual trees for *P. obovata* (Table 3.4) (indicated as low repeatability $Var_{Tree}/ Var_{Tree} + Var_{Residual} = 0.26$). However, examination of the data from the three replicate trees showed a consistent decline for all three trees. This excludes the possibility of one shoot being not representative. The result thus remains unexplained. For the control shoots, F_v/F_m was consistently higher for *P. obovata* than for *P. sitchensis* in all treatments. This is somewhat in contrast with the main experiment, where *P. sitchensis* had higher F_v/F_m at the treatment with lowest excitation pressure (HT-LL).

Estimated increase after recovery was 0.037 for *P. sitchensis,* and for *P. obovata* it was up to 0.052. These numbers are smaller than reported for *Picea glauca* in a recent study, where increases between 0.07 and 0.17 were reported after 30 minutes of recovery in the morning (Verhoeven, 2013). In that study, no recovery was found when air temperature was above zero the night before measurements were done, suggesting according to the author that sub-freezing temperatures are a prerequisite for the ΔpH-dependent sustained NPQ to persist (Verhoeven et al., 1998; Verhoeven et al., 2009; Verhoeven, 2013). This is in contrast to what have been found in the present study, where an effect of recovery was evident at 0°C and 10 °C. Another study found this effect at above-freezing temperatures as well (Demmig-Adams et al., 2006a). To my knowledge, no other studies have investigated relaxation in samples under different excitation pressures.

Verhoeven (2013) suggests that the flexible, ΔpH-dependent sustained NPQ is useful when temperatures fluctuate above and below zero, protecting the photosynthetic machinery in cold periods while making photosynthesis possible during warm spells. Periods favorable for photosynthesis can occur during winter in the coastal regions that *P. sitchensis* occupies and rapid adjustments would thus be favorable. Flexibility would be a less important trait for *P. obovata* originating from the cold interior of Siberia. Nevertheless, except for the LT-HL treatment, a trend for increased F_v/F_m was seen also for *P. obovata* (Figure 3.3), but the increases were minute for the HT-EHL and HT-LL-treatments.

Taken together, the increases in F_v/F_m after recovery for both species were minor, so the ΔpH-independent form of sustained NPQ is the dominant form of NPQ seen in these experiments. This means that even though a significant effect of recovery was found, any conclusions about the sustained non-photochemical quenching from the experiments reported here would be about the ΔpH-independent form.

4.4. No visual injury due to high excitation pressure

Winter chlorosis, defined as yellow coloration of foliage during the cold season that is reversible in summer, has been observed in spring (Baronius et al., 1991) when temperatures are still low and light is strong. Also, winter injury resulting in red-brown necrosis (not reversible) in *P. rubens* is reported to be strongest in southern and western aspects of foliage, and increasing with increasing height in the canopy (Peart et al., 1991). Molecular damage of light stress in winter and spring is known to include increased destruction and/or downregulation of synthesis of the D1-protein of the PSII core (Ensminger et al., 2004; Zarter et al., 2006a; Zarter et al., 2006b; Verhoeven et al., 2009). Since D1 binds chlorophyll, high degradation rates without corresponding high rates of D1-synthesis could lead to chlorophyll breakdown and subsequent bleaching of the needles if damage is severe. This led to the hypothesis that high excitation pressure cause oxidative damage leading to chlorosis and maybe also injury in conifers in spring.

Color changes were therefore monitored using a quantification method based on pixel counts of the different colors in pictures of the needles. Chlorosis and necrosis will appear as higher fraction of yellow and orange tissue, respectively (Strimbeck et al., 2007). Hence, both will become visible as a lower percentage of green color. No difference was shown for the four treatments (Figure 3.4), and no color difference was found in these samples compared to

samples from the day of harvest (data not shown). Green pixels constituted more than 85 % of the total needle area. This is the same proportion as reported for healthy needles of *P. rubens* in a previous experiment using the same method (Strimbeck et al., 2007). The results thus imply that the needles did not acquire any visible injury symptoms under the conditions applied. Still some D1 breakdown and other molecular damage to the photosynthetic apparatus could have occurred, but the damage should be considered limited at the leaf level. No visible color changes under increased excitation pressure thus signify that the observed ongoing non-photochemical quenching is sufficient for protection under the given stress. The stable F_0-levels (Figure 3.2) are also evidence for the absence of damage to the reaction centers. Depressed F_v/F_m and concomitantly increased F_0 is widely used and accepted as indicators of photodamage, probably due to degradation of the D1-protein (Ottander et al., 1995; Maxwell and Johnson, 2000).

Even though winter chlorosis did not develop in this experiment it cannot be ruled out that light stress is a cause for winter chlorosis. Under more extreme conditions, sustained NPQ might not be sufficient for protection of the photosynthetic apparatus and damage and chlorosis can follow. This was observed in seedlings of *Eucalyptus globulus* in Tasmania (Close et al., 1999). Plants that were not protected with shading cloth during a mild overnight frost and cold morning experienced high light intensity (700-800 μmol m^{-2} s^{-1}). The authors reported that 30 % leaf area had symptoms of photobleaching. Also, height growth measured two months later was reduced compared to shaded seedlings. As mentioned in section 4.1, light intensities experienced by conifers in field, especially in spring, can be far more extreme than in this experiment. Coincidentally, temperatures can stay well below freezing. For example, in Trøndelag Norway, where *Picea abies* is a dominant conifer, the temperature normal for February and March is -5.9 °C and -3.3 °C, respectively, with yearly mean temperatures often as low as -10 °C and -7 °C (Meterologisk Institutt, 2013). In Siberia, temperatures are even more extreme as mentioned in section 1.1. Field studies monitoring color change in shaded and exposed needles, or in more extreme growth chamber conditions, should be utilized for a better understanding of the phenomenon winter chlorosis in boreal conifers.

4.5. Evidence for contribution of the xanthophyll cycle

To confirm that the xanthophyll cycle and accordingly antenna quenching was involved in sustained NPQ observed in the present study, HPLC-analyses of pigments in the exposed shoots were done. The purpose was to determine the epoxidation status (EPS) for the different treatments, expressed as (V+0,5A)/(Z+A+V) where Z, A and V are zeaxanthin, antheraxanthin and violaxanthin, respectively (Thayer and Bjorkman, 1990). (Z+A)/Chl a+b are best correlated with NPQ for leaves from a single species, but only suitable for comparison for leaves within a species because of different chlorophyll content and leaf thickness of different species (Adams et al., 1995). Therefore, EPS is the preferred parameter for comparing the involvement of the xanthophyll cycle between species. However, no zeaxanthin was detected in the analyses which made calculation of EPS impossible. It is not likely that zeaxanthin was not present in the needles at all. Numerous studies of plants showing sustained NPQ report high pre-dawn levels of zeaxanthin (Adams et al., 1995; Verhoeven et al., 2009), including studies on conifers (Ottander et al., 1995; Adams and Demmig-Adams, 2004; Ebbert et al., 2005; Sveshnikov et al., 2006; Zarter et al., 2006b). No studies on pre-dawn pigment composition in plants with sustained NPQ have reported absence of zeanxanthin. Also, the reported changes in violaxanthin-levels indicate prescence of zeaxanthin. Separation of zeaxanthin from lutein can be difficult to achieve in HPLC. Co-elution of these xanthophylls could therefore occur and accordingly zeaxanthin may be masked of by lutein (Gilmore and Yamamoto, 1991). Fail to detect zeaxanthin in this experiment should not be affected by this, however, because absorption spectra were provided and thus identification of zeaxanthin should have been possible. Thus, the reason for the nonappearance of zeaxanthin in the samples remains elusive.

For this study, the pigment results are thus reported as V/Chl a+b, which should give an approximate mirror image of the parameter Z+A/Chl a+b. Species comparisons are therefore not performed. There are also problems with expressing xanthophyll as a fraction of the total chlorophyll content because this presupposes a constant chlorophyll content of the needles from the different treatments. Chlorophyll content has been shown to decrease during autumn (Ottander et al., 1995; Ensminger et al., 2004) and as a response to artificially lowered temperatures (Sveshnikov et al., 2006) in *Pinus sylvestris*. However, chlorophyll content has been found to be fairy constant during the cold period (Ottander et al., 1995; Ensminger et al., 2004) even under high irradiances in spring. Other studies report decreased chlorophyll

content during winter for leaves exposed to high irradiances (Sveshnikov et al., 2006). With conflicting evidence, it cannot be assumed that the chlorophyll content was the same between the different treatments in the study, which implies that caution must be taken when interpreting the results. Lower levels of chlorophyll in low temperature and/or high light would cause an overestimation of the violaxanthin levels in these treatments compared to the other treatments. However, no color changes was found in the needles from different treatments (Figure 3.4, see discussion below), which at least makes large changes in chlorophyll concentration unlikely.

Another factor that can influence the results is that the total amount of xanthophylls are shown to increase in situations with high excitation pressure (Ottander et al., 1995; Verhoeven et al., 1998; Adams et al., 2002; Savitch et al., 2002). If this is the case also in this experiment, there is a possibility that V/Chl a+b remains the same or gets higher even though the relative amount of violaxanthin to the total xanthophyll pool is decreased. This will underestimate the contribution of the xanthophyll cycle in NPQ. It is not possible to calculate the total xanthophyll pool per needle weight both because zeaxanthin was not detected and because the analyzes were not done quantitatively.

Nevertheless, (Z+A)/Chl a+b has been used in a number of studies of sustained non-photochemical quenching in winter (Bachmann et al., 2004; Ebbert et al., 2005; Demmig-Adams et al., 2006a; Zarter et al., 2006a; Zarter et al., 2006b). The pattern of changes in Z+A/Chl a+b have been shown to be similar to changes in de-epoxidation status (DEPS = (Z+A)/(V+A+Z)) for *Vinca minor* (Adams et al., 2002), but decreased chlorophyll content in the winter months led to a somewhat stronger increase in the Z+A/Chl a+b -parameter than DEPS-parameter, as expected. Based on the arguments above, the results obtained here using V/Chl a+b, will be used with care to infer information on the epoxidation status of the xanthophylls.

There was evidently an effect of temperature on the pre-dawn violaxanthin levels. Both for high and low light treatments, the violaxanthin level was significantly lower at low temperature (Figure 3.5). This indicates that more violaxanthin is converted to antheraxanthin and zeaxanthin when needles are exposed to low temperature. Since harvesting was done pre-dawn, the results show that high levels of zeaxanthin are retained even in darkness when temperature is low and contribute to the sustained quenching. This is fully in accordance with

previous studies on winter light stress acclimation responses in conifers (Adams and Demmig-Adams, 1994; Ottander et al., 1995; Ebbert et al., 2005; Verhoeven et al., 2009) and also observed in controlled laboratory experiments (Savitch et al. 2002; Sveshnikov et al. 2006). The same pattern is observed in other plants as well (Adams et al., 2002; Zarter et al., 2006a). These findings confirm a role for the xanthophyll cycle and concomitant antenna quenching in the sustained non-photochemical quenching observed in the experiment.

However, contrary to earlier observations (Ebbert et al., 2005; Sveshnikov et al., 2006; Verhoeven et al., 2009) no significant difference was observed in response to light intensity at either high or low temperature. A difference was expected because violaxanthin is supposed to be de-epoxidized to a higher extent when the excitation pressure rises under higher light intensities. Still, for both high an low temperature there is a trend showing lower levels of violaxanthin for the high light treatments. The disadvantage of the robust Kruskal Wallis test is a lower sensitivity, which can fail to reveal differences between the means (Walpole et al., 2007), which might be the case in this situation. Using ANOVA was not an option due to non-normal distribution of the data, and the assumption of normally distributed errors for the linear mixed model was violated. Even though the Kruskal Wallis test did not reveal any differences between the means for the light treatments, it is thus resonable to not fully reject the hypothesis of different violaxanthin levels for different light levels. Also the previous research mentioned above suggest such an interpretation of the data.

4.6. Expression of PsbS is independent of sustained NPQ-levels

The results presented in Figure 3.6 indicate that the protein PsbS is not involved in up-regulating the sustained non-photochemical quenching observed in this experiment. This helps clarify the apparent discrepancy regarding the role of PsbS in sustained non-photochemical quenching.

Previous studies have shown an increase in PsbS in wintertime. Savitch and co-workers (2002) showed increasing PsbS-levels in Scots pine (*Pinus sylvestris*) during winter hardening under controlled conditions. Ottander and colleagues (1995) also showed a strong increase in PsbS during winter for the same pine species from a natural stand. These results lead to the suggestion by Öquist and Huner (2003) that PsbS was involved in formation of a chlorophyll-xanthophyll-protein association that keep the xanthophylls de-epoxidized, as

mentioned in section 1.4. Ebbert (2005) also found increases in PsbS in winter for Douglas fir (*Pseudotsuga menziesii*).

In the results presented in this thesis, pre-dawn depressions in F_v/F_m indicate sustained non-photochemical quenching, and the extent of the sustained NPQ varied between the treatments. Also, the results from the recovery experiment demonstrate that the largest portion of the quenching observed is not relaxed at warm temperatures. The quenching is thus considered ΔpH-independent. If PsbS were an important protein inducing and/or supporting sustained non-photochemical quenching, higher optical densities of the protein would be expected in the needles showing lower F_v/F_m levels. However, no differences were found in PsbS levels between the treatments.

Also other studies have found no increase or even decreases in PsbS-levels when sustained NPQ increases in winter. In evergreen bearberry (*Arctostaphylos uva-ursi)* (Zarter et al., 2006a), the leaves that had a strong reduction in pre-dawn F_v/F_m in winter did not show a significant increase in PsbS-levels. On the other hand, shaded leaves experiencing less harsh winter conditions showing little reduction in pre-dawn F_v/F_m showed much higher PsbS levels in winter than in the summer. The authors interpret these results as an involvement of PsbS in the rapidly reversible NPQ that is increased in the shaded conditions but not when excitation pressure becomes too severe. The same conclusions were drawn in studies on conifers (Zarter et al., 2006b) (Busch et al., 2007). In central Siberia, PsbS-levels in Scots pine (*Pinus sylvestris*) declined in autumn and stayed low through the winter before it increased in late spring (Ensminger et al., 2004). This is an area with harsh winter conditions, experiencing mean daily temperatures typically around -20 °C in February and March. Rapidly reversible NPQ was shown to be low (Ensminger *et al* 2004).

Taken together, the results do not suggest a role for PsbS in sustained non-photochemical quenching in temperate and boreal spruce. However, as Zarter and colleagues (2006b) remark, it cannot be excluded that PsbS is involved or undergoes alterations in structure or function in the sustained mode but no evidence was found for the protein to be up-regulated and involved in the manner hypothesized by Öquist and Huner (2003). The increase in PsbS shown in earlier studies might be due to an increased need for rapidly reversible NPQ in the autumn or under other less harsh conditions. The molecular mechanism of sustained NPQ

thus remains elusive. There is some evidence pointing at sustained phosphorylation of D1 proteins of PSII-reaction centers during winter (Ebbert et al., 2005). Also, ELIP-like, or other stress-induced relatives of PsbS are speculated to be involved in long-term photoprotection (Zarter et al., 2006b), but further research is needed to verify these hypotheses.

It should be noted that there was large variation in the results obtained. *P. rubens* showed for example almost 1.5 times higher optical density at HT-LL compared to HT-HL, while *P. abies* showed optical density of 0.7 times that of HT-HL at HT-LL (Figure B1). In the case of *P. abies*, this could be observed in Figure 3.7 where lane 3 (HT-LL) showed a very weak band. This should be indicative of low concentration of PsbS at a tissue basis for this treatment. However, on the Coomassie-stained gel, weak bands of all the proteins were observed for this lane. Also for the other blots, one to three bands showed differences in Coomassie-staining. There was, however, no clear pattern of which treatment that had stronger or weaker Coomassie-stained bands. Therefore, it could be that there were different total pigment concentrations in the lysates, possibly due to incompletely dissolved protein pellet in the lysate buffer. Total protein concentration determination of the lysate, for example by BCA (bicinchoninic acid) protein assay, would therefore have been appropriate to do. However, too much beta-mercaptoetanol (BME) had been used in the extraction procedure to do a BCA assay. Additionally, time did not allow further analyses to be done. In conclusion, the results still show no differences in PsbS-expression, but less noise in the results would probably have been achieved if determination of total protein concentration had been done.

4.7. Is reaction center quenching involved?

The results for the component parameters of F_v/F_m showed no differences in F_0 between the treatments, while F_m decreased as the excitation pressure increased, in the same manner as F_v/F_m (Figure 3.2). This confirms previous findings in a similar growth chamber experiment (Lamontagne et al., 2000), while in a field study on *Pinus sylvestris* F_0 decreased from September to January and stayed at the same level until an increase was seen in April-May (Ottander et al., 1995). According to Bukhov et al (2001), no change in F_0 in the present experiment should mean that the quenching should be identified as reaction center quenching. They argue that reaction center quenching decreases F_m but not F_0, while antenna quenching decreases both F_m and F_0. This is not consistent with the pigment results discussed above, showing lower violaxanthin levels in the high excitation pressure treatments, which is

indicative of the xanthophyll cycle operating in antenna quenching.

Previous studies on conifers have concluded that reaction center quenching can take place during winter (Ivanov et al., 2002; Sveshnikov et al., 2006). This was investigated by means of thermoluminescence and the quenching of F_0 and F_m were not reported. Sveshnikov et al (2006) report that operation of the xanthophyll cycle occurred at the same time. This implies that the two mechanisms are not mutually exclusive. It is still unclear why F_0 quenching was not observed at all in the present experiment since antenna quenching appears to take place. However, the interpretation of changes in F_0 and F_m as indicators of antenna quenching and reaction center quenching is limited to Bukhov et al (2001) only. Actually, Stefanov and Therashima (2008) later rejected this method for distinction between antenna quenching and reaction center quenching. The presence of antenna quenching appears likely because of the changes in violaxanthin concentrations. The results presented here are thus not supportive for the distinction method described by Bukhov et al (2001). In summary, it cannot be concluded whether reaction center quenching takes place or not in the investigated shoots. To prove the presence of reaction center quenching, thermoluminescence studies should be performed. Antenna quenching seems likely due to changes in violaxanthin concentration.

5. Conclusion

This study revealed a strong decline in maximum photochemical efficiency of PSII for four boreal conifers in response to high excitation pressure caused by low temperature and high light. This indicates sustained non-photochemical quenching, which is an adaption protecting the conifers during winter and spring. It was confirmed that it was mainly ΔpH-independent, not ΔpH-dependent, sustained NPQ. Novel investigations of species-specific responses suggest adjustments of the photosynthetic machinery that are partly due to different ecological adaptations. Complete photosynthesis investigations should be performed to resolve the question on species-specific photosynthetic adaptions to low temperature conditions. The applied conditions did not cause any visible symptoms of winter chlorosis, but high excitation pressure should not be ruled out as a cause for winter chlorosis observed in field since conifers frequently experience harsher conditions than those employed in this study. Regarding the molecular mechanism of ΔpH-independent sustained NPQ, evidence was found for participation of the xanthophyll cycle, but not for participation of the protein PsbS. This contributes clarifying the apparent discrepancy regarding this protein in previous studies. Further analysis of protein expression in similar 2×2 factorial experiments is warranted to reveal the molecular mechanism for ΔpH-independent sustained NPQ.

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APPENDIX A

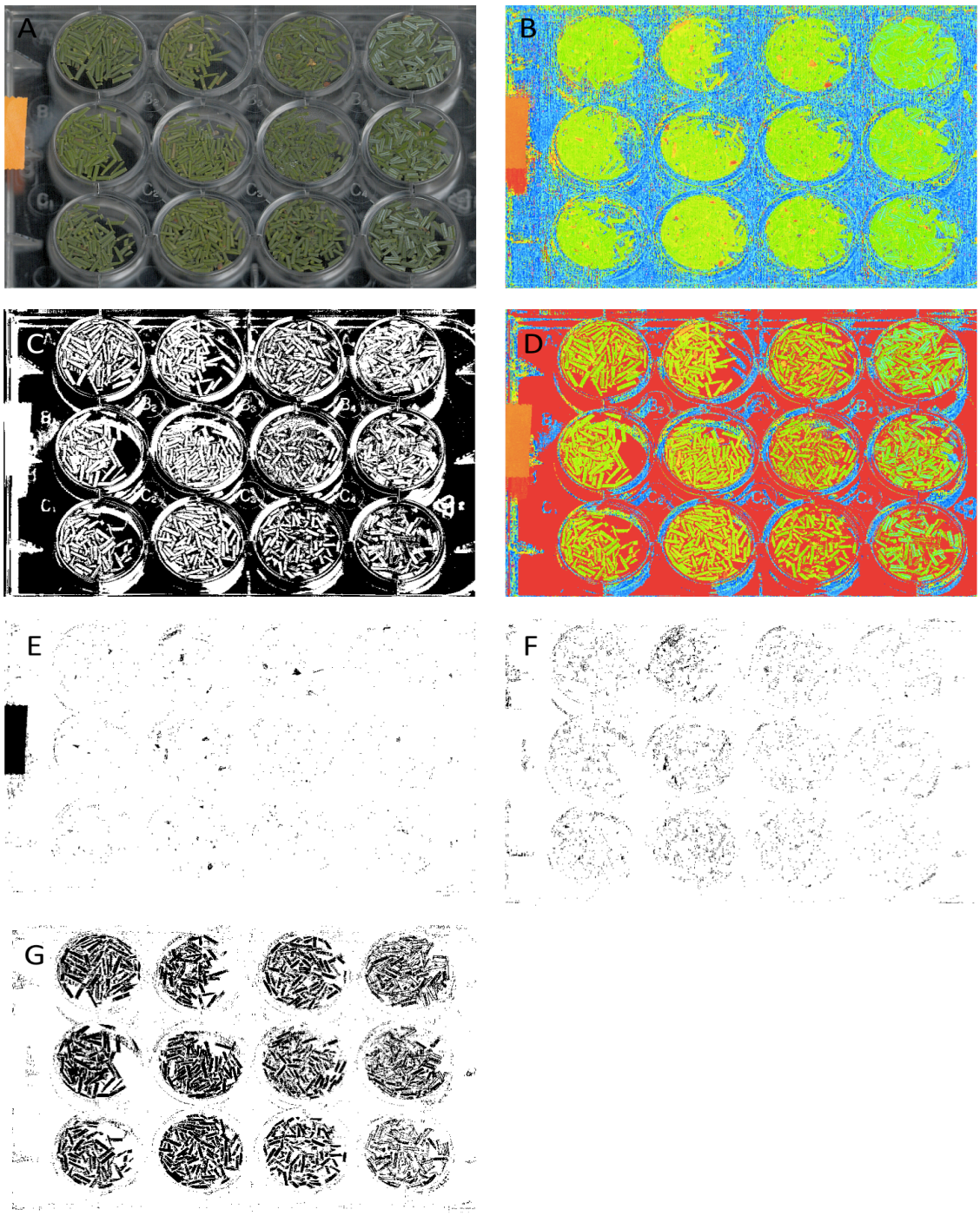

Figure A1: Example of the color analysis procedure. A: Original image of 12 samples scanned at 300 dpi. The image was separated into Hue, Saturation and Brightness slides. B: Hue slide C: Brightness slide with a threshold value of 100 in the grayscale LUT for producing a mask for the Hue-slide. D: The mask added to the Hue slide. Masked areas are in red color. E, F, G: Black areas are the areas selected using threshold values in the spectrum LUT of 15-35, 36-50 and 51-90 respectively, for selecting orange, yellow and green colored pixels.

APPENDIX B

Table B1: Factors included in candidate linear mixed models fitted by maximum likelihood. Comparisons of Akaike Information Criterion (AIC) for each model were performed to select the best model. In all models, Tree was included as a random factor (1|Tree).

Model ID	Factors included	|ΔAIC|
	One main effect	
1	Light + 1|Tree	1676
2	Temp + 1|Tree	896
3	Species + 1|Tree	1868
	Two main effects	
4	Light + Temp + 1|Tree	280
5	Light + Species + 1|Tree	1678
6	Species + Temp + 1|Tree	899
	Two main effects and their interaction	
7	Light + Temp + (Light × Temp) + 1|Tree	66
8	Light + Species + (Light × Species) + 1|Tree	1679
9	Light + Temp + (Species × Temp) + 1|Tree	258
	Three main effects	
10	Light + Temp + Species + 1|Tree	282
	Three main effects and one two-way interaction	
11	Light + Temp + Species + (Light × Temp) + 1|Tree	68
12	Light + Temp + Species + (Light × Species) + 1|Tree	263
13	Light + Temp + Species + (Temp × Species) + 1|Tree	258
	Three main effects and two two-way interactions	
14	Light + Temp + Species + (Light × Temp) + (Light × Species) + 1|Tree	43
15	Light + Temp + Species + (Light × Temp) + (Species × Temp) + 1|Tree	36
16	Light + Temp + Species + (Light × Species) + (Species × Temp) + 1|Tree	237
	Three main effects and three two-way interactions	
17	Light + Temp + Species + (Light × Temp) + (Light × Species) + (Species × Temp) + 1|Tree	8
	Full model	
18	Light + Temp + Species + (Light × Temp) + (Light × Species) + (Species × Temp) + (Light × Temp × Species) + 1|Tree	0

Table B2: Random and fixed effects estimated by restricted maximum likelihood. For fixed effects, the levels for the intercept are low temperature (0°C), high light (200 µmol m^{-2} s^{-1}) and species *Picea abies* (AB). The other levels are high temperature (10,0°C), low light (80 µmol m^{-2} s^{-1}) and species *Picea obovata* (OB), *P.rubens* (RU) and *P.sitchensis* (SI).

Random effects:		
	Variance	Std.dev.
Tree	0.0024	0.050
Residual	0.0046	0.068

Fixed effects:			
	Estimate	t-value	pMCMC
(Intercept, HL-LT-AB)	0.375	13.45	<0.001
Light LL	0.1675	14.31	<0.001
Temp HT	0.323	26.90	<0.001
Species OB	-0.051	-1.40	0.342
Species RU	0.017	0.41	0.434
Species SI	-0.093	-2.28	0.016
Light LL × Temp HT	-0.107	-6.23	<0.001
Light LL × Species OB	0.057	3.42	<0.001
Light LL × Species RU	0.028	1.73	0.095
Light LL × Species SI	0.070	4.25	<0.001
Temp HT × Species OB	0.026	1.52	0.138
Temp HT × Species RU	-0.028	-1.67	0.093
Temp HT × Species SI	0.038	2.22	0.031
Light LL × Temp HT × Species OB	-0.079	-3.31	0.002
Light LL × Temp HT × Species RU	-0.026	-1.09	0.289
Light LL × Temp HT × SpeciesSI	-0.008	-0.33	0.745

Table B3: Investigated models and their |ΔAIC|-values in the recovery experiment. |ΔAIC| indicates the model with lowest AIC. The data were analyzed separately for the two species. In all models, Tree was included as a random factor (1|Tree).

Model ID	**Factors included**	**|ΔAIC|**
P. sitchensis		
1	Treatment + 1|Tree	23
2	Treatment + Recovery + 1|Tree	0
Full model	Treatment + Recovery + Treatment×Recovery + 1|Tree	2
P. obovata		
1	Treatment + 1|Tree	37
2	Treatment + Recovery + 1|Tree	38
Full model	Treatment + Recovery + Treatment×Recovery + 1|Tree	0

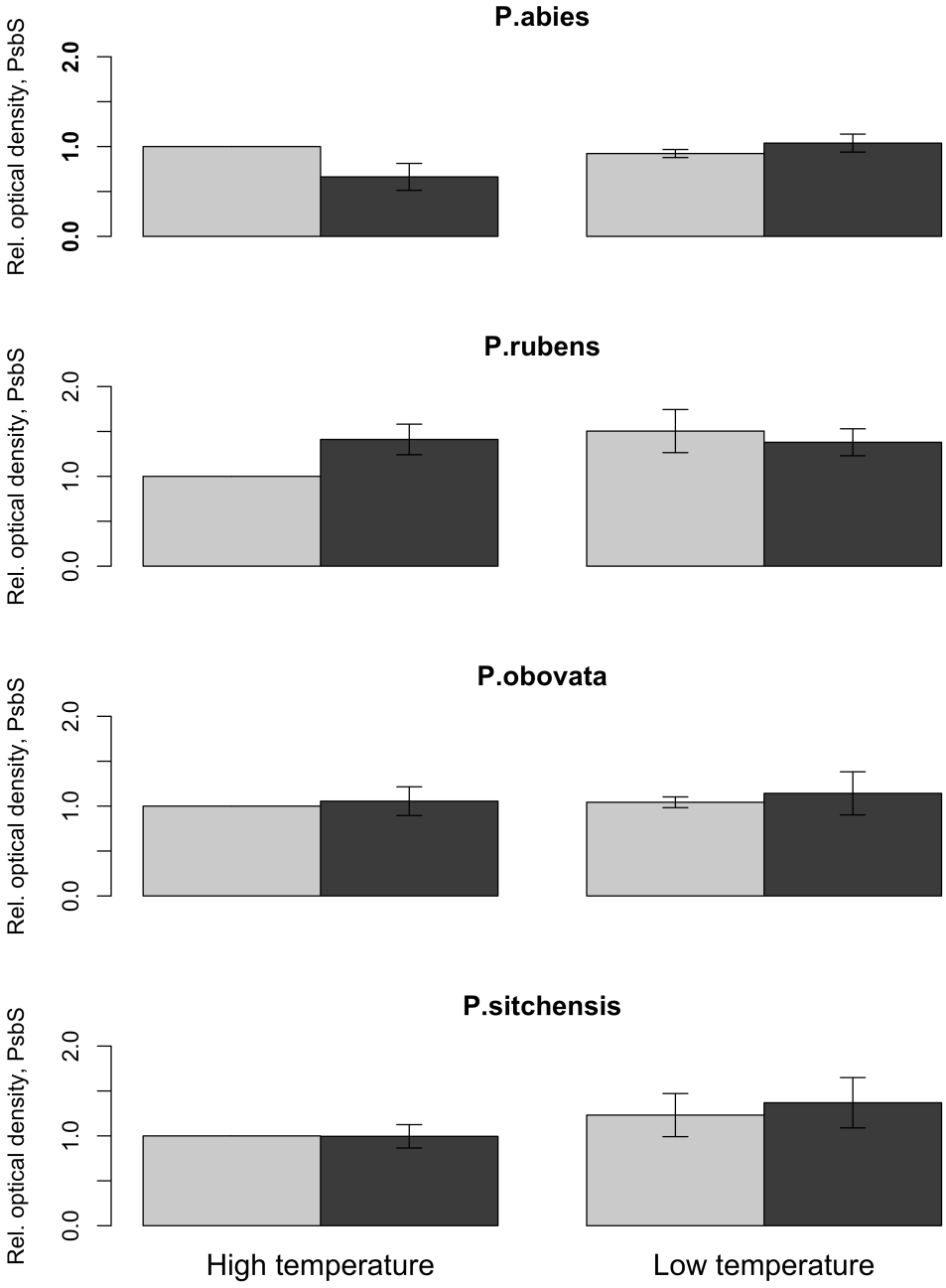

Figure B.1: Western blot expression results of the protein PsbS in shoots of *Picea* grown under four different combinations of high and low temperature (10/0 °C) and high (light shading) and low (dark shading) light (200/80 µmol m^{-2} s^{-1}) for 14 days. Relative band intensity was calculated as band intensity of the sample relative to the band intensity of the HT-HL-treatment for each species. All treatments for each species were run in the same blot. Replicates for the species were run on different blots. Error bars ±SE. n=3 except for *P.sitchensis* at HT-LL and LT-LL, and *P.obovata* at LL-LT, where n=2.