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Studying the diurnal and seasonal acclimation of photosystem II using chlorophyll-*a* fluorescence

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Academic dissertation

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for public discussion
in Lecture Hall 2, Info Centre Korona, Viikinkaari 11, Helsinki
on 29th of August 2008, at 12 o'clock noon.

Title of dissertation: Studying the diurnal and seasonal acclimation of photosystem II using chlorophyll-*a* fluorescence

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Dissertationes Forestales 69

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ISSN 1795-7389
ISBN 978-951-651-226-9 (PDF)

(2008)

Publishers:
Finnish Society of Forest Science
Finnish Forest Research Institute
Faculty of Agriculture and Forestry of the University of Helsinki
Faculty of Forestry of the University of Joensuu

Editorial Office:
The Finnish Society of Forest Science
Unionkatu 40A, FI-00170 Helsinki, Finland
http://www.metla.fi/dissertationes

Porcar-Castell, A. 2008. Studying the diurnal and seasonal acclimation of photosystem II using chlorophyll-*a* fluorescence. Dissertationes Forestales 69. 47 p. Available at http://www.metla.fi/dissertationes/df69.htm

A small fraction of the energy absorbed in the light reactions of photosynthesis is re-emitted as chlorophyll-*a* fluorescence. Chlorophyll-*a* fluorescence and photochemistry compete for excitation energy in photosystem II (PSII). Therefore, changes in the photochemical capacity can be detected through analysis of chlorophyll fluorescence. Chlorophyll fluorescence techniques have been widely used to follow the diurnal (fast), and the seasonal (slow) acclimation in the energy partitioning between photochemical and non-photochemical processes in PSII at the leaf level. Energy partitioning in PSII estimated through chlorophyll fluorescence can be used as a proxy of the plant physiological status, and measured at different spatial and temporal scales. However, a number of technical and theoretical limitations still limit the use of chlorophyll fluorescence data for the study of diurnal and seasonal acclimation processes in PSII. The aim of this Thesis was to study the diurnal and seasonal acclimation of PSII in field conditions through the development and testing of new chlorophyll fluorescence-based tools, overcoming these limitations.

A new model capable of following the fast acclimation of PSII to rapid fluctuations in light intensity was developed. The model was used to study the rapid acclimation in the electron transport rate under fluctuating light. Additionally, new chlorophyll fluorescence parameters were developed for estimating the seasonal acclimation in the sustained rate constant of thermal energy dissipation and photochemistry. The parameters were used to quantitatively evaluate the effect of light and temperature on the seasonal acclimation of PSII. The results indicated that light environment not only affected the degree but also the kinetics of response of the acclimation to temperature, which was attributed to differences in the structural organization of PSII during seasonal acclimation. Furthermore, zeaxanthin-facilitated thermal dissipation appeared to be the main mechanisms modulating the fraction of absorbed energy being dissipated thermally during winter in field Scots pine. Finally, the integration between diurnal and seasonal acclimation mechanisms was studied using a recently developed instrument *MONI-PAM* (Walz GmbH, Germany) capable of monitoring the energy partitioning in PSII both at the diurnal and seasonal time-scales.

Keywords: Energy partitioning, F_o, F_m, k_{NPQ}, k_P, field measurements, light reactions, photosynthesis, Scots pine, spring recovery.

Acknowledgements

These years have been a continuous gaining of experience and learning. I am greatly indebted to my supervisors Professors Pertti Hari and Eero Nikinmaa, and Doctor Eija Juurola who have always been ready to explain, guide and supervise. Specially, I am very grateful to Pepe for having transmitted, hopefully, part of his way of thinking and approaching the complexity of nature; as well as to Eero and Eija not only for being excellent supervisors but also friends.

Docent Jaana Bäck, Professor Frank Berninger and Doctor Ingo Ensminger, have not only acted as co-authors in my articles but also as instructors. My most sincere thanks for their patience and readiness. I also wish to thank Janne Korhonen and Doctor Erhard Pfündel for their fruitful participation in Study IV.

Docent Esa Tyystjärvi and Professor Federico Magnani are gratefully acknowled for their thorough work pre-examining the present thesis. In addition, Studies I, II and III benefited from Esa's eagerness to offer his expert and constructive criticism whenever asked.

I want to thank all the friends and colleages at the Department of Forest Ecology and rest of the Faculty, with whom the daily work, and the lunch breaks, have been most pleasant. Among many others, "muchos kiitos" to Martti, Maarit, Pasi, Jukka, Liisa, Teemu, Boris, Raja, Antti, Edu,... I am also very grateful to Jukka Lippu, Sirkka Bergström, and Varpu Heliara, always ready to answer questions and solve practical matters. Likewise, I want to thank all the staff in Hyytiälä and Smear for their effort making experimental data collection a simpler task: my special thanks to Veijo Hiltunen, Topi Pohja, Erkki Siivola, and Janne Levula.

I will always be indebted to those who made possible my first steps here in Finland back in 1999 as a MSc Student. Long time ago, yet so crucial moments. My most sincere gratitude to Professor Olavi Luukanen, Kari Leppänen (Helsinki Consulting Group), Magalis Marin (Mi Casita), Sakari Soini (Töölölainen Lehti), Pekka Puolakka, Laia Linsio, Eddie Glover, Hanna Mäenpää, and Aulis Lind.

Most importantly, I want to express my gratitute to my friends: Jose Mari, Rafel, Jose, Joan, Feliu, Mònica, Floren, Laia, Jussi, Alex, Markku, Aulis, Jüri, Nuria, Timo, Cristina, Janne, Eva, Jari, Hanna, Eduardo, Patrick, Remko, Daisy, Ed, Sven, Ritu, Massa, Arturo, Tere and Miquel. Thank you for your friendship all these years.

My warm thanks go to my beloved parents Clemente and Maria del Carmen, my dear sister Carmeta, and her family: Floren, Genís and Marcel; as well as, *requiéscat in pace*, my much-loved grandmother Vicenta and uncle Jaume. For their love, patience, and understanding during these years.

The Academy of Finland, and the University of Helsinki are acknowledged for funding this Thesis.

Annamari, and recently Leo, have always been a support, keeping my fit on the ground, and supplying that kind of imperceptible but essential energy that this work and I needed. Moltes gràcies! We finally did it!

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. Porcar-Castell A, Bäck J, Juurola E, Hari P, 2006. Dynamics of the energy flow through photosystem II under changing light conditions: a model approach. *Functional Plant Biology* 33(3): 229-239.

II. Porcar-Castell A, Juurola E, Nikinmaa E, Berninger F, Ensminger I, Hari P, 2008. Seasonal acclimation of photosystem II in *Pinus sylvestris*. I. Estimating the rate constants of sustained thermal energy dissipation and photochemistry. *Tree Physiology* 28: 1475-1482.

III. Porcar-Castell A, Juurola E, Ensminger I, Berninger F, Hari P, Nikinmaa E, 2008. Seasonal acclimation of photosystem II in *Pinus sylvestris*. II. Using the rate constants of sustained thermal energy dissipation and photochemistry to study the effect of the light environment. *Tree physiology* 28: 1483-1491.

IV. Porcar-Castell A, Erhard Pfündel, Janne FJ Korhonen, Eija Juurola, 2008. A new monitoring PAM fluorometer (*MONI-PAM*) to study the short- and long-term acclimation of photosystem II in field conditions. *Photosynthesis Research* 96: 173-179. *DOI: 10.1007/s11120-008-9292-3*

Albert Porcar-Castell was the author of the summary part of this thesis. He was the main responsible for the planning, data collection, data processing and analysis, and writing of all four articles. Pertti Hari prepared an early version of the model in STUDY I. The experimental design in STUDIES II and III was mainly planned by the co-authors. The canopy profile data in STUDY III was collected and analysed by Eija Juurola. In STUDY IV, Erhard Pfündel carried out the lab tests for the light and temperature response of the MONI-PAM, and participated in writing the technical description of the MONI-PAM. All co-authors contributed commenting and discussing the articles.

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Symbols and abbreviations

A- Leaf absorptance
a- Fraction of absorbed light captured by PSII
A_s- Reference A during summer
a_s- Reference *a* during summer
ATP- Adenosine 5'-triphosphate
Chl- Chlorophyll
D1- Protein associated with the reaction centre of PSII
E- Efficiency of thermal dissipation (Eq. 5), representing the fraction of active quenching sites in PSII
ETR- Electron transport rate
F- Fluorescence intensity
F_m- Maximum fluorescence measured after a saturating light pulse in dark-acclimated leaves
F_m'- Maximum fluorescence measured after a saturating light pulse in light-acclimated leaves
F_{ms}- F_m measured in the absence of sustained thermal dissipation (e.g. summer)
F_o- Minimum fluorescence measured in dark-acclimated leaves
FRET- Fluorescence resonance energy transfer
F_t- Current fluorescence level
F_v/F_m – Maximum quantum yield of photochemistry, where variable fluorescence $F_v = (F_m-F_o)$
h- Planck's constant ($6.62\ 10^{-34}$ Js^{-1})
I- Light intensity (µmol m^{-2} s^{-1})
I_{MB}- Constant light intensity of the modulated beam from the fluorometer
k_D- Rate constant of constitutive thermal energy dissipation (s^{-1}), or in Eq. 2, of total thermal energy dissipation
k_f- Rate constant of chlorophyll fluorescence (s^{-1})
k_n- Rate constant of regulated thermal dissipation (s^{-1}) by active quenching sites (S^{ON}) (see Eq. 5)
k_{NPQ} - Rate constant of regulated thermal dissipation (s^{-1})
k'_{NPQ} - Rate constant of regulated thermal dissipation (s^{-1}) relative to the sum of k_f and k_D
k_{PSII}- Rate constant of photochemistry (s^{-1})
k_P- Overall rate constant of photochemistry of a mixed population of open and closed RCs (s^{-1})
k'_P- Overall rate constant of photochemistry of a mixed population of open and closed RCs (s^{-1}) relative to the sum of k_f and k_D
k_T- Rate constant of energy transfer to non-fluorescing pigments in PSI, i.e. state transitions (s^{-1})
NADP- Nicotinamide adenine dinucleotide phosphate
P_{680}- Chlorophyll molecule located in the reaction centre of PSII with an absorption peak at 680 nm
PC- Plastocyanine
Pheo- Pheophytin
PSI- Photosystem I
PSII- Photosystem II
Q_A- Quinone A, in the model (Eq. 3) refers to the fraction of open RCs, also as Q in Eq. 5
Q_B- Quinone B
qE- Energy dependent quenching (of chlorophyll fluorescence)
qI- Photoinhibitory quenching (of chlorophyll fluorescence)
qT- State-transitions quenching (of chlorophyll fluorescence)
RC- Reaction centre, in Eq.3 refers to the fraction of functional or active PSII RCs
S- Quenching site or site where thermal dissipation takes place, S^{ON} active site, S^{OFF} inactive site
α- Light absorption efficiency parameter
β- Proportionality constant of the fluorometer detector
γ- parameter representing the reoxidation rate of the quinone pool
ε- Light extinction coefficient
λ_b- parameter linked to the building of regulated thermal dissipation (Eq. 6)
λr- parameter linked to the relaxation of regulated thermal dissipation (Eq. 6)
Φ- yield

1 INTRODUCTION

1.1 Motivation behind the Study

Photosynthesis is an essential process by which solar energy enters the biosphere. Photosynthesis acts as a pump of carbon from atmosphere into terrestrial and marine ecosystems. In general, contemporary global issues such as climate change, or the increasing energy demands of the worlds' population set the general motivation behind the study of photosynthesis. In particular, understanding the functioning of the photosynthetic process under field conditions, and how photosynthesis is controlled by environmental and physiological factors, is a cornerstone in plant and ecosystem ecology. For example, in order to evaluate the effect of climate change on plant performance, on interspecific competition and species composition, or on the carbon cycle, it is necessary to comprehend, among others, how photosynthetic capacity is controlled and regulated by environment in different plant species (Chapin III et al. 2002). To this purpose, new techniques and instruments are constantly appearing that facilitate the monitoring photosynthesis at different spatial and temporal scales. Yet, in order to optimally use these technologies and interpret the resulting data, new theoretical development that complements the technical development is required.

An important approach to estimate photosynthesis and its acclimation is the use chlorophyll-a fluorescence (hereafter chlorophyll fluorescence). Chlorophyll fluorescence is a non-destructive method to probe the energy partitioning in photosystem II (PSII). Chlorophyll fluorescence can be measured from some millimetres to several meters, and up to the near-future airborne and satellite measurements of passive chlorophyll fluorescence, i.e., based on sunlight induced fluorescence. Energy partitioning in PSII can be used as a proxy to follow the plant's physiological status (e.g. in response to drought, extreme temperatures, or the annual cycle), therefore chlorophyll fluorescence may provide easily acquirable data on plant status at different spatial and temporal scales. Recent developments in chlorophyll fluorescence instrumentation allow for high resolution measurements of the acclimation of the light reactions of photosynthesis. However, a number of technical and theoretical limitations still limit the amount of information that can be obtained from such measurements. These limitations, together with the important applications of chlorophyll fluorescence in the field of plant ecology were the main motivations behind the study.

1.1.1 Measuring the acclimation of photosynthesis

The photosynthetic process is composed of two separate sets of reactions: light reactions absorb light energy and use it to produce ATP and NADPH, while dark reactions utilize the energy stored in the ATP and NADPH to synthesise sugars from CO_2. The overall process can be summarised as (Taiz and Zeiger 2002):

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

A critical feature of photosynthesis is that energy input into the light reactions is linearly proportional to the incident light intensity but independent of temperature. In contrast,

energy utilization by the dark reactions is dependent on temperature but largely independent on light intensity. These differences are caused by the temperature independency of the biophysical energy absorption in the light reactions, combined with the temperature dependency of the enzymatic dark reactions (e.g. Öquist and Huner 2003). Similarly, other factors such as water or nutrient stress (e.g Niinemets et al. 2001), may also affect energy utilization without directly affecting light absorption. Consequently, plants are naturally exposed to imbalances between energy supply and energy consumption in photosynthesis, both during the course of the day and throughout the year. If energy supply exceeds energy consumption, the excitation pressure in the light reactions increases (Huner et al. 1996, 1998), which may lead to photooxidative damage of thylakoid membrane components. Excitation pressure in the photosystem has been regarded as a mechanism by which the leave senses the environment, and through which the leaf is capable of adjusting its photosynthetic parameters (Huner et al. 1996, 1998, Ensminger et al. 2006). However, excitation pressure does not control all acclimation processes in the light reactions, for example, photoinhibition of PSII reaction centres has been found to take place independently of excitation pressure (Matsubara and Chow 2004, Tyystjärvi et al. 2008). In summary, acclimation mechanisms in the light reactions tend to adjust the energy supply to the energy demand of the dark reactions.

The state of acclimation of photosynthesis can serve as a proxy to monitor changes in the overall plant physiological status, e.g. in response to stress or to the annual-cycle. Two main approaches have been used to follow the acclimation of photosynthesis under natural conditions. First, the exchange of CO_2, or occasionally O_2, between atmosphere and foliage has been measured by means portable enclosure chambers at the leaf-level (e.g. those commercialised by LiCor, Walz, PP-systems *inter alia*), permanent chambers at the shoot level (e.g. Hari et al. 1999), or eddy covariance techniques at a stand-level (e.g. Vesala et al. 1998), which measure the flux of CO_2 or O_2 between atmosphere and ecosystem. Another approach is to estimate the energy supplied by the light reactions of photosynthesis, which provides information on how much energy enters the overall photosynthetic process. This has been chiefly done using chlorophyll fluorescence techniques (Schreiber 1986, Maxwell and Johnson 2000), although other approaches such as photoacoustics have been tested under laboratory conditions (Buschmann 1999, Herbert et al. 2000).

Absorbed light energy is mainly used in photochemistry, resulting in the formation of ATP and NADPH. However, a variable fraction of the absorbed energy is always lost as heat and emitted as measurable chlorophyll fluorescence. Therefore, chlorophyll fluorescence carries information that can be used to estimate the rate of photochemistry, as well as the partitioning of absorbed energy between photochemical and non-photochemical processes. In the next chapters, I describe the origin of chlorophyll fluorescence, how it is linked and affected by acclimation in the light reactions of photosynthesis, and what are the main limitations challenging the use of chlorophyll fluorescence in the study of the acclimation of PSII under field conditions.

1.2 The biophysics of light absorption and energy partitioning by pigment molecules

Light is electromagnetic radiation that has both electric and magnetic components. Electromagnetic radiation is characterised by wavelength (λ) (meters), and frequency (ν) (s^{-1}). Radiation with wavelengths between 400-700 nm is photosynthetically active and its energy can be absorbed by plant pigments and transduced to chemical energy through photosynthesis. In 1900, Max Planck presented his quantum theory that estimated the total

radiative energy of a blackbody as the sum of the energies of a finite population of resonators composed of discrete energy elements (ε) (Eq.1) (Planck 1901). A few years later, Albert Einstein discovered that light is quantized, and energy carried by a quantum can only be absorbed or emitted as discretized quanta units (Einstein 1905), where a particle carrying a quantum of energy is called a photon. The energy of a photon (ε) depends on the frequency of the electromagnetic wave (ν), which is related to the wavelength (λ) as (Planck 1901, Lawlor 2001):

$$\varepsilon = h\nu = hc/\lambda, \qquad \text{(Eq. 1)}$$

where h is Planck's constant (6.62×10^{-34} J s) and c is the velocity of light *in vacuo* (3×10^{8} ms^{-1}).

From the quantum theory it follows that the absorption of a photon by an atom will take place if the energy carried by the photon equals the energy required to bring one of the atoms' electron to a higher energy state. Similarly, an emitted photon will carry an energy equal to the one lost by the atom when its electron returns to the ground state. In the case of molecules, the electrons of the constituent atoms interact giving raise to complex energy levels and subsequent absorption and emission spectra (Lawlor 2001). Pigments are molecules that absorb light in the visible range. Chlorophyll-a for example has maximum absorption peaks at 453 and 642 nm, corresponding to wavelengths of what we call blue and red light, respectively (Scheer 2003). In contrast, due to the electronical configuration of the chlorophyll molecule, photons in the range 520-570 nm are not so easily absorbed and therefore leaves and vegetation containing chlorophyll appear green.

When a pigment molecule absorbs a photon, one of its electrons is raised to a higher energy state or orbital. If the electron is excited to a state higher than the first orbital (e.g. S_2), the energy is rapidly and efficiently lost by thermal relaxation and internal conversion and dissipated as heat (Clegg 2004), and the electron relaxes to the first excited state (S_1) (Fig. 1). It is from the first excited state that the excitation energy of the electron (exciton) is partitioned at the molecular level. The exciton can be converted to a photon of fluorescence, dissipated as heat, transferred to a neighbouring molecule through Förster resonance energy transfer (FRET) (Förster, 1951), or used to produce a chlorophyll triplet state. Formation of chlorophyll triplet states will thus be enhanced when the excitation lifetime increases (i.e. the time between photon absorption and dissipation of its energy). Triplet states play an important role since they can lead to photooxidative damage in the thylakoid membranes, through the production of singlet oxygen (Formaggio et al. 2001, Krause and Jahns 2004). Therefore, maintenance of a short lifetime is one of the main roles of the acclimation of photosystem II (PSII). In the next chapter, I present the structure of PSII and look at the energy partitioning at the macromolecular level of PSII.

1.3 Structure, function and biophysical processes of photosystem II

The photosynthetic pigments responsible for the absorption of light are associated with two different protein complexes: photosystem II (PSII) and photosystem I (PSI). As it will be explained later, PSII is of special interest due to its chlorophyll fluorescence properties, which allow the estimation of the energy partitioning and its acclimation.

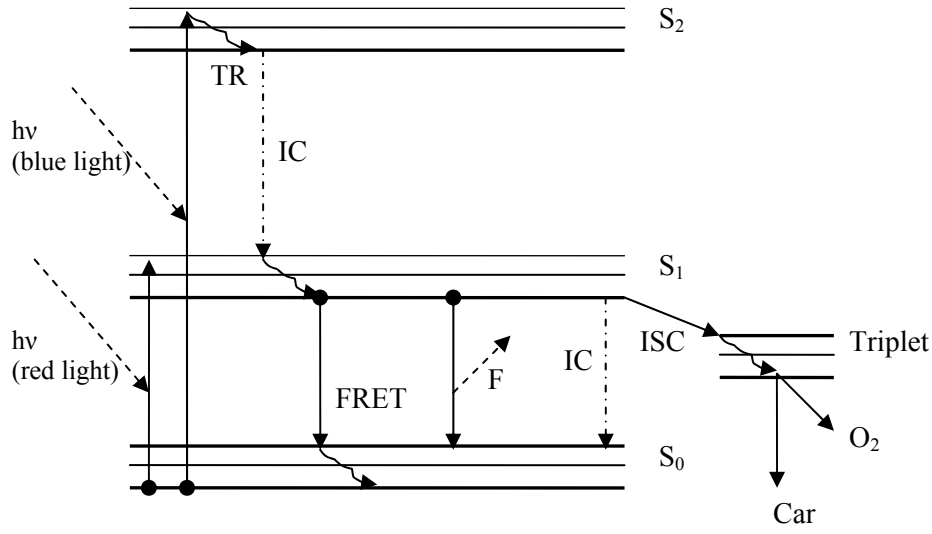


Figure 1. Modified Jablonski diagram showing the ground and excited states of a chlorophyll molecule. The vertical axis represents the energy. A photon (hv) carries a certain amount of energy which is given by the frequency ν, according to Planck's Law (Eq. 1). Once the electron in the ground state (S_0) is excited to either the first (S_1) or second (S_2) excited states, part of the energy is rapidly and effectively lost by thermal relaxation (TR), to the lowest vibrational energy level within the excited state, or by internal conversion (IC) to the first excited state (S_1), producing heat. From the first excited state (S_1), the excitation energy may relax to the ground state by internal conversion (IC) producing heat, may be emitted as a photon of red shifted light or fluorescence (F), may be transferred to a neighbouring molecule through Förster resonance energy transfer (FRET), or may lead to the formation of a triplet state, through intersystem crossing (ISC) that may react with oxygen forming dangerous triplet oxygen, or be deactivated by a carotenoid molecule (Car). Modified from Clegg (2004). In photosynthesis, FRET is the key process by which excitation energy is transferred within the pigment antenna and eventually captured by the reaction centre to be used in photochemistry.

Photosystem II is normally present in the thylakoid membrane as a dimer (Nield et al. 2000). In each PSII monomer (Fig. 2) the reaction centre (RC) is the responsible for the primary charge separation between a reaction centre chlorophyll-a (P_{680}) and Pheophytin (Pheo). The RC is composed of a D1/D2 protein heterodimer which contains 6 Chl*a* and 2 Pheo molecules, (Rhee et al. 1998). In addition, closely linked to the RC, the manganese cluster catalyzes water oxidation and acts as electron donor to P_{680} (Zouni et al. 2001). The chlorophyll binding protein complexes CP43 and CP47 surround the RC and make up the core region of PSII. CP43 and CP 47 contain Chl*a* molecules and β-carotene (Rhee et al. 1998, Barber et al. 2000). The core antenna functions as a light absorption unit and as an excitation energy bridge between the outer antenna and the reaction centre. Surrounding the core, three minor monomeric complexes CP24, CP26 and CP29 bind both chlorophyll -*a* and -*b* as well as carotenoids (Bassi et al. 1993) making up the inner antenna region of PSII. Finally a number of major light harvesting complexes LHCII make up the outer antenna of

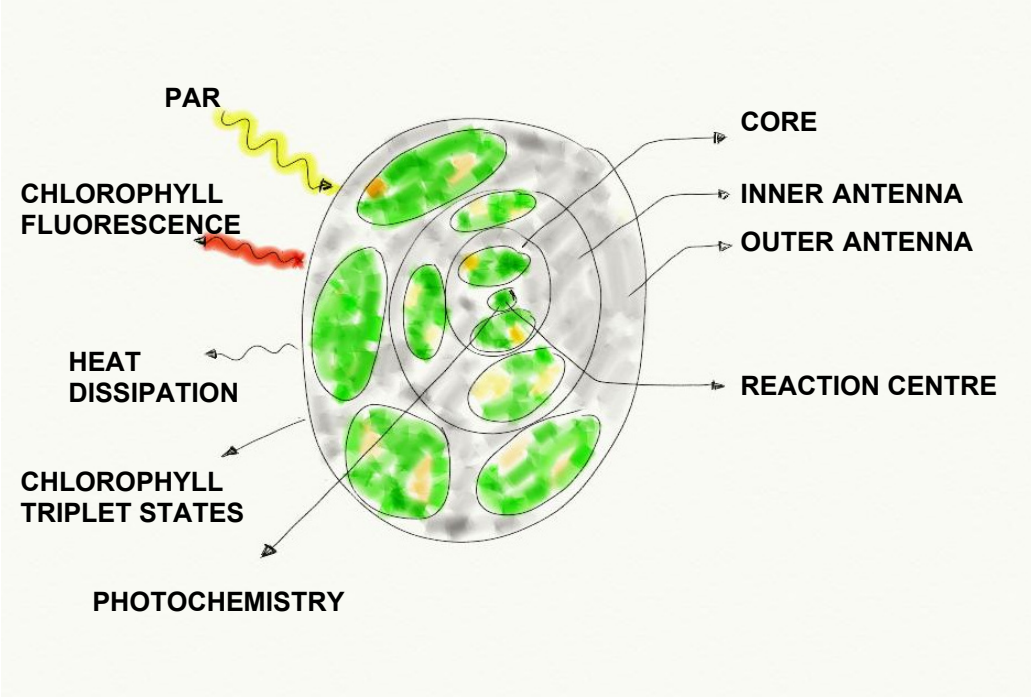


Figure 2. Monomeric structure of photosystem II (PSII) adapted from van Amerongen and Dekker (2003), with main domains. Green ovals represent the main protein complexes containing chlorophyll and carotenoids as well as the PSII reaction centre as described in the text. The main routes of energy are indicated in the figure.

PSII which is responsible for the absorption of more than half of the photons in PSII (Anderson and Andersson 1980) and bind approximately half of the thylakoid membrane chlorophyll (Liu et al. 2004).

The LHCII are typically found as trimers. Each monomer binds both chlorophyll -*a* and -*b* (Liu et al. 2004). In addition, each monomer has four carotenoid binding sites, out of which one is able to bind a xanthophyll-cycle carotenoid (Kühlbrandt et al. 1994, Liu et al. 2004). This xanthophyll-cycle binding site (L2) is of particular interest since it has been associated with the regulated component of thermal dissipation in the thylakoid membrane, which is one of the key acclimation processes in PSII that I discuss below (Chapter 1.5).

The exact mechanism by which thermal dissipation is regulated in the PSII antenna remains still controversial (Kanervo et al. 2005). A first mechanism could be the direct excitation transfer from chlorophyll to a xanthophyll-cycle pigment and the dissipation of the exciton as heat, which seems plausible given the orientation and distance of chlorophyll molecules located around the xanthophyll-cycle binding site L2 in LHCII (Liu et al. 2004); a second mechanism could involve a conformational change induced by the xanthophyll-cycle pigment that promotes then the dissipation of excitation energy as heat (Formaggio et al. 2001); thirdly, Psbs proteins found in the outer antenna have also been related to the thermal dissipation process (Li et al. 2000), and finally, structural changes and aggregation of LHCII trimers have been shown to promote excitation energy transfer between trimers

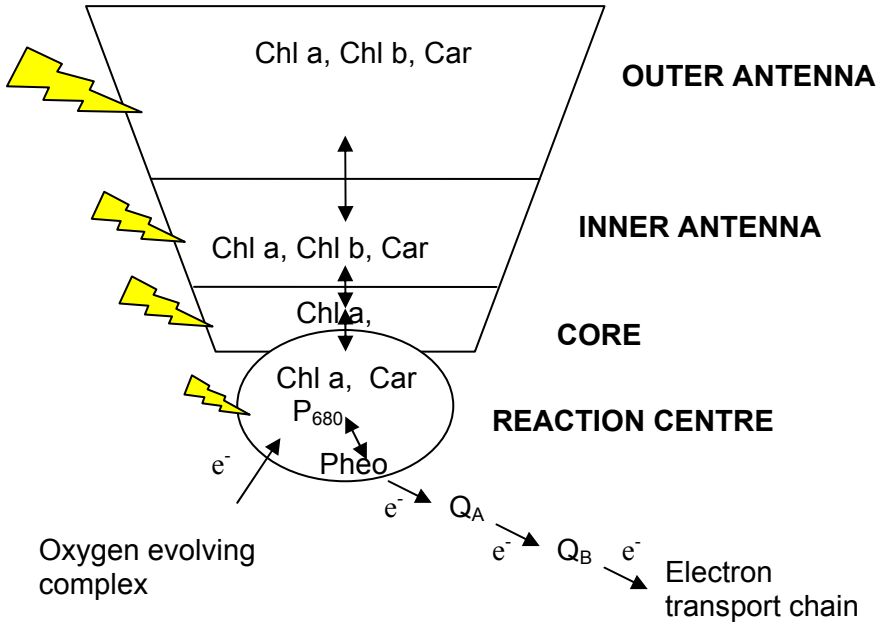


Figure 3. Excitation energy transfer in PSII. After light absorption by pigments in PSII, excitons move freely through the antenna until they reach the reaction centre chlorophyll P_{680}. Once P_{680} is excited, one of its electrons is transferred to pheophytin (Pheo) and further to quinone A (Q_A) and quinone B (Q_B), initializing the electron transport that eventually leads to the formation of ATP and NADPH by the light reactions of photosynthesis. In order to return to its original state, oxidized P_{680}^+ accepts an electron from the oxygen evolving complex. Finally, in the case Q_A is still reduced when another exciton reaches the reaction centre, the probability of primary charge separation between P^*_{680} and Pheo will drastically decrease (Schatz et al. 1988), promoting the lengthening of the lifetime of excitation and the potential formation of chlorophyll-triplet states.

(Liu et al. 2004), as well as sustained thermal dissipation states, for example in overwintering evergreen trees (Ottander et al. 1995, Gilmore 2000). Overall, the mechanism by which the fraction of excitation energy being dissipated as heat is modulated, is likely to be composed of several processes. These processes operate at different time-scales, interacting during the acclimation process, and including both biochemical and structural changes (Gilmore et al. 1995, Gilmore 1997, Horton et al. 2005, Demmig-Adams and Adams III, 2006). I discuss these mechanisms further below.

After light absorption, photons absorbed by accessory carotenoid pigments and chlorophyll-*b* are rapidly transferred to chlorophyll-*a* (Formaggio et al. 2001). From chlorophyll-*a* the excitation energy is partitioned at the molecular level between the different energy-consuming processes (Fig. 1), including the transfer of excitation to

neighbouring chlorophyll -*a* molecules through FRET. At the level of PSII, the overall time between photon absorption and trapping by the reaction centre is around 300 ps, which suggests that there is a large number of energy-transfer steps prior to trapping (Jennings et al. 1993). Eventually, the exciton reaches P_{680} where the electron is transferred to Pheo (Fig. 3). At this step the electron can be transferred from $Pheo^-$ to the primary quinone acceptor Q_A or recombined and captured again by $P^+_{680,}$ following the reversible radical pair model (Schatz et al. 1988). Efficient electron transfer from $Pheo^-$ to Q_A requires that Q_A is in the oxidized form and able to take up an electron (Schatz et al. 1988). Upon charge recombination ($Pheo^- P^+_{680} \rightarrow$ Pheo P^*_{680}) , the exciton continues visiting Chl*a* molecules and P_{680}, increasing the probability of excitation energy being lost as heat, emitted as chlorophyll fluorescence or producing a chlorophyll-*a* triplet state.

1.4 The light reactions of photosynthesis and their connection with dark reactions

When excitation energy reaches the reaction centre of PSII, and the reaction centre chlorophyll is excited P_{680}*, the excitation is rapidly used to reduce the primary electron acceptor pheophytin (Pheo) in a process known as charge separation (Schatz et al. 1988, Krause and Weis, 1991) (Fig. 3). The electron is then used to reduce quinone A (Q_A) and quinone B (Q_B) initializing the electron transport chain, and leading to charge stabilization, while leaving P_{680}^+ oxidized (Fig. 3). In order to return to its original state, oxidized P_{680}^+ accepts an electron from the oxygen evolving complex (OEC). The splitting of water is a multi-step process involving successive oxidation steps that results in the production of electrons, molecular oxygen and protons that eventually will be used in ATP synthesis. During the electron transport process (Fig. 4), the reducing power energy is used by the **Cytochrome b_6f complex** to pump protons from the stromal into the lumenal side of the thylakoid membrane decreasing in this way the lumen pH. The electron is eventually captured by plastocyanine (PC) which acts as the electron donor to PSI. In turn, the excitation energy captured by photosynthetic pigments associated with PSI is used by the reaction centre chlorophyll P_{700} to donate an electron and reduce the primary acceptor of PSI A_0. Oxidized P_{700}^+ receives then an electron from reduced PC, returning to the original state P_{700}. The resulting electron and reducing power are eventually used by the enzyme ferredoxin-NADP oxidoreductase to reduce $NAPD^+$ into NADPH. In parallel, the osmotic energy of protons accumulated in the thylakoid lumen is used to produce ATP, from ADP + P_i, when protons are pumped out of the lumen by **ATP synthase**. In summary, light energy is used by PSII and PSI working in series to produce NADPH (reducing power) and ATP (metabolic energy), the precursors required by the dark reactions of photosynthesis to fix atmospheric carbon. See Blankenship (2002) for details.

PSII and PSI reaction centres are able to absorb light at slightly different wavelengths up to 680nm (PSII) and up to 700nm (PSI), due to differences in the chlorophyll spectral forms (van Grondelle and Gobets 2004). Therefore, changes in light quality may affect differently the rate of energy transduction in PSII compared to PSI. If PSII is overexcited compared to PSI, the intersystem electron carriers will tend to be reduced and unable to accept further electrons from PSII, since reoxidation by PSI is slower than reduction by PSII, and the contrary will happen when light excites PSI more than PSII. These imbalance would cause a decrease in the overall quantum yield of electron transport. To avoid this type of imbalance, the light reactions of photosynthesis have a rapid acclimation mechanism known as state-transitions capable of balancing the rate of energy capture between PSII and PSI within a few minutes (Müller et al. 2001). This mechanism uses the redox state of the plastoquinone pool as clue to sense the imbalances (Allen 1992, Allen 2003). When PSII is

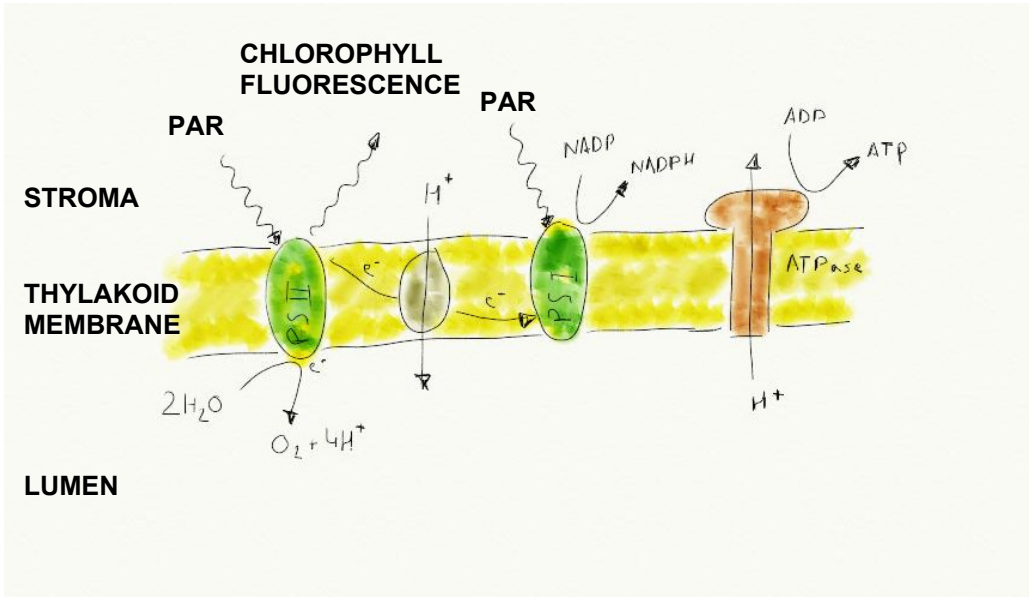


Figure 4. Scheme depicting the linear electron transport in the light reactions of photosynthesis in the thylakoid membrane. Light energy absorbed by photosystem II is used to split the molecule of water and initialise electron transport. Linear electron transport between the photosystems is used to pump protons into the thylakoid lumen which are eventually used by ATP synthase to produce ATP from ADP + P_i. Finally, light energy absorbed by PSI is used to produce reducing power by reducing NADP into NADPH.

overexcited, part of the light harvesting complexes (LHCII) associated with PSII are phosphorylated by a thylakoid protein kinase and migrate to regions rich in PSI, thus balancing PSII and PSI absorption cross-section areas and energy capture rates. However, state-transitions are probably not a significant process in higher plants, in particular under high light intensities (Rintamäki et al 1997, Müller et al. 2001). Similarly, if the imbalance between energy absorbed by PSII and PSI is long lasting, for example as a result of shading by new growth, the stoichiometry and absorption cross-section areas of PSII relative to PSI (PSII:PSI) can also acclimate to the new conditions, resulting in a different ratio of PSII:PSI reaction centres, and probably also for the PSII:PSI absorption cross section (Rintamäki et al. 1997, Kanervo et al. 2005, Fan et al. 2007).

Apart from linear electron transport, cyclic and pseudocyclic electron transport routes also exist that result in the production of ATP with no NADPH formation. In the water-water cycle (Mehler 1951, Asada 1999) electrons are eventually transferred by PSI to oxygen instead of $NADP^+$, while protons are still pumped to the thylakoid lumen promoting formation of ATP. The water-water cycle is thought to protect PSI from photooxidation. It also participates in the generation of a low lumen pH required for pH-dependent thermal dissipation process, and serves to adjust the ratio of ATP to NADPH (Asada 1999). Similarly, cyclic electron transport through PSI also generates ATP without NADPH production. The cyclic route is thought to be insignificant in the presence of linear electron transport (Asada 1999). However, it might play a significant and protective role under conditions when PSII and linear electron transport are downregulated (Ivanov et al. 2001, Kanervo et al. 2005).

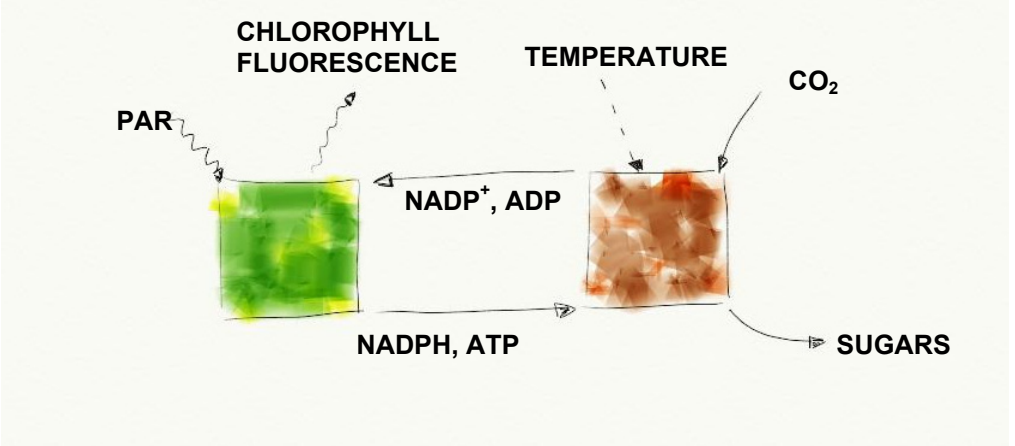


Figure 5. Scheme of the photosynthetic light-dark reactions energy system working in series. Light energy is used in the light reactions of photosynthesis to produce NADPH and ATP from $NADP^+$ and ADP. In contrast, the NADPH and ATP are used by the dark reactions of photosynthesis to reduce atmospheric CO_2 into sugars, resulting in regeneration of $NADP^+$ and ADP. Importantly, rates of energy transduction and energy consumption by light and dark reactions, respectively, are affected by environmental variables that most of the time change in an independent fashion: i.e. light and temperature.

Light and dark reactions of photosynthesis also work in series (Fig. 5), and ATP and NADPH produced in the light reactions of photosynthesis are used by the Calvin-Benson cycle to reduce atmospheric CO_2 into sugars. Therefore, for the optimal utilization of resources there should be a balance between energy supply by the light reactions and energy utilization by the dark reactions of photosynthesis. Yet, energy imbalances occur almost continuously between light and dark reactions. For example, if stomata are closed as a result of momentary water stress, the concentration of CO_2 within the leaf will tend to decrease, slowing down carbon assimilation by the dark reactions, and decreasing the utilization of ATP and NADPH. In contrast, the light reactions of photosynthesis continue producing ATP and NADPH normally, and the substrate ADP and $NADP^+$ becomes limiting. This limitation produces a feedback that results in a decrease in the thylakoid membrane proton conductivity through ATPase (Kramer et al. 2004), the accumulation of protons in the thylakoid lumen, the slowing down of electron transport, and the reduction of the intersystem electron transport carriers. If the electron transport chain is reduced, the rate of charge separation between P^*_{680} and Pheo decreases drastically (Schatz et al. 1988) leading to a lengthening of the excitation lifetime in PSII, and increasing the probability of hazardous chlorophyll triplet states formation. The degree of imbalance between energy supply and energy utilization has been described in terms of excitation pressure in PSII (Huner et al. 1996, Huner et al. 1998, Öquist and Huner 2003). The question arises: How does PSII acclimate to maintain a low excitation pressure?

1.5 Acclimation in the energy partitioning in photosystem II

As introduced in the previous chapter, differences between the pattern of light intensity and temperature, water stress, or the metabolic sink strength, produce energy imbalances that

increase the excitation pressure on PSII and may lead to photooxidative damage of the photosynthetic machinery, among others. To cope with these imbalances, plants have evolved several acclimation mechanisms that operate at a similar time-scale than that at which the imbalance takes place.

1.5.1 Short-term or diurnal acclimation

At the short-term or diurnal time-scale, a major acclimation mechanism in PSII is the **xanthophyll-cycle and pH-dependent thermal dissipation**, which quickly adjusts (seconds-minutes) the fraction of absorbed light being dissipated as heat (Krause and Weis 1991, Adams III and Demmig-Adams 1994, Demmig-Adams 1998, Müller et al 2001). During summer, for example, leaves may close their stomata momentarily in the afternoon in order to reduce water loss, resulting in a decrease in CO_2 concentration available for carboxylation and subsequently slowing down the Calvin cycle and the regeneration of ADP + P_i (Fig. 5), causing accumulation of protons in the thylakoid lumen. Accumulation of protons decreases the lumen pH, and triggers the protonation of specific PSII proteins and the enzymatic de-epoxidation of violaxanthin into antheraxanthin and zeaxanthin (Demmig-Adams et al. 1996, Gilmore 1997, Müller et al. 2001). This process is thought to be bi-modal, where the protonation occurs rapidly whereas the de-epoxidation reactions are slower (Gilmore 1997, Müller et al. 2001, Morosinotto et al. 2003, Ensminger et al. 2006). As a result, the fraction of thermal dissipation at the antenna of PSII, and probably also of PSI (Morosinotto et al. 2003), increases. This acclimation process tends to adjust the electron transport and ATP and NADPH supply to the prevailing needs, reducing the risks associated with excess light.

Conversely, when energy utilization by the dark reactions increases, the increased demand of ATP will enhance the membrane permeability to protons increasing the lumen pH. Increased lumen pH promotes the epoxidation of zeaxanthin back to violaxanthin and de-protonation of PSII proteins, resulting in a decrease in the levels of thermal dissipation in PSII. Xanthophyll-cycle and pH-dependent thermal dissipation generally relaxes within a few minutes in the dark. The xanthophyll-cycle and pH-dependent mechanism has been proposed to be very flexible in coping with changes in relative demands of ATP and NADPH while maintaining the required levels of protection to excess light (Kramer et al. 2004). Similarly the combination of fast protonation (seconds) with epoxidation de-epoxidation reactions (seconds-minutes) gives further temporal flexibility to the mechanism (Müller et al. 2001, Horton et al. 2005).

1.5.2 Long-term or seasonal acclimation

At longer time-scales, imbalances between energy supply and energy utilization may be more sustained. For example, decreased metabolic activity, induced either by the annual cycle, water stress, low temperatures, or nutrient deficits, may result in a sustained decrease in energy utilization by the dark reactions of photosynthesis (Huner et al. 1998, Niinemets et al. 2001, Öquist and Huner 2003). Sustained energy imbalances require sustained acclimation processes, which include several mechanisms.

One important way by which plants adjust the amount of energy absorbed by the light reactions is by **adjusting the leaf chlorophyll contents** (Öquist et al. 1978, Ottander et al. 1995, Vogg et al. 1998, Ensminger et al. 2006). If the number of PSII units remains constant, a decrease in chlorophyll contents will reduce the photosystem effective absorption cross-section area and the probability of photons being captured by a reaction centre and used in photochemistry (Huner et al. 1998, Ensminger et al. 2006).

Another important mechanism, is the adjustment in the **sustained thermal dissipation** of absorbed light, which allows PSII to be continuously engaged in thermal dissipation. This mechanism is needed for example to cope with early morning subfreezing temperatures combined with high irradiance, typical in boreal Scots pine needles during spring. Subfreezing temperatures would impair the fast enzymatic de-epoxidation of violaxanthin (Eskling et al. 2001) required to adjust the fraction of xanthophyll-cycle and pH-dependent thermal dissipation to the increasing morning irradiance. In contrast, sustained thermal dissipation allows PSII to be readily engaged in thermal dissipation during cold mornings, providing a safer mode of protection. This mechanism involves the same xanthophyll-cycle described above, however the thermal dissipation capacity is sustained in the dark and in the absence of a low thylakoid pH (Verhoeven et al. 1998, 1999), which is thought to be facilitated by a structural reorganization of the light harvesting proteins (Ottander et al. 1995, Gilmore and Ball 2000, Ensminger et al. 2006, Busch et al 2007) and is commonly accompanied with an increase in the pool of xanthophyll-cycle pigments and sustained high levels of de-epoxidation (Ottander et al. 1995, Demmig-Adams and Adams III 1996, Havaux 1998, Havaux and Niyogi 1999, Ensminger et al. 2004). Also, sustained thermal dissipation has been associated with non-functional reaction centres in PSII (Krause et al. 1988, Lee et al. 2001, Ivanov et al. 2002), which I discuss next in connection to photoinhibition.

Photoinhibition and recovery of PSII reaction centres is another process that affects the energy partitioning in PSII at a time-scale of hours to days. I will use the definition of photoinhibition by Tyystjärvi et al. 2008, where photoinhibition is defined as the reaction by which the photochemical electron transport activity of PSII is lost in such a way that *de novo* synthesis of the reaction centre D1 protein is required in order to regain the photochemical activity. Several hypothesis for the molecular mechanism of photoinhibition exist. Overreduction of the plastoquinone pool and of the primary electron acceptor Q_A taking place under excess light is known as the acceptor-side mechanism of photoinhibition, where triplet state formation by the radical pair P^{+}_{680} $Pheo^{-}$ promotes singlet oxygen $^1O_2$ formation which subsequently may damage the reaction centre D1 protein (Vass et al. 1992). However, the extent of photoinhibition has been found to be proportional to light intensity, occurring also under dim light conditions (Tyystjärvi and Aro 1996). Furthermore, total inhibition of the Calvin-Benson cycle in pea leaves by D,L-glyceraldehyde resulted in lower levels of photoinhibition, as measured by oxygen evolution, compared to control leaves treated with lincomycin, an inhibitor of chloroplast-encoded protein synthesis (Hakala et al. 2005). These findings do not fully support the acceptor-side mechanism of photoinhibition nor the expected role of excitation pressure increasing photoinhibition. Recently, experimental evidence seems to point out to a new mechanism that involves the donor-side of PSII, and particularly the manganese cluster, as the original target of photoinhibition (Hakala et al. 2005, Tyystjärvi 2008). Once the oxygen evolving complex becomes inactive, triplet state formation by the radical pair P^{+}_{680} $Pheo^{-}$ would promote damage to the reaction centre D1 protein (Tyystjärvi 2008). Yet the exact molecular mechanisms of photoinhibition remain still unknown, in particular under natural field condition. Under natural growing conditions, damage and recovery of the reaction centres take place simultaneously (Aro et al. 1993). Therefore, photoinhibited reaction centres will only accumulate when rate of damage exceeds the rate of recovery by *de novo* synthesis of the D1 protein, such as under cold winter conditions, when low temperatures inhibit recovery of damaged reaction centres (Anderson and Aro 1994). Photoinhibited or inactive reaction centres have been proposed to participate in the sustained thermal dissipation of excitation energy in PSII (Krause et al. 1988, Anderson and Aro 1994, Lee et al. 2001, Ivanov et al. 2002, Matsubara and Chow, 2004), protecting the

remaining functional reaction centres. However, the mechanisms by which inactive reaction centres participate in sustained thermal dissipation of excitation energy seem to differ between leaves that have undergone seasonal acclimation in field conditions compared to leaves photoinactivated at room temperature (Matsubara and Chow, 2004), therefore this mechanism still needs to be confirmed under field conditions.

1.6 What does the state of acclimation of PSII tell us?

Given the linkage between light and dark reactions described in the previous chapters, the state of acclimation of PSII carries information both on the instant photosynthetic carbon assimilation rate as well as on the overall physiological status of the plant. Over the short-term, the electron transport rate (ETR) will be rapidly adjusted to the prevailing ATP and NADPH needs by the dark reactions, in response to the short-term or diurnal acclimation mechanisms. Whereas over the long-term, changes in energy partitioning in PSII can interpreted as the response to long-term stress factors affecting the plant, or to the natural variation in metabolic activity induced by the plant's annual cycle. Therefore seasonal acclimation of PSII can be used as a proxy of the plant physiological status.

However, electron transport rate (ETR) and carbon assimilation may be uncoupled under certain conditions due for example to alternative energy sinks (Baker and Oxborough 2004). In particular, photorespiration is well known to run the Calvin-Benson cycle with oxygen as substrate instead of CO_2, partly uncoupling ETR from carbon assimilation (Genty et al. 1990, Harbinson et al. 1990). Therefore factors uncoupling energy supply from CO_2 assimilation need to be considered when attempting to estimate CO_2 assimilation from ETR, or extrapolate the acclimation of PSII to that of photosynthetic carbon assimilation.

1.7 Chlorophyll fluorescence: a tool to follow the acclimation of photosystem II

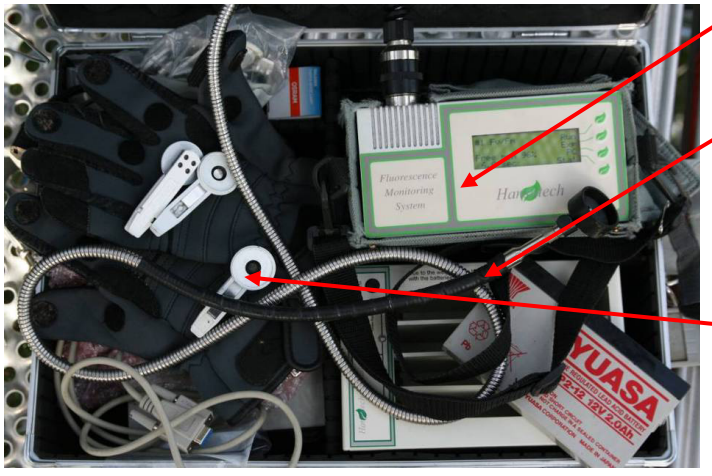
Monitoring of the energy partitioning and its acclimation in PSII under natural conditions has become relatively easy using portable field fluorometers (Fig. 6). Chlorophyll fluorescence is measured remotely, from a few millimetres with conventional portable fluorometers (Schreiber 1986, Maxwell and Johnson 2000), to several meters (Flexas et al. 2000, Moya et al. 2004), or up to the near-future satellite measurements of passive sun-induced chlorophyll fluorescence (Grace et al. 2007). In addition, new chlorophyll fluorescence monitoring systems (e.g. the one presented in STUDY IV), provide chlorophyll fluorescence data extending over a wide time-scale. Therefore, studying the acclimation of PSII through the interpretation of chlorophyll fluorescence data requires theoretical tools capable of covering different spatial and temporal scales. Next, I discuss the main techniques and protocols currently used for probing PSII fluorometrically, as well as the limitations that I dealt with in the present work.

As explained above, once a photon is absorbed by the antenna of PSII, the excitation energy may undergo different fates: i) it can be reemitted as a photon of chlorophyll fluorescence, ii) it can be dissipated thermally by internal conversion and thermal relaxation, iii) it can produce a chlorophyll triplet state, iv) it can be absorbed by pigments from the PSII antenna that at the moment are associated with PSI (state-transitions), v) or it can be used in photochemistry through the photochemical charge separation process, which largely depends on the availability of oxidized acceptors (Q_A). Generally, the rate of

chlorophyll triplet state production is not considered to be a significant process *in vivo* compared to the other pathways (Barber et al. 1989). Furthermore, due to the stability of excited P^{+}_{700}, the oxidized reaction centre of PSI is able to trap excitation energy and dissipate it as heat (Nuijs et al. 1986). Thus, chlorophyll fluorescence emission by PSI contributes to a constant level to the total observed fluorescence (Pfündel 1998), and very little to the observed variable fluorescence ($F_v = F_m - F_o$). Therefore, assuming a lake antenna organization model (Dau 1994) with free transfer of excitation energy between photosynthetic units, the rate of fluorescence emission will be proportional to the rate of light absorbed by PSII (I_a) and to the quotient of the rate constant of fluorescence (k_f), divided by the rate constants of all other processes competing for excitation energy: overall thermal dissipation (k_D), transfer to non-fluorescent pigments through state-transitions (k_T), and overall photochemistry (k_P), as (Krause and Weis 1991):

$$F = I_a \frac{k_f}{k_f + k_D + k_T + k_P} \qquad \text{(Eq. 2)}$$

Experimental estimation of energy partitioning in PSII is based on Eq.2 combined with a series of techniques used to shut-down specific energy-consuming processes. Under normal conditions, after a leaf is transferred from darkness into light, a fluorescence peak is obtained after approximately 1s (Vredenberg 2000). This phenomenon is termed Kaustky effect, after the first qualitative observations by Hans Kaustky and A. Kirsch in 1931 (Kaustky and Kirsch 1931). The peak is explained by the rapid reduction of the plastoquinone pool followed by a gradual increase in the reoxidation rate during the next minutes as a result of the activation of carbon assimilation by the dark reactions of photosynthesis (Maxwell and Johnson 2000) (see development of F_t in Fig. 7). Reduction and oxidation of the quinone acceptor Q_A will decrease and increase k_P, respectively, explaining the observed peak in fluorescence F (Eq. 2). The quenching of chlorophyll fluorescence by photochemistry or oxidized quinone is commonly addressed in fluorescence terminology as **photochemical quenching** or qP. A few second upon



Main unit. Includes the fluorescence detector, light sources, software, power unit, and data storage.

Optical fiber cable. Supplies actinic and modulated light to the leaf and transmits fluorescence from the leaf to the main unit.

Leaf-clip. Used to hold the leaf together with the optical fiber. The sliding metal window allows to dark-acclimate the leaf before measurement

Figure 6. Example of portable field fluorometer unit (FMS-2, Hansatech, UK).

illumination, the xanthophyll-cycle and pH-dependent thermal dissipation mechanisms activate the thermal dissipation of excitation energy, thus increasing the rate constant of thermal dissipation (k_D) (Eq. 2), this fluorescence quenching is commonly referred in fluorescence terminology as **energy dependent quenching** or qE. Another form of fluorescence quenching, commonly referred to **state-transitions quenching** or qT, is due to the dissociation of part of the LHCII from PSII to the non-fluorescent PSI, resulting in a decrease in the observed fluorescence yield. Finally, the sustained quenching of the chlorophyll fluorescence signal is referred as **photoinhibitory quenching** or qI.

Acclimation in energy partitioning can be quantitatively tested with pulse-amplitude modulated (PAM) fluorometry combined with a saturating pulse technique (Schreiber 1986, 2004). Typically, a leaf clip is used to keep the leaf sample and the optical fiber cable together. The optical fiber cable supplies actinic and modulated light to the leaf and transmits the chlorophyll fluorescence arising from the leaf sample into the instruments detector (Fig.6). The saturating pulse technique consists of supplying the leaf sample with a short pulse of saturating light such that it reduces all quinone electron acceptors, and k_P in Eq. 2 becomes zero. This technique is commonly combined with dark-acclimation, which consist of dark-acclimating the leaf for a period of time long enough to relax all the fast reversible xanthophyll-cycle and pH-dependent thermal dissipation (minimum k_D). After the dark-acclimation period, and under dim light, photochemistry operates at its optimum

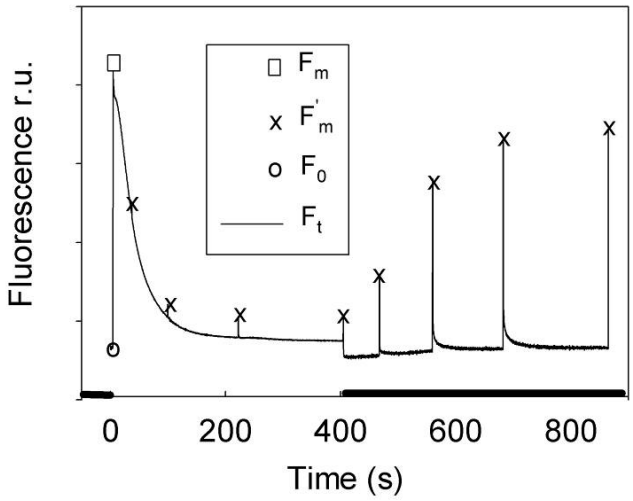


Figure 7. Chlorophyll fluorescence transient in leaves of *Betula pendula* Roth. obtained with a FMS-2 (Hansatech Ltd., UK), the system uses a modulated beam light-source of constant intensity to measure chlorophyll fluorescence. Dark thick horizontal line represents periods with dim light illumination. Otherwise light intensity was 1200 $\mu molm^{-2}s^{-1}$. Upon transfer from low light to high-light illumination the initially minimum chlorophyll fluorescence intensity (F_o), arising from the constant modulated beam light, increases and the current fluorescence intensity (F_t) peaks during the first seconds of illumination due to the Kautsky effect. The initial maximum fluorescence intensity (F_m) and maximum fluorescence intensity in the light (F_m') are obtained after supplying the leaf with a saturating light pulse. Decrease of F_m' under illumination and subsequent recovery in the dark are caused by increase of reversible thermal dissipation during illumination and its subsequent recovery in the dark.

since all electron acceptors are oxidized and capable of photochemical quenching of excitation energy, i.e., k_P is at its highest (Eq. 2), thus the recorded fluorescence intensity at minimum (F_o). Upon illumination the current fluorescence intensity (F_t) will increase as part of the above described Kaustky effect. If a saturating pulse is then supplied to the leaf, k_P will become zero and the maximum fluorescence intensity can be recorded (F_m). Subsequent saturating pulses yield a lower maximum fluorescence intensity under illumination (F_m'), since the fast-reversible thermal dissipation processes activate under illumination and increase the fraction of absorbed energy being dissipated as heat (increase in k_D), resulting in decrease in fluorescence (Eq. 2). Later on, if the leaf is again transferred to dim light conditions F_m' will gradually recover in response to the relaxation in thermal dissipation (decrease in k_D), (Fig. 7).

Estimation of the minimum and maximum fluorescence intensities F_o and F_m after dark acclimation, is used to estimate the maximum quantum yield of photochemistry (Kitajima and Butler 1975) as $F_v/F_m = (F_m-F_o)/F_m$. Similarly, the operating quantum efficiency of PSII (Genty et al. 1989) in light acclimated leaves can be estimated as $F_v'/F_m' = (F'_m-F_t)/F'_m$, and other parameters such as NPQ (Bilger and Björkman 1990), NPQ= (F_m-F_m')-1, have been used to follow the short-term acclimation of regulated thermal dissipation in PSII.

Using the saturating pulse technique to estimate the energy partitioning in PSII has some limitations. If we seek to investigate the acclimation of PSII to different light intensities and supply saturating pulses at high frequencies, the saturating pulse itself will cause acclimation in PSII therefore making it impossible to separate the effect of the saturating pulse from that of the natural illumination. This drawback makes it impractical to directly use the saturating pulse technique to follow the rapid adjustments in energy partitioning in PSII at high time resolution (e.g. seconds). Therefore a different approach is needed to follow the rapid acclimation of PSII. In addition, an important requirement to compare the fluorescence intensity over time is that the factors affecting light absorption by the leaf sample remain constant (e.g. leaf area under examination), so that all changes in fluorescence intensity can be attributed to the acclimation of PSII. However, monitoring of the same leaf area over prolonged periods of time is technically challenging. Importantly, changes in chlorophyll contents will also affect the light absorption capacity, limiting the interpretation of chlorophyll fluorescence data at a seasonal time-scale. This is the main reason behind the general lack of chlorophyll fluorescence parameters to follow the seasonal acclimation in PSII.

2 AIM OF THE STUDY

The aim of this thesis was to study the diurnal and seasonal acclimation of PSII in field conditions through the development and testing of new chlorophyll fluorescence-based tools. Specific objectives were:

- To study the dynamics of short-term or diurnal acclimation in the energy partitioning in photosystem II to the rapid fluctuations in light intensity, by developing a mathematical model based on the current understanding of the short-term acclimation processes and chlorophyll fluorescence.
- To study the long-term or seasonal acclimation in the energy partitioning in photosystem II to the seasonal changes in light and temperature, by developing a mathematical expression to estimate the rate constants of sustained thermal dissipation and photochemistry from chlorophyll fluorescence data.

- To quantitatively evaluate the role that light and temperature play in the seasonal acclimation of PSII in field conditions.
- To study the interaction between diurnal and seasonal acclimation of photosystem II in field conditions using a new monitoring-PAM (*MONI-PAM*).

3 MATERIALS AND METHODS

3.1 Theoretical Framework

3.1.1 Theoretical model of the acclimation of PSII

Idealization and abstraction of ideas from conceptual models of reality is a powerful methodological tool to investigate complex biological systems and the processes that constitute their functioning (Tuomivaara et al. 1994). A conceptual model of reality comprises the state-of-the-art knowledge on the given genuine processes. Through abstraction and idealization, the most relevant processes from the conceptual model are selected to produce a theoretical model of the reality that captures the essential features of the studied phenomena (Fig. 8). Theoretical models are used to derive mathematical models intended to describe the processes occurring in the real biological system. Mathematical models are thus an idealised, abstracted, and simplified view of reality, yet they provide a powerful tool to interpret empirical data and test hypothesis on the genuine processes, given a set of boundary conditions. In this thesis I applied this model-based approach to the interpretation of chlorophyll fluorescence data in order to study the acclimation of PSII to the environment. In Fig. 8 I present my theoretical model of the processes affecting the energy partitioning and acclimation of PSII. I used this theoretical model to derive mathematical expressions (STUDY II), and a model (STUDY I), that utilize chlorophyll fluorescence data to obtain key parameters describing the acclimation of PSII.

3.1.2 Boundary conditions: Time-scales

Acclimation in PSII and in the light reactions of photosynthesis occurs at different time-scales (Table 1). The rate constants of chlorophyll fluorescence (k_f) and constitutive thermal dissipation (k_D) can be assumed to remain constant. In contrast, the rate constant of photochemistry (k_P) varies in response to both rapid changes in the proportion of available electron acceptors (Q_A) (ms-s), as well as slow changes in the fraction of functional reaction centres (RC), (hours-weeks). Similarly, the rate constant of regulated thermal dissipation (k_{NPQ}) varies in response to both the fast-reversible and sustained thermal dissipation, which operate at time-scales of seconds to minutes, and days to weeks, respectively. The rate constant of state-transitions (k_T) varies at a time-scale of seconds to minutes, while more sustained changes in the ratio of the relative absorption cross-section area of PSII to that of PSI (*a*) would take place at a time-scale of days to weeks. Finally, adjustments in pigment contents can be observed at a time-scale of days to weeks. Overall, the acclimation of PSII is the result of the continuous and integrated effect from several acclimation processes that operate at different time-scales. I used a set of temporal boundary conditions to reference the mathematical models, dividing the acclimation of PSII into short-term or diurnal acclimation (s-min), and slow or seasonal acclimation (hours-weeks).

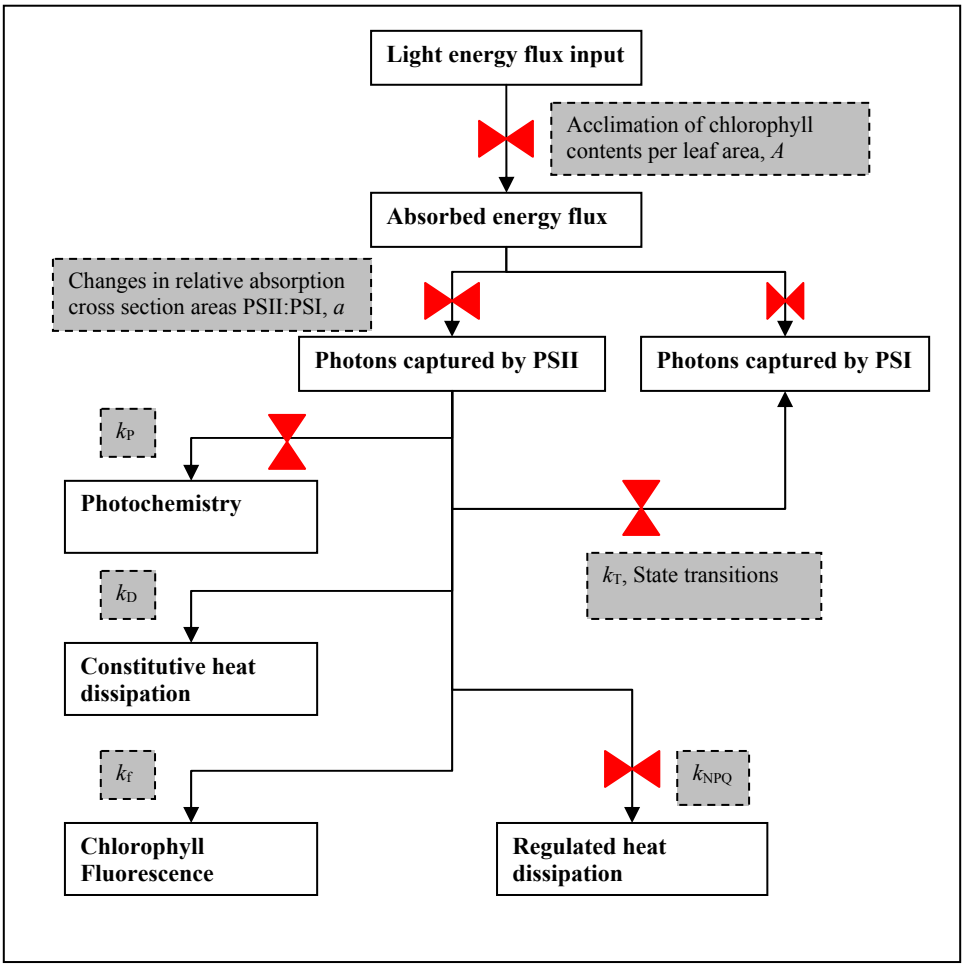


Figure 8. Theoretical model of the acclimation in PSII. Main fluxes of energy in PSII, including the energy partitioned into PSI. The main components of the acclimation are indicated by the red valve symbols. Rate constants and parameters associated with each process are indicated. *A* is the leaf absorptance, *a* is the fraction of absorbed light captured by PSII, and k_f, k_D, k_P, k_T and k_{NPQ} are the rate constants associated with fluorescence, constitutive thermal dissipation, photochemistry, state transitions, and regulated thermal dissipation.

3.1.3 General chlorophyll fluorescence equation to describe the acclimation of PSII

Based on the theoretical model presented in Fig. 8, the boundary conditions set by the diurnal and seasonal time-scales (Table 1), and assuming a lake antenna organizational model with perfect connectivity between photosynthetic units (Dau 1994), I derived and used the following mathematical expression (Eq. 3) as the general mathematical model to describe the effect of acclimation of PSII on the intensity of measured chlorophyll fluorescence :

Table 1. Approximate time-scales at which the variables in our theoretical model of PSII change

	Time-Scale					
	SHORT-TERM/ DIURNAL				LONG-TERM/ SEASONAL	
Variables	ms	s	min	hour	day	week
k_f	<----------CONSTANT ---------->					
k_D	<----------CONSTANT ---------->					
k_P	<-----Q_A------->			<---------- RC---------->		
k_{NPQ}	<-------Fast-reversible NPQ-->				<----sustained NPQ----->	
k_T, a	<-State transitions--->				<---Changes in a ---->	
A	<-Changes in Pigments->					

$$F_t = \beta\ I_{\mathrm{MB}}\ A\ \ a\frac{k_{\mathrm{f}}}{k_{\mathrm{f}} + k_{\mathrm{D}} + k_{\mathrm{NPQ}} + k_{\mathrm{P}}} = \beta\ I_{\mathrm{MB}}\ A\ \ a\ \frac{k_{\mathrm{f}}}{k_{\mathrm{f}} + k_{\mathrm{D}} + k_{\mathrm{NPQ}} + k_{\mathrm{PSII}}[Q_{\mathrm{A}}][RC]} \quad \text{(Eq. 3)}$$

where, F_t is the current fluorescence intensity recorded by a conventional modulated fluorometer, β is a proportionality constant that depends on the fluorometer detector, I_{MB} is the constant light intensity of the modulated measuring light (Schreiber 1986), A is the leaf absorptance, a is the fraction of absorbed light captured by PSII, k_f, k_D, k_{NPQ} and k_P are the rate constants of fluorescence, constitutive thermal dissipation, regulated thermal dissipation, and photochemistry, respectively. In turn, the overall rate constant of photochemistry (k_P), represents the overall rate constant of a mixed population of reaction centres (RCs) with open/functional and closed/damaged RCs (Kornyeyev and Hendrickson 2007), and can also be expressed in terms of the rate constant of photochemistry (k_{PSII}) (Shinkarev and Govindjee 1993), multiplied by the fraction of open/functional reaction centres. In Study II, I proposed two new parameters to identify the fraction of open reaction centres [Q_A], as in Kitajima and Butler (1975), and the fraction of functional reaction centres [RC]. Both parameters can take up values ranging from zero to one, and represent the proportion of reaction centres that are able to conduct photochemical electron transport. Similarly, acclimation in the rate constant of regulated thermal dissipation (k_{NPQ}) will take place in response both to adjustments in the fast xanthophyll-cycle and pH-dependent thermal dissipation, as well as to the slow changes in sustained thermal dissipation. In addition, in a similar fashion as for k_P, k_{NPQ} could as well be denoted by a rate constant multiplied by parameters influencing the capacity of regulated thermal dissipation, such as the de-epoxidation of xanthophyll-cycle pigments, or the fraction of damaged reaction centres 1-[RC]. However, since these mechanisms remain elusive I chose to consider them implicitly in k_{NPQ} (Eq. 3).

In Study I, the short-term or diurnal acclimation of PSII was studied by modelling the rapid and light-induced adjustments in k_{NPQ}, as well as the changes in [Q_A], whereas slow changes in [RC] were assumed to remain constant at this time-scale (Table 1). In contrast, in Studies II and III dark-acclimated fluorescence data (F_o and F_m) were used, leaving only the sustained component of regulated thermal dissipation embedded in k_{NPQ}. Similarly, in dark-acclimated leaves all electron acceptors can be assumed to be re-oxidized and [Q_A]=1, while [RC] may range between one and zero. I used dark-acclimation as the criterion to differentiate between short-term or diurnal acclimation processes (reversible upon dark-acclimation), and slow or seasonal acclimation processes (sustained upon dark-acclimation) (Table 1).

Rate constants and parameters from Eq. 3 can be estimated using chlorophyll fluorescence data. Subsequently, once the rate constants of each of the energy consuming processes in PSII are known, the yield of each of the processes (*i*) can be estimated as:

$$\Phi_i = \frac{k_i}{k_\mathrm{f} + k_\mathrm{D} + k_\mathrm{NPQ} + k_\mathrm{P}} \qquad \text{(Eq. 4)}$$

where *i* can be f, D, NPQ or P.

3.1.4 Rapid adjustments in energy partitioning in PSII to fluctuations in light

We assumed that when a photon of light is absorbed by a chlorophyll molecule the chlorophyll molecule in the ground state ($Chla^{\mathrm{OFF}}$ or $Chlb^{\mathrm{OFF}}$) becomes excited and the exciton rapidly results in an excited chlorophyll-a molecule ($Chla^{\mathrm{ON}}$). Thus the number of excitons in a population of PSII units with a given total chlorophyll content (Chl_T), can be estimated through the following differential equation, as:

$$\frac{\mathrm{d}Chla^{\mathrm{ON}}}{\mathrm{d}t} = \alpha\, I\, Chl_T - k_\mathrm{f}\, Chla^{\mathrm{ON}} - k_\mathrm{d}\, Chla^{\mathrm{ON}} - k_\mathrm{n}\, E\, Chla^{\mathrm{ON}} - k_\mathrm{p}\, Q\, Chla^{\mathrm{ON}} \qquad \text{(Eq. 5)}$$

where the first term represents the rate of light capture, determined by the light intensity *I*, the amount of chlorophyll, and a light absorption efficiency parameter α, and the following terms represent the consumption of excitons by each of the main energy-consuming processes in PSII: fluorescence, constitutive thermal dissipation, regulated thermal dissipation, and photochemistry, in the same order as in Eq. 5. In addition, I introduced an efficiency term *E* that represented the fraction of active quenching sites (ON) [$E = S^{\mathrm{ON}}/(S^{\mathrm{ON}}+S^{\mathrm{OFF}})$], denoting the efficiency of thermal dissipation in the PSII population. Similarly, Q (Q_A in Eq. 3) represented the fraction of oxidized electron acceptors (OFF) as [$Q = Q^{\mathrm{OFF}}/(Q^{\mathrm{ON}}+Q^{\mathrm{OFF}})$]. The rate constants in STUDY I correspond with those in Eq. 3 as: $k_\mathrm{f}=k_\mathrm{f}$, $k_\mathrm{d}=k_\mathrm{D}$, $k_\mathrm{n}=k_\mathrm{NPQ}$, and $k_\mathrm{p}=k_\mathrm{PSII}$.

The adjustments of *E* and *Q* to light intensity were modelled as:

$$\frac{\mathrm{d}\,E}{\mathrm{d}t} = \frac{\lambda_\mathrm{b}\; Chla^{\mathrm{ON}}\; S^{\mathrm{OFF}}}{S^{\mathrm{OFF}} + S^{\mathrm{ON}}} - \frac{\lambda_\mathrm{r}\; Q\; S^{\mathrm{ON}}}{S^{\mathrm{OFF}} + S^{\mathrm{ON}}} \qquad \text{(Eq. 6)}$$

and

$$\frac{\mathrm{d}Q}{\mathrm{d}t} = \frac{\gamma\, Q^{\mathrm{ON}}}{Q^{\mathrm{ON}} + Q^{\mathrm{OFF}}} - \frac{k_\mathrm{p}\; Q\; Chla^{\mathrm{ON}}}{Q^{\mathrm{ON}} + Q^{\mathrm{OFF}}} \qquad \text{(Eq. 7)}$$

where λ_b, λ_r, and γ are parameters linked to the building of regulated thermal dissipation, its relaxation, and the reoxidation of the quinone pool equivalents, respectively (STUDY I). These parameters were estimated following the changes in measured chlorophyll fluorescence intensity (f') upon illumination of dark-acclimated leaves and subsequent dark-acclimation, according to:

$$f = I_{\mathrm{MB}}\ Chl_T\ \alpha\ \frac{k_{\mathrm{f}}}{k_{\mathrm{f}} + k_{\mathrm{d}} + E\ k_n + Q\ k_{\mathrm{p}}} \qquad \text{(Eq. 8)}$$

where I_{MB} is the constant light intensity of the fluorometer modulating beam, and f is proportional to the measured fluorescence signal F (given the proportionality constant β). This equation was used to derive information on the acclimation of PSII through chlorophyll fluorescence measurements, as well as to parameterise and test the model.

Adjustments in the energy partitioning of a process i were subsequently estimated as:

$$\Phi_i = \frac{k_i}{k_{\mathrm{f}} + k_{\mathrm{d}} + E\ k_{\mathrm{n}} + Q\ k_{\mathrm{p}}} \qquad \text{(Eq. 9)}$$

where i can be f, d, n or p.

3.1.5 Estimating the rate constant of sustained thermal energy dissipation and photochemistry

A main limitation to the interpretation of seasonal chlorophyll fluorescence data is caused by the effect that seasonal changes in pigment contents have on light absorptance, and subsequently, on the measured fluorescence signal (Eq. 3). In a first attempt to correct for these changes I expressed leaf absorptance (A) as a function of the total chlorophyll content (Chl) and a leaf light extinction coefficient (ε), according to Parson and Nagarajan (2003), and assuming that carotenoids do not participate significantly in light absorption (Bassi and Cafarri 2000), as:

$$A = 1 - 10^{-\varepsilon\ Chl} \qquad \text{(Eq. 10)}$$

The mathematical expression (Eq. 3) derived from the theoretical model presented in Fig. 8, combined with Eq. 10 allows us to use chlorophyll fluorescence data to estimate the rate constants of sustained thermal dissipation (k_{NPQ}) and photochemistry (k_{P}), as (STUDY II):

$$k_{\mathrm{NPQ}} = \left(\frac{F_{\mathrm{m}s}}{F_{\mathrm{m}}} \frac{A\ a}{A_s\ a_s} - 1 \right) \left(k_{\mathrm{f}} + k_{\mathrm{D}} \right) \qquad \text{(Eq. 11)}$$

and

$$k_{\mathrm{P}} = k_{\mathrm{PSII}} \left[RC \right] = \left(\frac{F_{\mathrm{m}s}}{F_{\mathrm{o}}} - \frac{F_{\mathrm{m}s}}{F_{\mathrm{m}}} \right) \frac{A\ a}{A_s\ a_s} \left(k_{\mathrm{f}} + k_{\mathrm{D}} \right) \qquad \text{(Eq. 12)}$$

where the values with the "*s*" subindex refer to the summer reference levels.

3.2 Experiments and setup

3.2.1 Short-term chlorophyll fluorescence measurements

Leaves from seven-years-old European alder [*Alnus glutinosa* (L.) Gaertn.] trees, planted near the Viikki Science Campus, were used in STUDY I to monitor the rapid adjustments in energy partitioning in PSII to fluctuations in light. After dark acclimation *in situ* for two hours with dark-acclimation clips (Hansatech, UK), leaves were illuminated for several minutes and subsequently brought back to darkness. The acclimation of PSII was followed by recording the dark-acclimated chlorophyll fluorescence levels F_o, F_m, and the successive light acclimated F_m' using a portable FMS-2 fluorometer (Hansatech, UK). Maximum chlorophyll fluorescence yields (F_m and F_m') were obtained by supplying a saturating-light pulse of 3500 $\mu molm^{-2}s^{-1}$ at the leaf surface. Data were used to estimate the parameters in Eqns 6 and 7.

3.2.2 Long-term chlorophyll fluorescence measurements

Three forty-five-years-old planted Scots pine trees [*Pinus sylvestris* (L.)] growing at SMEAR II station (Station for Measuring Forest-Ecosystem-Atmosphere Relations) in southern Finland, were used to monitor the spring recovery of PSII *in situ* in STUDIES II, III and IV. Additionally, seven-years-old Scots pine trees growing a few hundred meters from SMEAR-II were used in STUDY III to study the effect of different levels of shading on the seasonal acclimation of PSII under spring recovery. During the previous autumn, a shading structure was mounted around the tree and shading was provided with different layers of grey net. The seasonal acclimation of PSII was monitored approximately twice a week in STUDIES II and III, by dark acclimating the needles for two hours followed by measurement of minimum and maximum chlorophyll fluorescence levels F_o and F_m, respectively, using the saturating pulse technique with a portable FMS-2 fluorometer (Hansatech, UK). The fluorescence data were used to estimate the maximum quantum yield of photochemistry (F_v/F_m) (Kitajima and Butler 1975), as well as the new parameters developed in STUDY II (Eqns. 11 and 12). Finally, a new field monitoring fluorometer (*MONI-PAM*, Heinz Walz GmbH, Germany) was used to study the seasonal and diurnal acclimation of PSII in field Scots pine growing at SMEAR II station during spring recovery of photosynthesis. The instrument was tested in STUDY IV. The new instrument measured the same needles for many days and recorded the current fluorescence F_t, the maximum fluorescence F_m', PAR and temperature every few minutes.

3.2.3 Environmental data

Radiation and temperature data from SMEAR-II station were used in STUDY II (Vesala et al. 1998). In addition, the seasonal and diurnal variation in light intensity and temperature was followed in Study III with PAR sensors (Li-Cor, Lincoln, Nebraska), and ventilated Pt100 radiation protected thermoelements, respectively, recording at 30 seconds intervals. Finally, in study IV, the temperature and PAR sensors integrated in the *MONI-PAM* were used.

3.2.4 Biochemical determinations

Two-pairs of needles were collected from each of three trees per treatment (i.e., monitoring trees STUDY II, or shading treatments in STUDY III), approximately twice a week in order

to follow the seasonal adjustments in chlorophyll contents, as well as in xanthophyll-cycle pigments. Needle samples were collected and immediately frozen in liquid nitrogen. Samples were later ground with mortar and pestle in liquid nitrogen and lyophilized. Pigment extractions were performed in 100% acetone, buffered with $NaHCO_3$, for 2 hours at 4 °C in the dark. Separation of pigments was done with high-performance liquid chromatography (HPLC), as described in Ensminger et al. (2001).

4 RESULTS AND DISCUSSION

4.1 Diurnal acclimation of PSII studied using chlorophyll fluorescence

The model developed in STUDY I was able to reproduce the rapid acclimation of the energy partitioning in PSII upon changing the illumination conditions of the leaf (Fig. 9). Some limitations rose due to the first approximations used in the model, for example I used a constant parameter to estimate the reoxidation of the plastoquinone pool (γ) (Eq. 7). Therefore, upon illumination of dark-acclimated leaves the fluorometrically measured maximum photochemical yield (Genty et al. 1989) decreased rapidly and recovered during the following minutes until reaching a steady-state, however our model could not reproduce this pattern. The dynamics in the photochemical yield upon illumination could be explained as the effect of the slow enzymatic activation of the dark reactions and opening of stomata after dark-acclimation (Pearcy et al. 1997, Allen and Pearcy 2000, Maxwell and Johnson 2000), which could temporarily limit the reoxidation of the plastoquinone pool. Similarly, measured yield of thermal dissipation also peaked upon illumination of dark-acclimated leaves before attaining a steady-state (STUDY I). These patterns give evidence of the feedback that the dark reactions of photosynthesis exert on the energy partitioning in PSII. The model presented here was a first step towards the modelling of the acclimation of PSII, and did not include adjustment in the rate of reoxidation of the electron acceptors caused by

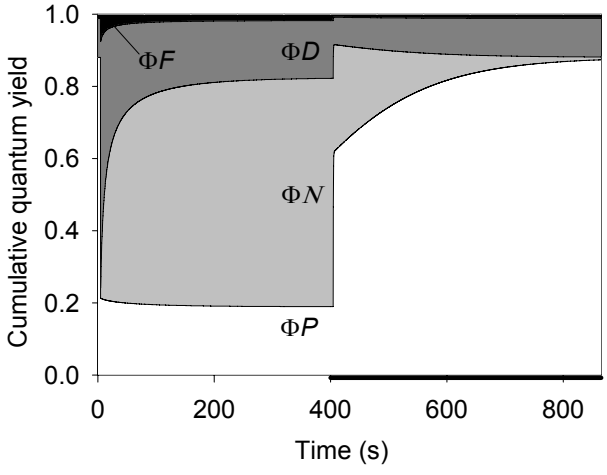


Figure 9. Modelled changes in the energy partitioning in PSII between fluorescence (ΦF), constitutive thermal dissipation (ΦD), regulated thermal dissipation (ΦN), and photochemistry (ΦP) in previously dark-acclimated alder leaves. The thick horizontal bar indicates dark period (PPFD=0.001 μmol m^{-2} s^{-1}), otherwise the illumination PPFD was 1200 μmol m^{-2} s^{-1}.

the dark reactions feedback. It was suggested that future versions of the model could be linked to the acclimation of the dark reactions through the re-oxidation rate of the plastoquinone pool, represented by the parameter γ. Overall, the dynamics of acclimation in energy partitioning presented in Figure 9 indicate that acclimation of PSII to changes in light intensity is not instantaneous and it takes several minutes before a new steady-state is attained.

Light reactions of photosynthesis, and in particular PSII, acclimate to rapid fluctuations in light intensity by adjusting the partitioning of energy between photochemical and non-photochemical processes, causing rapid adjustments in the electron transport rate (ETR), and ATP and NADPH formation. Availability of ATP and NADPH is the limiting step in photosynthetic carbon assimilation under low light intensities, or under conditions where the energy partitioning in the light reactions is diverted to non-photochemical processes. Currently available models for photosynthetic carbon assimilation (e.g. Farquhar et al. 1980, Gross 1982, Hari et al. 1986, Pearcy et al. 1997, Allen and Pearcy 2000, Hari and Mäkelä 2003) include the effect of enzymatic and stomatal limitations on carbon assimilation. Yet, the rapid dynamic adjustment of ETR to the light environment is ignored, and ETR, or ATP and NADPH formation, are estimated from light response measurements obtained at the steady-state after some minutes of illumination. However, under natural conditions, the ETR will seldom reach the steady state since leaves are exposed to frequent fluctuations in light intensity. Consequently, using a steady-state modelling approach for the estimation of ETR exclusively as a function of light intensity might lead to over or underestimations in carbon assimilation under fluctuating light.

I addressed this issue in **STUDY I** and used the model to compare the performance of a steady-state approach with our dynamic approach under fluctuating light. The results indicated that for fluctuations in light occurring in less than one second (e.g. leaf fluttering) a steady-state approach would lead to underestimations in the ETR compared to our dynamic approach. In contrast, if the fluctuations occur at intervals longer than 5 seconds (e.g. clouds) the steady-state approach would tend to overestimate the ETR (STUDY I). Similarly, steady-state and dynamic models of the dark reactions of photosynthesis, the latter including enzymatic and stomatal controls, have also been compared under fluctuating light (Gross 1982, Roden and Pearcy 1993, Pearcy et al. 1997, Naumburg and Ellsworth 2002). These studies also concluded that due to the utilization of the stored energy pools during the periods of low light, the steady-state approach underestimated carbon assimilation under rapid sunflecks. In contrast, due to the dynamics of stomatal and enzymatic controls on photosynthesis, the same approach overestimated the carbon assimilation under slow fluctuations in light intensity. It was thereby tempting to suggest that part of the observed effect of fluctuating light on carbon assimilation could originate from the dynamic acclimation of PSII.

Overall, the model presented here was able to follow the rapid acclimation of PSII to changes in light intensity occurring in a time-scale of seconds to minutes, based on parameters obtained from chlorophyll fluorescence measurements. This model provides a new tool for the study of the short-term or diurnal acclimation of PSII to the natural fluctuations in the light environment that plant leaves meet in the field.

4.2 Seasonal acclimation of PSII studied using chlorophyll fluorescence

4.2.1 New Parameters: The rate constant of sustained thermal energy dissipation and photochemistry

The seasonal development of the maximum quantum yield of photochemistry (F_v/F_m) (Fig. 10a) indicated a strong seasonal downregulation in PSII of Scots pine, typical for overwintering boreal conifers (Ottander et al. 1995, Ensminger et al. 2004). Three main phases could be qualitatively differentiated according to the pattern of variation of F_o and F_m (Fig. 10). During phase I, both F_o and F_m were lower compared to the summer levels, denoting certain level of sustained thermal dissipation, since thermal dissipation competes for excitation energy both when measuring F_o and F_m (Butler and Kitajima 1975, Demmig-Adams et al. 1989). Later, during phase II, both F_o and F_m levels rapidly increased, denoting relaxation in the levels of sustained thermal dissipation. And finally, the differences in pattern of variation of F_o and F_m during phase III, with F_o remaining high or even decreasing slightly, and F_m still increased strongly, may denote both further relaxation in sustained thermal dissipation as indicated by the increase in F_m, combined with an increase in photochemistry, as indicated by the absence of increase in F_o. Increased photochemical efficiency may be due to the recovery of damaged reaction centres (Krause 1988, Yamane 1997), or to increased connectivity between antenna and core or between reaction centres (Schreiber and Armond 1978, Krause and Weis 1984, Yamane 1995). Both of these result in a decrease of F_o, since photochemical utilization of excitation energy competes with fluorescence at the F_o state.

The chlorophyll fluorescence parameter F_v/F_m [$F_v/F_m = (F_m-F_o)/F_m$] (Kitajima and Butler 1975, Krause and Weis 1991) denotes the maximum quantum yield of photochemistry in dark-acclimated leaves. The parameter F_v/F_m uses maximum and minimum chlorophyll

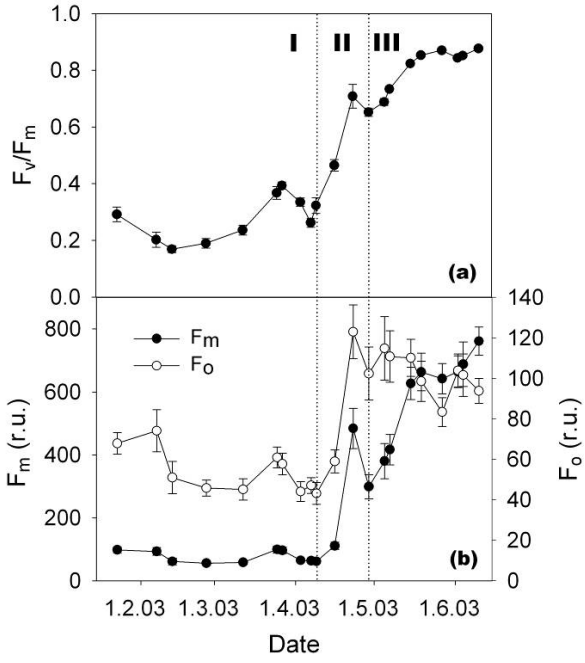


Figure 10. Seasonal changes in the maximum quantum yield of photochemistry (F_v/F_m) (a); and seasonal changes in minimum (F_o) and maximum (F_m) chlorophyll fluorescence intensities (b); in overwintering Scots pine. Mean ±SE (n=9).

fluorescence values obtained from dark-acclimated leaves. Therefore F_v/F_m is not affected by the rapid or diurnal acclimation of PSII and it has been widely used to monitor the seasonal acclimation of PSII (Adams III and Demmig-Adams 1994, Ottander et al. 1995, Ensminger et al. 2004, Slot et al. 2005). Yet, although F_v/F_m successfully serves as an overall indicator of the downregulation state of PSII, it does not provide information on whether changes in the maximum quantum yield of photochemistry are caused by photochemical limitations, i.e. a decreased capacity for photochemical electron transport, or by non-photochemical processes, i.e. an increase in the sustained thermal dissipation in PSII. At a diurnal time-scale, there are a large number of chlorophyll fluorescence parameters that allow differentiating between adjustments in photochemical and non-photochemical acclimation processes and their yields (cf. Krause and Weis 1991, Roháček 2002). However, these parameters cannot be used to follow the seasonal acclimation of PSII since they are obtained from light-acclimated leaves and thus include both the effect of diurnal and seasonal acclimation processes. In STUDY II two new chlorophyll fluorescence parameters were developed to estimate the seasonal changes in the rate constants of photochemistry and sustained thermal dissipation in PSII, (Eqns 11 and 12).

Qualitative comparison of the pattern of F_o and F_m has been widely used to indicate sustained thermal dissipation in PSII or inactivation or photodamage of PSII reaction centres. The qualitative analysis is based on the principle that changes in photochemical capacity of electron transport affect F_o but not F_m, whereas changes in non-photochemical thermal dissipation affect both F_o and F_m (Krause 1988, Barber et al. 1989, Demmig-Adams et al. 1989, Ögren and Sjöström 1990, Dau 1994, Ottander et al. 1995, Verhoeven et al. 1996, Yamane et al. 1997), however no quantitative approach had been developed before. A main limitation has probably been rooted in the assumptions behind the seasonal comparison of chlorophyll fluorescence data (Logan et al. 2007). Seasonal changes in F_o and F_m may be due to acclimation of PSII, but also to changes in the leaf area under examination, or in leaf absorptance. Therefore parameters developed at this time-scale require consideration of these factors. The new parameters developed in STUDY II included the effect of seasonal changes in leaf absorptance (Eqns. 10-12), and special care was taken to maintain the leaf area under examination constant throughout the measuring period.

Estimation of k_{NPQ} and k_P facilitated the quantitative interpretation of F_o and F_m, and the estimation of photochemical and non-photochemical capacities in PSII. The rate constants in Figure 11 are expressed relative to the sum of k_f and k_D, thus if k_P is 3 it denotes that the rate constant of photochemistry is three times as high as those for fluorescence and constitutive thermal dissipation combined. Overall, seasonal changes in k_P (i.e. obtained from dark-acclimated leaves), can be related to changes in the fraction of open or functional RCs ([RC] in Eq. 3), since after dark-acclimation all PSII electron acceptors can be assumed to be in an oxidated state ($[Q_A=1]$). However, some considerations need to be taken into account.

The degree of connectivity between PSII units, which regulates the transfer of excitation energy between connected RCs (Joliot and Joliot 1964, Kitajima and Butler 1975, Trissl and Lavergne 1994, Oxborough and Baker 2000), regulates the extent to what excitation can move from innactive RCs to active RCs. In Eq. 3 I assumed a lake antenna organization model (Dau 1994), where excitation can move freely between PSII units. The lake model assumption will affect the correlation between the estimated [RC] and the actual proportion of active or functional RCs, more specifically [RC] would overestimate the actual fraction of functional reaction centres. Thus, changes in [RC], as embed in k_P, reflect adjustments in photochemical electron transport efficiency, but the degree of connectivity, and any seasonal changes in it, needs to be considered when trying to link [RC] with the

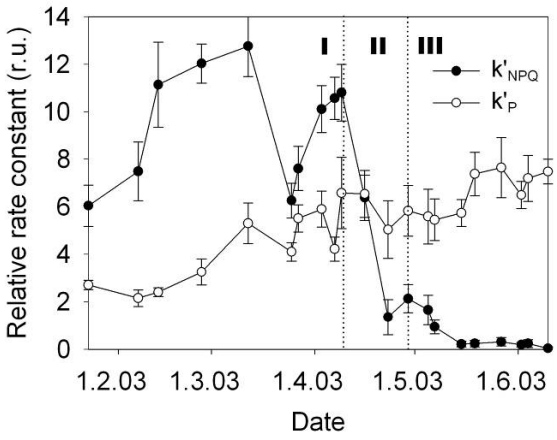


Figure 11. Seasonal changes in the rate constant of thermal dissipation (k_{NPQ}') (black dots) and the rate constant of photochemistry (k_P') (white dots), expressed relative to the sum of k_f and k_D. Mean ±SE (n=9).

actual fraction of functional reaction centres. The relationship between the fraction of functional reaction centres, estimated through oxygen yield resulting from a single-turnover saturating flash (Chow et al. 1991), and the chlorophyll fluorescence parameter ($1/F_o - 1/F_m$) have been found to correlate over a large range of conditions (Havaux et al. 1993, Lee et al. 2001, Matsubara and Chow 2004). The parameter $1/F_o - 1/F_m$ is the short-term equivalent of the parameter k_P derived here (see Eq. 12), where k_P includes the effect of seasonal changes in light absorptance using summer F_m as reference. Therefore, given constant connectivity between PSII units during the season, I would expect that k_P correlates with the fraction of functional reaction centres.

Seasonal changes in k_{NPQ} reflect the adjustments in the efficiency of sustained thermal dissipation, which has been linked to processes such as structural changes or aggregation of light harvesting complexes and sustained retention of zeaxanthin (Adams WW III and Demmig-Adams 1994, Ottander et al. 1995, Verhoeven et al 1998, 1999, Öquist and Huner 2003, Ensminger et al. 2004, Busch et al. 2007), as well as to thermal dissipation by non-functional reaction centres (Krause et al. 1988, Anderson and Aro 1994, Lee et al. 2001, Ivanov et al. 2002, Matsubara and Chow, 2004). The role of these mechanisms in the field will be discussed in the next chapter. Finally, it is important to understand that in the same way as fast changes in $[Q_A]$ and slow changes in [RC] influence k_P in natural conditions, also the rate constant k_{NPQ} will include reversible and sustained thermal dissipation components when estimated under illumination. Yet the purpose in STUDIES II and III was to estimate only the sustained component of thermal dissipation and I used dark-acclimated leaves.

Overall, the newly developed parameters captured the information carried by the seasonal trends in F_o and F_m, facilitated the quantitative analysis of the seasonal acclimation of PSII and the estimation of the energy partitioning (Eq. 4), and complemented F_v/F_m.

4.2.2 Seasonal acclimation of PSII to light and temperature

The results from overwintering Scots pine, from STUDIES II and III, revealed that the overall downregulation state of PSII, in terms of maximum quantum yield of photochemistry measured fluorometrically as F_v/F_m, was not only lower during the colder

periods compared to summer, but also lower in more exposed needles compared to the more shaded ones, consistent with the excitation pressure hypothesis (Huner et al. 1996, Huner et al. 1998), as well as with previous observations (Strand and Öquist 1985, Strand and Lundmark 1987, Ottander and Öquist 1991, Sveshnikov et al. 2006). Dark reactions of photosynthesis are strongly regulated by temperature, whereas light absorption by light reactions of photosynthesis is directly proportional to light intensity and independent of temperature, therefore it could be expected that light and temperature largely determine the imbalances between energy supply and energy consumption, and the resulting excitation pressure in PSII (Huner et al. 1996, Huner et al. 1998).

In STUDIES II and III we evaluated the effect of light and temperature not only on the overall acclimation or down-regulation state of PSII, but on each photochemical and non-photochemical process, using the parameters k_{NPQ} and k_P developed in STUDY II. Differences in downregulation of PSII between shaded and exposed leaves have been related both to differences in sustained thermal dissipation capacity: exposed needles typically present larger xanthophyll-cycle pools and de-epoxidation status (Demmig-Adams and Adams III, 1996, Demmig-Adams 1998, Adams et al. 2001, Sveshnikov et al. 2006); as well as to differences in the proportion of damaged or inactive reaction centres: exposed needles present higher proportion of damaged reaction centre compared to shaded needles (Karpinski et al. 1994, Langvall and Örlander 2001, Sveshnikov et al. 2006).

Our results indicated that the light environment to which the needles were exposed had a clear effect on the level on sustained thermal dissipation: more exposed needles had higher rate constants of sustained thermal dissipation during winter (Fig. 12a). Later on, towards mid April, the sustained thermal dissipation decreased drastically in all treatments, along with increasing temperatures, remaining low after that. I concluded that the drastic decrease in the sustained thermal dissipation in mid April (Fig. 12a) was consistent with the structural de-aggregation of protein complexes in the thylakoid membrane, which would trigger the change in the kinetics of thermal dissipation, shifting from sustained thermal dissipation into reversible pH-dependent thermal dissipation. Comparison of k_{NPQ} with the de-epoxidation state of the xanthophyll-cycle pigments before and after the shift gave further support to the hypothesis. Structural changes in the thylakoid membrane of overwintering Scots pine needles were first observed by Martin and Öquist (1979), and later on Verhoeven et al. (1999) proposed that aggregation of thylakoid membrane protein complexes during winter would be a requisite for maintaining a high level of sustained thermal dissipation in the absence of low lumen pH. Aggregation of protein complexes facilitates the thermal dissipation of excitation energy protecting the remaining chlorophyll in this way (Ottander et al. 1995, Gilmore and Ball 2000, Busch et al. 2007). The role of zeaxanthin in this process has not been elucidated. Zeaxanthin might cause the required conformational change needed for the aggregation (Bassi and Caffari 2000), while aggregation of protein complexes could in turn facilitate the sustained retention of zeaxanthin in a de-epoxidized state. Furthermore, observed changes in the amounts of other proteins, e.g. PsbS, and their role in thermal energy dissipation add more complexity of the mechanism, involving also processes with more slow kinetics (cf. Ensminger et al. 2006).

I analysed the kinetics of response of k_{NPQ} to temperature, and found that shaded needles adjusted the efficiency of thermal dissipation to changes in temperature faster than exposed needles during winter. Subsequently, the kinetics of response increased drastically after the hypothetical structural change in the thylakoid membrane. This is consistent with previous observations under controlled conditions, where levels of thermal dissipation in shaded leaves of *Euonymus kiautschovicus* transferred from cold to warm conditions relaxed faster compared to leaves that had been growing under high light (Verhoeven et al. 1998). I concluded that the observed change in the kinetics of acclimation to temperature in needles

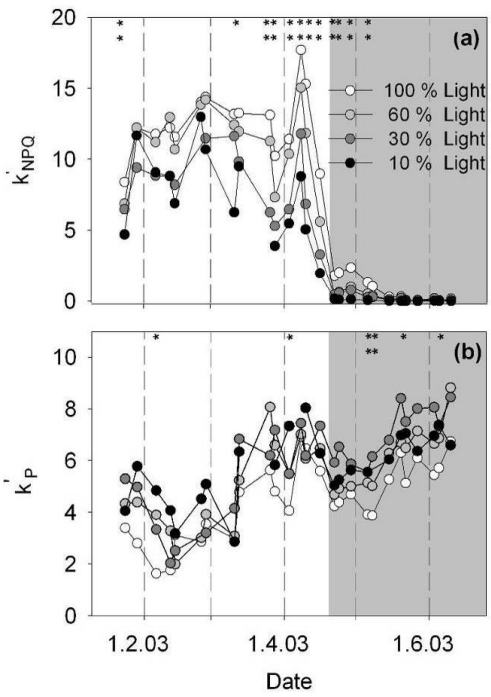


Figure 12. Seasonal changes in the rate constants of thermal dissipation (k'_{NPQ}) (a), and photochemistry (k'_P) (b), expressed relative to the sum of k_f and k_D. The grey area indicates the period after the change in the form of thermal dissipation from sustained to reversible takes place. Significant differences between the treatments with 100% and 10% of light are indicated by * ($p<0.05$) or ** ($p<0.01$) (T-test, two-tailed, n=5).

of overwintering Scots pine during spring recovery as well as the differences in kinetics between shaded and exposed needles are consistent with changes in aggregation state of thylakoid membrane protein complexes.

Decreased photochemical electron transport capacity during winter, as induced by damage to the PSII RC D1 protein, has been widely reported in evergreens (Ögren and Öquist 1984, Ottander and Öquist 1991, Adams et al. 1994, Ottander et al. 1995), which agrees with our results where lower k_P values were obtained during winter gradually recovering towards summer (Fig. 11 and 12b). Furthermore, more severe photoinhibition has been observed in exposed needles compared to shaded ones (Karpinski et al. 1994, Langvall and Örlander 2001, Sveshnikov et al. 2006). On the one hand, these findings would appear to support the excitation pressure hypothesis (Huner et al. 1996, Huner et al. 1998), in conjunction with the acceptor-side mechanism of photoinhibition (Vass et al. 1992), where more exposed needles present higher levels reduction of the primary electron acceptor Q_A, enhancing triplet state formation by the radical pair P^+_{680} $Pheo^-$, and leading to singlet oxygen $^1O_2$ production and subsequent damage to the reaction centre D1 protein. However, no evidence has been found in support of excitation pressure regulated photoinhibition (Hakala et al. 2005). On the other hand, observed photoinhibition is the result between simultaneous damage of reaction centres and recovery by *de novo* synthesis of the reaction centre D1 protein (Aro et al. 1993). Damage of reaction centres has been found to be directly proportional to absorbed light (Tyystjärvi and Aro 1996), while recovery of reaction centres is largely dependent on temperature (Anderson and Aro 1994, Lee et al. 2001). These findings would explain both the lower k_P during winter compared to summer since low temperatures limit recovery of damaged reaction centres, as well as the higher damage observed in more exposed leaves compared to more shaded ones, since

more exposed leaves have higher influx of photons and consequently higher rates of damage. Therefore, the observed effect of light and temperature on the rate of photoinhibition and recovery of reaction centres in overwintering trees could be also explained by this mechanism.

Interestingly, no consistent differences between k_P (proportional to [RC]) from exposed and shaded needles was observed during winter (Fig. 12b). An explanation could be that differences in connectivity between shaded and exposed needles interfere with [RC]. Namely, if needles exposed to full sunlight had higher connectivity compared to needles in the shade, [RC] would overestimate the real fraction of functional reaction centres to a higher extent than in shaded needles. However, plants grown in high light usually have smaller grana and less connectivity between PSII units compared to plants grown in low light (Lee et al. 2001), and not *vice-versa*. A more plausible explanation would be that the higher rates of thermal energy dissipation of excitation energy in exposed needles compared to shaded needles (Figure 12a) prevented further photoinhibition in exposed needles. The relative protection of regulative thermal energy dissipation mechanisms (or NPQ) on photoinhibition has been suggested to be only of secondary importance, conferring only 20-30% protection against photoinhibition (Tyystjärvi et al. 2005, Sarvikas et al. 2006). However, these studies were carried out under controlled conditions and with *Arabidopsis* as model, and might differ from overwintering field Scots pine. Extrapolation of these mechanisms from lab studies into the field has been debated (Matsubara and Chow, 2004). The NPQ mechanism induced in lab experiments is linked to the fast reversible pH-dependent and zeaxanthin mechanism, or to the more sustained reaction centre dissipation of thermal energy. In contrast, according to our results the mechanism of thermal dissipation in overwintering evergreens is largely related to the sustained zeaxanthin retention and structural changes in the thylakoid membrane. This mechanism may have significantly higher efficiency in thermal energy dissipation compared to that observed in *Arabidopsis*, conferring higher protection against photoinhibition.

Thermoluminescence studies with Scots pine seedlings under controlled conditions have recently indicated that thermal dissipation by damaged reaction centres could be enhanced in overwintering Scots pine (Ivanov et al. 2002, Sveshnikov et al. 2006). However, my results did not provide evidence on this process to a significant extent, since no inversely proportional correlation between k_P and k_{NPQ} was observed, as should be if damaged reactions centres significantly enhanced the levels of sustained thermal dissipation. Once more, the strong correlation between k_{NPQ} and the de-epoxidation state of the xanthophyll-cycle pigments suggested that zeaxanthin-facilitated thermal dissipation appears to be the main actor in the seasonal modulation of the thermal dissipation capacity in overwintering Scots pine in the field.

4.3 Integrating diurnal and seasonal acclimation of PSII: The New *MONI-PAM*

Estimating the energy partitioning in PSII through chlorophyll fluorescence data requires the use of a series of assumptions concerning the optical properties of the leaf sample. For example, the estimation of F_v/F_m can be done in a few seconds with a conventional field fluorometer, where the sample is fixed relative to the fluorometer, and the effect of the sample optical properties on the fluorescence parameter cancels out. In contrast, changes in the yield of thermal dissipation are commonly measured by comparing the maximum fluorescence level measured in the dark-acclimated state (F_m) with that measured at the light acclimated state (F_m'), for instance NPQ = (F_m/F_m') – 1 (Bilger and Björkman 1990).

Figure 13. Monitoring-PAM probe (*MONI-PAM,* Walz, Germany) attached with the Moni-PAM tree-holder (Topi Pohja, Hyytiälä) measuring chlorophyll fluorescence in Scots pine needles in the field. The instrument is able to measure exactly the same leaf area over a prolonged period of time extending from the diurnal to the seasonal time-scale.

Estimation of NPQ and estimation of the new parameters presented in STUDY II require that the optical properties of the sample remain constant during the measurement so that their effect cancels out. This largely limits the monitoring of acclimation of PSII in field conditions at the diurnal, and especially at the seasonal time-scale (Logan et al. 2007), since the same leaf area should be measured continuously in order to discriminate the effect of the optical properties of the sample. In STUDY II, we presented two new parameters to follow the seasonal acclimation in photochemical and non-photochemical processes in PSII. The parameters included the effect of seasonal changes in absorbance induced by the seasonal acclimation in pigment contents. Yet, special care was needed to continuously measure approximately the same leaf area throughout the monitoring period in order to be able to compare F_o and F_m data throughout the monitoring experiment.

Furthermore, estimating the energy partitioning in PSII in the field has been largely limited by logistic reasons. In the best of the cases, measurements of F_v/F_m every few days, have been combined with laboratory measurements of detached leaves, in an attempt to derive information on the seasonal acclimation from short-term measurements of PSII (cf. Ensminger et al. 2004). Field measurements of the energy partitioning in PSII under natural conditions and over long periods of time have not been feasible.

In **STUDY IV** we presented and tested the potential of a new monitoring-PAM (Moni-PAM) intended for long-term monitoring of the energy partitioning in PSII in the field, which overcomes the above mentioned technical and logistic limitations (Fig. 13). The instrument was able to record the prevailing fluorescence level F_t and the maximum fluorescence level after a saturating light pulse (F_m') every 5-10 minute intervals during several days. During the night, the prevailing fluorescence F_t can be assumed to correspond to the dark-acclimated F_o, and F_m' would correspond with F_m, allowing the estimation of

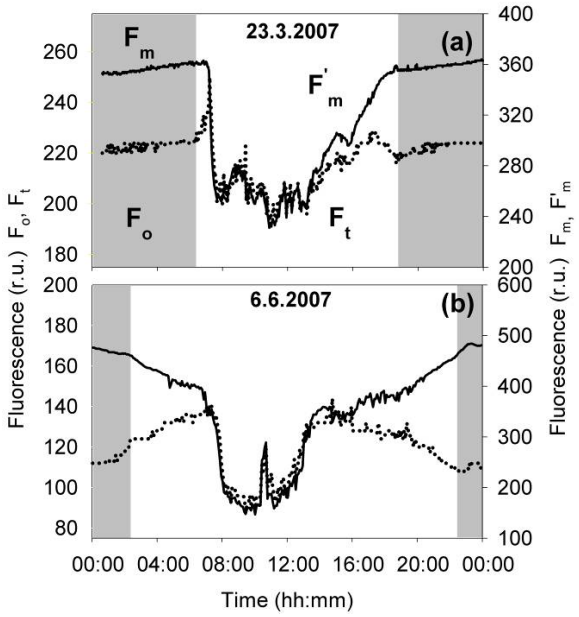


Figure 14. Diurnal changes in the maximum chlorophyll fluorescence intensity (F_m', or F_m during the night), and current chlorophyll fluorescence intensity (F_t, or F_o during the night), measured with *MONI-PAM* (Heinz Walz GmbH, Germany), before (a) and after (b) the spring recovery of photosynthesis in top needles of a Scots pine tree. Grey areas represent night time (ambient PPFD= 0).

F_v/F_m as well as facilitating the calculation of k_P and k_{NPQ} (Eqns 10 and 11). Interestingly, the data provide information ranging from the fast diurnal to the slow seasonal acclimation of PSII, which can be used to study the integrated effect of both diurnal and seasonal acclimation of the energy partitioning in PSII. For example, the results showed that during cold days in winter (Fig. 14a), F_t began to increase immediately after sunrise, whereas F_m' started to decrease approximately 45 min later, indicating that reduction in the electron transport chain occurs immediately after sunrise (increase in F_t), while the delay in the decrease in F_m' represents the time-lag in the activation of the pH-dependent and xanthophyll-cycle mechanism of thermal dissipation. In contrast, in summer, both the reduction of the electron transport chain and the activation of the pH- and xanthophyll-cycle dependent thermal dissipation took place immediately after sunrise (Fig. 14b). These differences in kinetics of the response of the acclimation in the thermal dissipation capacity between cold winter days and warm summer days is consistent with the structural changes taking place during spring recovery of photosynthesis discussed in STUDY II.

Finally, the fact that F_m' showed a diurnal pattern also during March, with lower noon values and recovery towards the evening (Fig. 14a) indicates that fast reversible thermal dissipation also participates in the overall acclimation of PSII before the spring recovery of photosynthesis, (note that temperatures reached 7°C during noon in the same day, and the diurnal pattern of F_m' might be much more flat at below zero temperatures). Therefore, both diurnal and seasonal acclimation mechanisms in PSII are intimately integrated.

I concluded that new technologies, such as the MONI-PAM presented here, will serve to facilitate the future understanding of the integration between diurnal and seasonal acclimation processes in field conditions, for example through the estimation of the new parameters developed in STUDY II.

5 CONCLUSIONS

In the four papers presented here I developed, tested and used new chlorophyll fluorescence tools intended to improve the study of the diurnal and seasonal acclimation of PSII in field conditions. The new model presented in STUDY I facilitated the study of the rapid short-term acclimation of PSII to changing illumination conditions. The model made possible to quantitatively study the effect that rapid fluctuations in light intensity had on the acclimation of PSII and the ETR, and I found out that the use of a steady-state approach to model the ETR may lead to over or underestimations under natural conditions in the field. At the longer seasonal time-scale, the new parameters developed in STUDY II improved the study of the seasonal acclimation of photochemical and non-photochemical processes in PSII, complementing in this way the extensively used F_v/F_m parameter. A key step towards this goal was the integration of the seasonal changes in light absorption into the interpretation of seasonal chlorophyll fluorescence data. The new parameters served to study the processes involved in the spring recovery of the photosynthetic light reactions and its kinetics, and I found that the xanthophyll-cycle appears to be the main mechanism used by Scots pine during winter and spring recovery to adjust the partitioning of energy in PSII, while the data supported the hypothesis that different levels of aggregation in protein complexes, induced by the light environment, were responsible for the differences in the kinetics of acclimation. These results have important implications at the crown level, where different parts of the crown present different degrees of acclimation and different kinetics of response to temperature. Finally, the new instrument presented in STUDY IV provided information on the combined effect of diurnal and seasonal acclimation of PSII, giving field evidence that diurnal acclimation operates on top of the seasonal acclimation to adjust to the prevailing excitation pressures found in natural conditions in the field.

In conclusion, the new tools presented in this Thesis were able to overcome existing major limitations to the interpretation of leaf-level chlorophyll fluorescence data. The new model, parameters, and instruments facilitate the study of diurnal and seasonal acclimation of photosystem II in field conditions.

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