I certify that the work presented in this thesis is my own, except for where acknowledged. The research was undertaken at the Research School of Biology, Australian National University.

This thesis does not incorporate without acknowledgment and appropriate references, any previously published material.

Alonso Zavaleta Fernández de Córdova
We can easily forgive a child who is afraid of the dark; the real tragedy of life is when men are afraid of the light.

Plato
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Credits

Even though a section such as this in the thesis is not very common, the author would like to give credit to the contributors and collaborators of the present work.

The overall thesis was overseen by Prof. Chow.

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Chapter 2 was directed by Prof. Shunichi Takahashi

Chapter 3 was directed by Dr. Michael Cheah (who helped me in designing the experiment and wrote the multicomponent analysis script) and by Prof. Hiroyuki Mino (whom mainly directed the writing of the Chapter).

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The initial idea of pursuing the project was from the late Prof. Warwick Hillier.
Publications

Here I certify that Alonso Zavafer and Alonso Zavaleta Fernandez de Cordova are the same person. I have adopted the name Alonso Zavafer for indexation purposes for on-line data bases. Here is a list of my current list of publications related to this thesis.


ABSTRACT

Light is required for photosynthesis to occur, but it also has a deleterious effect by damaging the photosynthetic machinery, in particular Photosystem II (PSII). Photoinhibition of PSII is a complex process, balancing between photoinactivation, protective and repair mechanisms. Current understanding of photodamage is limited to competing hypotheses where the photosensitizer is either photosynthetic pigments or the Mn₄CaO₅ cluster itself, with little consensus on the mechanisms and consequences of PSII photoinactivation. Using different spectroscopic techniques, the author evaluated the mechanisms of photodamage and photoprotection of PSII under visible light.

In the first Chapter a critical examination is presented of current evidence about the photodamage mechanisms, in particular that related to the action spectra of photodamage. The second Chapter examines if the damage to the Mn₄CaO₅ cluster is applicable to PSII under visible light as manganese absorbs visible light only weakly. The third Chapter was intended to determine which of the three main events of PSII photodamage occurs first: (1) Inactivation of the oxygen evolution; (2) inactivation of the PSII RC; (3) and release of Mn ions. The fourth Chapter evaluates whether quenching of excessive excitation does protect PSII from photodamage, as the recent rise of paradigms that advocate that photodamage is independent of excessive excitation has questioned the relevance of quenching mechanisms. Finally, the fifth Chapter discusses and integrates the result of the present thesis in the context of the current hypothesis to explain PSII photodamage.

The main results of this work are: (1) visible light damages Mn₄CaO₅ cluster prior to photodamage to the PSII reaction center; (2) the two step model under visible light is explained mostly by limitations on the acceptor side; (3) release of Mn ions is not the cause of PSII inactivation but a later consequence; and (4) the discovery of a long-lived fluorescence quencher that accumulates faster than the light-induced loss of PSII efficiency shows that the induction of this quencher is a preventive mechanism to reduce the excitation pressure and not the consequence of photodamage. Upon critical examination of the previously reported evidence and the new discoveries in the present work, the hybrid mechanism of photoinactivation, sensitized mostly by photosynthetic pigments and in minor way the Mn₄CaO₅, is the most plausible explanation to PSII photodamage under visible light.
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<td>$^1$O$_2$</td>
<td>Singlet oxygen</td>
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<td>$^3$P680</td>
<td>Triplet state of P680</td>
</tr>
<tr>
<td>BBY-Particles</td>
<td>PSII-enriched membranes</td>
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<td>Chl $\alpha$ fluorescence</td>
<td>Chlorophyll $\alpha$ fluorescence</td>
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<td>CR$^1$O$_2$</td>
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<td>Cyt b$_0$f</td>
<td>Cytochrome b$_0$f</td>
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<tr>
<td>DCPIP</td>
<td>Di-chlorophenolindophenol</td>
</tr>
<tr>
<td>DPC</td>
<td>Di-phenyl-carbazide</td>
</tr>
<tr>
<td>EA</td>
<td>Electron Acceptor (only used in Chapter 3)</td>
</tr>
<tr>
<td>ED</td>
<td>Electron Donor (only used in Chapter 3)</td>
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<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence Lifetime Imaging Microscopy</td>
</tr>
<tr>
<td>$F_0$</td>
<td>Fluorescence Intensity when all RC are open in PSII</td>
</tr>
<tr>
<td>$F_V/F_M$</td>
<td>Maximum efficiency of PSII</td>
</tr>
<tr>
<td>LHC</td>
<td>Light-harvesting complex that serves either photosystem I or photosystem II</td>
</tr>
<tr>
<td>Mn$_4$CaO$_5$</td>
<td>Manganese calcium cluster</td>
</tr>
<tr>
<td>NPQ</td>
<td>Non-photochemical quenching</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>OEC</td>
<td>Oxygen Evolving Complex</td>
</tr>
<tr>
<td>P680</td>
<td>Pigment Centre of PSII</td>
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<td>PC</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>Pheo</td>
<td>Pheopythin</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PPBQ</td>
<td>Phenyl-para-benzoquinol</td>
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PSI      Photosystem I
PSII     Photosystem II
QA       Quinone A
QB       Quinone B
qE       Energy-dependent quenching
qI       Photoinhibitory quenching
qT       State transition quenching
qZ       zeaxanthin-dependent quenching
RC       Reaction centre
ROS      Reactive Oxygen Species
T50      Half lifetime
TCSPC    Time Correlated Single Photon Counting
Tyr      Tyrosine Z
WT       Wild Type
Yz       Tyrosine Z
τAV      Fluorescence Average Lifetime
Chapter 1: INTRODUCTION TO LIGHT INDUCED DAMAGE TO PHOTOSYSTEM II

*The information of the present text has been published in Zavafer et al. 2015 a*

1.1. Photosynthesis

Oxygenic photosynthesis is the most important biochemical process on planet Earth. At a global scale, it sustains most ecosystems; it is the source of the oxygen we breathe and it enables the CO₂ fixation reactions (Berkner and Marshall, 1965, Falkowski, 1994). Photosynthetic reactions are some of oldest metabolic pathways, presumed to have appeared around 2500 million years ago, just after fermentation reactions (Hartman, 1998). After oxygenic photosynthesis had appeared, the Earth’s atmosphere changed, giving rise to the present 21% oxygen, the ozone layer and to respiratory metabolic pathways (Berkner and Marshall, 1965, Falkowski, 1994, Blankenship and Hartman, 1998). Later, during the carboniferous, photosynthesis activity was so high that it gave rise to all the biomass from which fossil fuels originated (Beerling et al., 1998).

Photosynthesis could be defined as the conversion of solar energy into biochemical energy (Blankenship and Hartman, 1998). It can be categorized in two types: anoxygenic and oxygenic photosynthesis. The difference depends on whether oxygen is produced as a by-product of photosynthetic reactions (Blankenship and Hartman, 1998). This thesis focuses only on oxygenic photosynthesis; therefore, for convenience in this text, oxygenic photosynthesis will be addressed simply as photosynthesis, unless otherwise stated. Photosynthesis occurs in most cyanobacteria, algae and higher plants, although some species have lost this capacity (Young and Wolfe, 1997).

The light phase of photosynthesis occurs at the thylakoid membrane (Anderson et al., 1988). The chemical composition of the thylakoid membrane is highly conserved in all plants, algae and cyanobacteria (Rutherford and Faller, 2003). Photosynthesis begins at the reaction centres of protein complexes termed Photosystems (Blankenship and Hartman, 1998). In the reaction centres the absorbed light is transformed into chemical energy. A special pigment molecule in each reaction centre donates an electron to the primary acceptor (Hillier and Messinger, 2005). In oxygenic photosynthesis there are two types of photosystems, Photosystem I and II (PSI and PSII respectively) (Blankenship and Hartman, 1998).
PSI and PSII work in an independent but cooperative manner (Trebst, 1974). Each photosystem has an internal electron transport chain and light harvesting antenna complexes (Horton and Ruban, 2005, Trebst, 1974). The antenna complexes are believed to enhance the light absorption of photosynthesis (Horton and Ruban, 2005). Since both photosystems perform their activity in tandem, it is generally described as a linear process. After electrons are pumped from PSII, a mobile pool of plastoquinone (PQ) ensures electron transport between the photosystems. This occurs when reduced PQ donates the electrons to a protein complex called cytochrome b_{6}f (Cyt b_{6}f) (Trebst, 1974). At Cyt b_{6}f electrons flow that contributes to the formation of a proton gradient that can be used by the ATP synthase for the synthesis of ATP (Steinberg-Yfrach et al., 1998). Also, Cyt b_{6}f donates the electrons to a water soluble molecule called plastocyanin (PC) which acts as primary electron donor to PSI (Gorman and Levine, 1965).

Like PSII, PSI donates an electron, using the energy of light, to a series of acceptors inside its structure. Outside PSI, the electrons are transferred to the ferredoxin (Fd) which then transfers the electrons to NADP oxidoreductase, which reduces the molecule NADP^{+}, the end acceptor of the electron transport chain (Anderson, 1981). Alternatively, electrons can be diverted from ferredoxin to Cyt b_{6}f in what is called the cyclic electron transport in order to accumulate more protons for ATP synthesis (Heber and Walker, 1992).

However, photosynthesis is intrinsically a suicidal process (Van Gorkom and Schelvis, 1993, He and Chow, 2003, Chow et al., 2005) since all exposure to light will eventually cause inhibition of the photosynthetic machinery generally termed photoinhibition (Melis, 1999). Photoinhibition encompasses a series of processes that will be addressed in depth in the next sections in this Chapter.
Figure 1.1 Diagram of the thylakoid membrane and the energy cascade. The arrows represent the fluxes of energy for the electron transport chain or for the protons. PQ represents the mobile pool of quinones, LHC represents light harvesting complexes (for PSII LCHII and for PSI LHCI). CF₁ and CF₀ represent domains of the ATP synthase. OEC represents the oxygen evolving complex or water splitting site. Note that PSII usually locates in the stacks and the OEC is oriented towards the lumen. Image taken and modified from Atwell et al. (1999)

1.2 The history of photoinhibition

The detrimental effect of light on plants is a long recognized process. The first scientific reports about photoinhibition could be found in 1896 (Ewart, 1896, Powles, 1984). For the first half of the 20th century reports of photoinhibition focused mostly on the effect over the biomass (Powles, 1984). It was not until 1956 that Bessel Kok (Kok, 1956) published a report where he proposed the term photoinhibition. Ten years later Jones and Kok (Jones and Kok, 1966a, 1966b) published the first action spectrum of photoinhibition in spinach thylakoid membranes. In their studies they observed a very strong peak in the UV region, while the action spectrum between 400 to 700 nm resembled more the absorption spectrum of photosynthetic pigments.
After these two discoveries it was clear that the most affected parts of the photosynthetic machinery during photoinhibition were related to the light reactions (Powles, 1984, Melis, 1999). In 1984, it was finally discovered that PSII was the most affected part during photoinhibition (Kyle et al., 1984). In fact, Kyle et al. identified that a subunit of PSII, the D1 protein, was degraded during photoinhibition. This suggested that the primary site of damage was located in the PSII reaction centre. After the discovery of Kyle et al., many groups focused their energies on understanding the photoinhibition process in detail between the late 80’s and early 90’s (Melis, 1999a). Thanks to that research the three main events of photoinhibition are known in great detail: (1) PSII photoinactivation, (2) photodamage and (3) the repair of the PSII complex (see Figure 1.2). Also it is well accepted that photoinhibition is increased when photosynthetic organisms are under environmental stress such as salt, heat, dehydration, etc (Takahashi and Murata, 2008).

1.3 The Plastoquinone:Water oxidoreductase (Photosystem II)

Before examining in the controversial topic of photoinhibition it is necessary to understand the structure and function of PSII. PSII is an enzyme responsible for the water splitting reaction (Hillier and Messinger, 2005). It is arguably the most important enzyme on the planet Earth (only to be compared with another photosynthetic enzyme such as RUBISCO). PSII provides the initial steps of the linear electron transport of photosynthesis and it is the enzyme responsible
for the evolution of oxygen, which is a side product of the water splitting reaction (Wydrzynski and Satoh, 2006).

**Figure 1.3 Diagram of PSII.** (a) The PSII components are positioned according to their redox potentials (modified from Pearson Education, Inc.1). (b) Diagram of the photosystem II monomer. Each letter labels one of the PSII subunits (Taken from Wei-Zhong He & Richard Malkin REF2)

PSII is well conserved in cyanobacteria, algae and higher plants, in contrast to other photosynthetic complexes (Rutherford and Faller, 2003), such as the antennae (Green and Pichersky, 1994). The generally accepted hypothesis is that PSII originates from the bacterial reaction centre type II (Blankenship and Hartman, 1998) present in some autotrophic prokaryotes that perform anoxygenic photosynthesis. PSII is a large protein complex formed by more than 20 subunits and 99 cofactors, having an estimated size of 350 kDa (Shen, 2015).

Over the last two decades several studies have been conducted in order to resolve the three-dimensional structure of PSII (Umena et al., 2011, Kamiya and Shen, 2003, Shen, 2015). Based on those studies the basic parts that comprise the PSII or PSII core are known. Due to its complexity, the active site of PSII has been dissected into three main regions: acceptor side, donor side and reaction centre (Williamson et al., 2011). The compositions of these three regions vary according to researchers; the following terminology is based on Wydrzynski (2008).

The photosynthetic reactions of PSII start at the reaction centre. As its names states, the reaction centre is where the primary photosynthetic reaction takes place. The basic components in the reaction centre are a special “pair” of chlorophylls termed P680 (based on

---

1 http://www.uic.edu/classes/bios/bios100/lectures/10_14_the_Z_scheme-L.jpg
2 http://www.life.illinois.edu/crofts/bioph354/images/psii_cartoon.gif
their absorption peak in the red region, which is actually 682 nm), a molecule of pheophytin (Pheo), a dimer of subunits D1 and D2 and Cyt b559. The reaction starts when excitation occurs at P680, either by direct absorption of light or by energy transferred from the photosynthetic antennas. The excitation induces what is called **photoinduced charge separation**: when the P680 is excited (P680*), its redox potential is so negative that it can donate an electron to the primary electron acceptor Pheo. This first event starts the process of energy conversion between light energy into chemical energy.

The reduced Pheo is a highly reactive species that donates its electron to what is called the acceptor side (Klimov, 2003). The **acceptor side** comprises primarily the two plastoquinone electron acceptors of PSII, QA and QB. First the electron travels from the Pheo molecule to QA, which is a bound quinone located at the D2 protein. In normal conditions, QA is singly reduced and it passes its electron to QB. By contrast, it is believed that QB is a mobile molecule that resides at the D1 protein and it requires two electrons in order to proceed forward in the electron transport chain. Upon reduction, $Q_B^{2-}$ picks up two protons from the stroma; $Q_BH_2$ then diffuses from its binding site to join the pool of PQ molecules, which are free to diffuse within the thylakoid membrane.

It is important to mention that historically QA has been considered the primary electron acceptor (Duysens and Sweers, 1963) in the PSII instead of Pheo (Klimov, 2003). This is because the average lifetime of Pheo$^-$ is relatively short compared to that of QA (Klimov, 2003). Furthermore, it is the redox state of QA that determines whether the reaction centre trap is open (QA available to be reduced) or closed (QA unavailable to be reduced) (van Kooten and Snel, 1990). This is particularly important because the ability to reduce QA is what determines the reaction centre activity (**RC activity**).

The immediate consequence of **primary photochemistry** (charge separation and electron transport) in PSII is a deficit of electrons at P680$^+$. This is compensated by the electron donation on the **donor side** (Hillier and Messinger, 2005, Wydrzynski, 2008). The donor side is composed of the Mn$_4$CaO$_5$ cluster and a reactive tyrosine (Y$_z$). The ultimate electron donor is the water molecule that is split at the Mn$_4$CaO$_5$ cluster. The exact mechanism for water oxidation is still not known; however, it is known that the by-product of this is molecular oxygen and protons and that four oxidising equivalents need to be accumulated at the Mn$_4$CaO$_5$ cluster. The accumulation of these four equivalents occurs during four transitions between different redox configurations at the Mn$_4$CaO$_5$ cluster termed **S-state transitions**. (Hillier and Messinger, 2005, Dau and Haumann, 2008) (Figure 1.4). Once the four equivalents are accumulated a molecule of water is split and the electrons are transferred to P680, via Y$_z$. 
Figure 1.4 The Joliot-Kok’s S-State cycle in the OEC. The arrows represent the transitions between each of the S states. Between two consecutive steps the lifetime for each transition is shown. The photons required for each transition and the products at each S-state are also shown. (Taken from Dau and Haumann (2008)).

The structure of the Mn₄CaO₅ cluster is of vital importance in relation to photoinhibition as will be explained in the next section. The consensus is that the Mn atoms are linked to the proteins by coordination bonds termed oxo-bridges (Umena et al., 2011). The exact link between the proteins and the atoms is still a matter of debate because each S state has different redox state for each of the four Mn atoms and there is no universal consensus on what is the configuration in each state (Cardona et al., 2015).

In sensu stricto, the reaction centre (RC), the primary electron acceptor Qₐ, the Mn₄CaO₅ and the Y₂ comprise the oxygen evolving complex (OEC) (Wydrzynski, 2008). Without any of this, no oxygen evolution can occur. However, in most works in the literature not specialized in the OEC, the term OEC is applied just to the Mn₄CaO₅ cluster itself (for example, Ohnishi et al. (2005), Murata et al. (2007), Takahashi and Murata (2008), Murata et al. (2012) ). In this thesis the former will be used; however, the OEC activity would be referred only to the capacity to evolve oxygen and therefore the activity on the donor side. The combined reaction at the RC and OEC will be termed Total PSII activity.
Figure 1.5 3D structure of the OEC based on crystal structure obtained by Umena et al. (2011)

All of these PSII components have been proposed as possible primary sites of PSII damage or related to the mechanisms of inhibition. In the following section this is discussed in greater detail.

1.4 Terminology

Photoinhibition, photodamage and photoinactivation have been used interchangeably in the past by different authors. There is no consensus on the meaning of the terms and in every field (in example ecology, physiology, biophysics, etc.) the concept means different things (for a discussion see Campbell and Tyystjärvi (2012)). However, some authors make a clear distinction between photoinhibition, photoinactivation and photodamage (Melis, 1999, Murata et al., 2007, Takahashi and Murata, 2008). Following the recommendations of Campbell and Tyystjärvi (2012), in this text each of the concepts will be defined and all evidence presented in this thesis will be evaluated by this conceptual frame work.

1.4.1. Photoinhibition

Photoinhibition is the most commonly used in the literature. It has been used to name the loss of photosynthetic activity, the loss of PSII activity, PSII damage, etc. In this work photoinhibition is defined as the phenomenon in which net photosynthetic activity is lost due
to a collection of different processes that are induced by light. This is based on the etymological Greek (phot – light) and Latin (inhibitio – forbidden) or restricted which translates as prohibited by light (Zavafer et al., 2015a). Based on Osmond and Grace (1995), two types of photoinhibition are recognized: *dynamic* and *chronic*. Dynamic photoinhibition is defined as the loss of maximum efficiency as result of dynamic changes of the photosynthetic apparatus; they are reversible and do not imply any damage. Chronic photoinhibition is the photoinhibition as a result of direct damage of the photosynthetic machinery. It is clearly observable when the rate of photodamage is higher than the rate of photorepair. It corresponds to the loss of photosynthetic activity observed in the presence of a photorepair mechanism.

### 1.4.2. Photoinactivation

In the present text photoinactivation is defined as loss of photosynthetic activity due to process or processes that is induced by light. This is based on the literal translation from Greek-Latin that means “cannot do because of light”.

### 1.4.3. Photodamage

Photodamage is another term that has been used as a synonym of photoinhibition. Since this term implies damage, it is more narrowly defined as loss of photosynthetic activity, where chemical modification of the photosynthetic apparatus is observed. Photodamage is considered as a subset of photoinactivation processes. This damage is irreversible and can only be compensated for by photorepair. It always occurs in the background of photosynthesis. The chemical modifications are caused by light itself such as photobleaching, photooxidation or photoreduction. In previous works, photodamage has been used to call the process of D1 protein degradation (Rintamäki et al., 1996, Haußühl et al., 2001, Aro et al., 1993a). Since D1 degradation is catalysed by an enzyme (Kato and Sakamoto, 2009) and it is not a direct effect of light but a consequence of the PSII damage (Aro et al., 1993a), D1 degradation will not be considered as photodamage. This process can only be measured upon inhibition of repair mechanisms using protein synthesis inhibitors such as lincomycin and chloramphenicol (Melis, 1999, Murata et al., 2007).

### 1.4.4 Photodamage efficiency

The photodamage efficiency is defined as the parameter used to determine the degree of damage of PSII. Several parameters have been described in the literature to address how efficiently photodamage is occurring. Probably the most commonly used parameter is the rate
constant of photoinhibition ($k_{pi}$), which is defined as the rate coefficient for the exponential loss of PSII activity. It is widely accepted that under the inhibition of photorepair, $k_{pi}$ is directly proportional to the PSII damage. Derivative parameters to $k_{pi}$ that have been used are the quantum yield of photoinactivation ($\Phi_{pi}$) defined as the initial number of PSII centres inactivated per unit time when repair is blocked, divided by the quanta incident on the sample per unit time (flux) ($J_i$). Other parameters and derivatives have been used to measure the efficiency of photodamage; Campbell and Tyystjärvi (2012) offer a comprehensive review about the topic.

1.4.5 Photorepair

The process of repair of a photodamaged photosystem will be termed photorepair. In the case of PSII this involves the active process of degradation of D1 protein, the synthesis of a new protein and its assembly back to the functional state (Kato and Sakamoto, 2009). An additional step in photorepair, which must be considered, involves the re-assembly of the Mn₄CaO₅ cluster, but since this process is independent of ATP, it would be addressed as photoassembly (Hoganson and Babcock, 1988).

1.4.6 Photoprotection

Photosynthetic organisms have developed strategies to cope with photoinhibition or photodamage. These strategies are referred to as photoprotective mechanisms. There is still a lot of controversy as to whether these mechanisms protect from photodamage or only from photoinhibition (by protecting photorepair); further, its extent of protection has been questioned (Takahashi and Badger, 2011). Nevertheless, it is well accepted in the field that the photoprotective mechanisms relieve some of the effects of light-induced stress. Three supergroups of photoprotective mechanisms could be found: (1) macroscopic, (2) microscopic and (3) molecular. Macroscopic effects encompass mechanisms such as leaf movement and phototropism (positive or negative) (Huang et al., 2014). Microscopic mechanisms are the ones related to chloroplast movement (Kong and Wada, 2014). Molecular mechanisms comprise a diverse series of strategies such as synthesis of filtering compounds such as in the case of UV photoinhibition (Hakala-Yatkin et al., 2010), synthesis of pigments that filter out visible light (Niyogi, 1999), molecular rearrangement of the thylakoid membrane (Iwai et al., 2014), production of scavengers of reactive oxygen species (ROS) (Niyogi, 1999) and the so-called non-photochemical quenching (NPQ) (Horton and Ruban, 2005).
1.4.6.1 Non-photochemical Quenching

Traditionally, NPQ is defined as dissipation of excessive light energy (Horton et al., 1996). Phenomenologically, it is the quenching of the chlorophyll α fluorescence that is not caused by primary photochemistry (Ruban et al., 2012). It is widely believed that this is an intrinsic photoprotective mechanism based on the fact that quenching of fluorescence reflects the dissipation of excited states of the chlorophylls (since chlorophyll fluorescence is a consequence of excitations) (Horton et al., 1996, Horton and Ruban, 2005, Ruban et al., 2012). It comprises a large collection of different processes involving the photosynthetic antenna complexes (Demmig-Adams and Adams lll, 1992). Not all photosynthetic organisms present the same type of NPQ mechanism (Niyogi, 1999), but all NPQ mechanisms involve: the generation of a proton gradient, the migration of the photosynthetic antenna (state transitions) and the de-epoxidation of violaxanthin to form zeaxanthin (the xanthophyll cycle). It has been assumed that NPQ has several components, the most important one being the quenching of excitation energy associated with energization of the thylakoid membrane by the proton gradient (qE) (Amarnath et al., 2012).

1.5 The problems of PSII photodamage

Photodamage of PSII has been one of the most controversial topics in the field of photosynthesis research (Vass, 2012, Tyystjärvi, 2008). To date there has not been conclusive evidence about: (1) the role of excessive light energy, (2) the primary site of damage, (3) the mechanism of PSII damage and (4) the action spectrum of photodamage. Even though there have been claims from (Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Nishiyama and Murata, 2014) that there is only one mechanism of photodamage, most groups still remain unconvinced (Tyystjärvi, 2008, Oguchi et al., 2011a, Oguchi et al., 2011b, Vass, 2011, Tyystjärvi, 2012, Vass, 2012, Schreiber and Klughammer, 2013). Some authors have suggested that photodamage is a cooperative process of multiple mechanisms interacting together (Oguchi et al., 2011a, Oguchi et al., 2011b, Tyystjärvi, 2012, Vass, 2012, Schreiber and Klughammer, 2013).

The only conclusions that have been accepted by all groups (Melis, 1999, Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Oguchi et al., 2011a, Oguchi et al., 2011b, Vass, 2011, Tyystjärvi, 2012, Vass, 2012, Schreiber and Klughammer, 2013) are the following:
• The rate coefficient of photoinhibition ($k_{PI}$) for PSII is directly proportional to the irradiance

• One PSII is inactivated after approximately $10^7$ photons have been absorbed by the tissue

• UV light induces more damage than any wavelength of the visible spectrum

• UV light has two targets: primary effect on the Mn$_4$CaO$_5$ cluster and alterations at the acceptor side of PSII.

In contrast to UV photodamage, visible light photodamage remains controversial and there is disagreement among different authors over the last 25 years of research. In the next sections each of the unresolved questions of photoinhibition will be examined in detail. It is important to mention that in the present thesis only reports where there is distinctive photodamage (absence of photorepair) are considered (see section 1.4.5). This is because studies where only photoinhibition was monitored are difficult to interpret. Also, only photodamage induced by the visible spectrum (400 – 700 nm) will be considered, since UV photodamage mechanisms are well resolved phenomena (Tyystjärvi, 2008, Tyystjärvi, 2012, Vass, 2012).

1.6 Is excessive light energy absorbed by pigments important for PSII photodamage?

The role of excessive light energy is probably the most important question concerning PSII photodamage. Excessive light energy absorbed by photosynthetic pigments refers to the amount of light absorbed by PSII that is not used in primary photochemistry (Melis, 1999, Ruban et al., 2012). Traditionally, it has been long accepted that excessive energy absorbed by photosynthetic pigments is the cause of PSII photodamage (Powles, 1984, Chow, 1994, Melis, 1999, Ruban et al., 2012). According to this hypothesis, photodamage should be minimal below the saturation point of irradiance and significantly higher above the saturation (Melis, 1999) as shown in Figure 1.6. Therefore, some sort of exponential or sigmoidal curve are to be expected when $k_{PI}$ is plotted as a function of irradiance.

The excessive light energy theory cannot be supported by the abundant experimental evidence of different groups that $k_{PI}$ versus irradiance shows proportionality (Jones and Kok, 1966a, Mattoo et al., 1984, Tyystjärvi et al., 1994, Park et al., 1995, Tyystjarvi and Aro, 1996, Baroli and Melis, 1996, Park et al., 1996, Lee et al., 2001, Nishiyama et al., 2001, Allakhverdiev and Murata, 2004, Santabarbara et al., 2002, Hendrickson et al., 2005). There is a reciprocal relationship between duration of illumination and irradiance in the PSII (Nagy et al., 1995). As a consequence this means that what is important is the number of photons absorbed and not the rate of their absorption (Atwell et al., 1999, Tyystjärvi, 2008, Tyystjärvi, 2012).
Figure 1.6 Excessive light energy concept. (a) The concept of excessive light energy. (b) Possible results of \( k_p \) vs light intensity (irradiance). The black line corresponds to the situation where the photodamage depends on the light dosage (photon exposure), not on the irradiance per se. The blue line represents the expected behaviour if excessive light energy were the cause of PSII damage. The magenta curve corresponds to multiple mechanisms acting at the same time independently. The yellow line corresponds to strong cooperativity during PSII photodamage. Panel A is taken from (Ruban et al., 2012)

In order to explain this behaviour two hypotheses have been put forward: (1) The first one proposes that photodamage is independent of excessive light energy, since linearity is interpreted as simple probability of a photon to inactivate a PSII irrespective of the rate of photosynthesis (Hakala et al., 2005, Tyystjärvi, 2008, Tyystjärvi, 2012). Additionally, further evidence (Nishiyama et al., 2001, Nishiyama et al., 2006, Murata et al., 2007, Murata et al., 2012) has suggested that the excessive light energy is an important factor for the inhibition of the PSII repair only. (2) The other hypothesis states that the linearity may be explained by excessive light energy since the mechanism in low and high light are different (Tyystjärvi, 2008, Tyystjärvi, 2012, Vass, 2012); this is possible because of a joint effect of two distinct mechanisms of charge recombination and singlet oxygen (\( ^{1}\text{O}_2 \)) mediated photodamage, (Vass and Cser, 2009, Vass, 2011, Vass, 2012). Each of these hypotheses proposes different primary sites of photodamage and mechanisms that will be explained in the following sections.
1.7 Where is the primary site of PSII photodamage by visible light?

The primary site of damage is the second most important question in the photodamage of PSII. The primary site of damage refers to the component of the PSII complex that is damaged first and that triggers the subsequent photodamage sequence. In this case two regions of PSII have been proposed as the primary sites of photodamage.

The most accepted damage site has been the acceptor side; e.g. some of the papers published in the last 10 years with more than 50 citations (Zhou et al., 2007, Wilhelm and Selmar, 2011, Dai et al., 2009, Heber et al., 2006, Cruz et al., 2005, Ahn et al., 2008). Note that all of these papers are from groups that are not researching directly in PSII photodamage but they accept the acceptor side model. The acceptor side hypothesis was one of the first ones to appear. This was mainly suggested by the results of Kyle et al. (1984) that show that the D1 protein was degraded during photoinhibition. Since the Q_B site is located in this area, it has been long accepted that photodamage induces chemical modifications that are irreversible on the acceptor side. A large body of literature suggests that under high light irreversible modifications may occur at Q_A (Aro et al., 1993b, Vass and Aro, 2008, Melis, 1999, Ohad et al., 1990, Vass et al., 1992, Macpherson et al., 1993, Keren et al., 1997, Šetlík et al., 1990) and Q_A level (Kyle et al., 1984, Vass et al., 1992, Vass and Aro, 2008, Melis, 1999, Ohad et al., 1990). As a result of these modifications, Q_A and Q_B cannot be reduced and then the activity of the RC is impaired. Interestingly this hypothesis is compatible with the idea that excessive light energy does cause photoinhibition, since damage occurs in conditions where electron transport is impaired. This has been supported mostly in isolated systems such as BBY particles and thylakoid membranes (Tyystjärvi, 2008, Vass, 2011, Tyystjärvi, 2012, Vass, 2012) and in some in vivo systems such as Chlamydomonas cells (Ohad et al., 1990) and Tobacco mutants (Allahverdiyeva et al., 2005).

The other candidate is the donor side (not to be confused with the donor side mechanisms that will be reviewed in the next section). This hypothesis suggests that chemical modification occurs first at the level of the Mn₄CaO₅ cluster (Hakala et al., 2005, Ohnishi et al., 2005), and then it is followed by the loss of PSII RC activity. This hypothesis has been demonstrated to be true especially for UV light, while it is still controversial for the visible light spectrum (Tyystjärvi, 2008, Vass, 2012). Strong evidence has been presented by two independent groups that clearly showed that under the blue portion of the light spectrum there is more damage in thylakoid membranes of pumpkin (Hakala et al., 2005) and thermophilic cyanobacteria (Ohnishi et al., 2005). However, the evidence for this hypothesis has been inconclusive in the red regions of the visible spectrum.
According to Hakala et al. (2005) damage to the Mn cluster is accompanied by the release of 1 Mn atom per cluster into the lumen. This is also supported by the convergence of the UV-blue region photoinhibition peak seen in several systems and the absorption spectrum of model Mn compounds (Hakala et al., 2005, Hakala et al., 2006, Wei et al., 2011). Furthermore, enzymes with Mn centres and Mn compounds suffered chemical changes (photodamage) when illuminated with light in the UV-blue region (Hakala et al., 2006). Also, several works (Ohnishi et al., 2005, Nishiyama et al., 2006, Murata et al., 2007, Murata et al., 2012, Tyystjärvi, 2008) suggested that the damage to the Mn₄CaO₅ cluster is the only compatible explanation of the linearity of \( k_{pi} \) versus irradiance. Even though this site seems to be the dominant component of photodamage in the blue region of the visible spectrum, this still does not explain what happens under red illumination (Tyystjärvi, 2008).

A third hypothesis has suggested that there are two sites of photodamage: the donor and the acceptor site (Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b, Schreiber and Klughammer, 2013, Vass, 2012). Depending on the experimental conditions, one site or the other would be the dominant; both mechanisms could act independently or cooperatively. In the next section, each one of the mechanisms associated with these three hypotheses will be explored in greater detail.

1.8 What is the mechanism of PSII photodamage?

In the literature eleven possible mechanisms have been reported to explain photodamage to either the donor or the acceptor side. Each of these mechanisms offers some degree of explanation of the experimental data, but no single model has been able to explain all results in the literature. For convenience, the eleven mechanisms are divided in two categories: Excitation pressure dependent and independent (see Figure 1.8). The main difference between the two groups is whether excessive light energy does or does not cause photodamage.
Figure 1.7 Diagram of the available mechanisms of PSII based on the excitation pressure. On the right hand side in red is presented their number (Latin characters) and in parenthesis the year when they were proposed originally. In blue is presented the primary site of damage that each mechanism proposes.

1.8.1 Excitation pressure-dependent hypotheses

The excitation pressure dependent group can be divided in three subgroups: Acceptor side limitations, Donor side limitations and the “revised charge recombination and \textsuperscript{1}O\textsubscript{2} mediated model”. In the acceptor side limitation mechanisms, great emphasis is placed on the fact that all the energy that is not used in primary photochemistry due to limitations in the electron transport (on the acceptor side) is the cause of damage (Murata et al., 2007, Tyystjärvi, 2008, Vass, 2012). This group of mechanisms have been the most accepted in the field (Aro et al., 1993b, Vass and Aro, 2008, Melis, 1999, Ohad et al., 1990, Vass et al., 1992, Macpherson et al., 1993, Keren et al., 1997, Šetlík et al., 1990, Kyle et al., 1984, Zhou et al., 2007, Wilhelm and Selmar, 2011, Dai et al., 2009, Heber et al., 2006, Cruz et al., 2005, Ahn et al., 2008).
1.8.1.1. Acceptor side limitations

Mechanism (i) was one of the first ones to be proposed (Vass et al., 1992, Vass and Styring, 1993). It centres on the idea that when PSII is illuminated under strong light in anaerobic conditions, QA remains largely reduced and can be stabilized by double reduction or protonation. The phenomenological consequences are the increase in the F₀ value of Chl a fluorescence and the inhibition of O₂ evolution (Kirilovsky and Etienne, 1991, Vass et al., 1992, Hundal et al., 1990, Sundby and Schiött, 1992). The rise of F₀ has been interpreted as accumulation of protonated singly and doubly reduced QA or PSII that has lost QA (Vass et al., 1992). This model has been demonstrated to occur in PSII-enriched membranes (BBY-particles), which are fragments of thylakoid granal membranes with digested top, bottom, lateral and stromal sections. Since this model occurs in the absence of oxygen, it is unlikely to occur under natural conditions (Tyystjärvi, 2008).

Mechanism (ii) is an extended version of (i) where in aerobic conditions the doubly reduced QA (QAH₂) does not accumulate (Vass et al., 1993) and assumes that photodamage PSII occurs by accumulation of ¹O₂ produced after the stable formation of QA⁻ (Hideg et al., 1998, Hideg et al., 2001) (Hideg and Vass, 1996, Spetea et al., 1997). Clear evidence for this mechanism has not been presented; however, this could be supported by the fact that there is ¹O₂ production in leaves (Hideg et al., 1998, Hideg et al., 2001) and thylakoid membranes (Hideg and Vass, 1996, Spetea et al., 1997) when they are photodamaged and the production rate depends on the extent of photodamage. On the other hand, a clear link between ¹O₂ and photoinactivation is still missing (Tyystjärvi, 2008). Furthermore, according to Nishiyama et al., ¹O₂ does not cause photodamage but it is implicated in the inhibition of photorepair (Nishiyama et al., 2004, Nishiyama et al., 2006). However, the ¹O₂ observed by Nishiyama et al. (2004) was artificially generated outside complete cells. In such conditions it is likely that a highly reactive species such as ¹O₂ may be consumed by reacting with other cell components. Furthermore, according to (Macpherson et al., 1993) the ¹O₂ that inactivates the PSII is generated inside the PSII core and it is not accessible for ¹O₂ scavengers.

Mechanisms (i) and (ii) are valid if, and only if, the illumination of the sample is above the PSII saturation level. So, these mechanisms do not explain why photodamage occurs at low light intensities.

Mechanism (iii) Keren et al. (1995) offered an explanation based on the idea that recombination reactions of the acceptor side with the S-states S₂ and S₃ are inversely proportional to the light intensity. This recombination leads to ³P₆₈₀ (triplet P₆₈₀) that reacts
with oxygen to generate $^3$O$_2$. Then $^3$O$_2$ oxidizes the machinery in the RC. This is circumstantially supported by the fact that the efficiency of D1 degradation increases towards low light intensities. However, this result diverges from the well-known first order kinetic behaviour, since their results obtain mostly show a zero order kinetic. In the opinion of Tyystjärvi (2008) this exaggerates the observed effects.

Mechanism (iv) Song et al. (2006) is the only mechanism that suggests that limitations on the acceptor side affects the Mn$_4$CaO$_5$ cluster. This mechanism has been observed in BBY-particles that have been exposed to strong light (1000 µmol photons m$^{-2}$ s$^{-1}$). In this mechanism limitation on the acceptor side favours the accumulation of superoxide (O$_2^-$) and because O$_2^-$ can interact with components of the thylakoid membrane to produce H$_2$O$_2$. Either O$_2^-$ or H$_2$O$_2$ may diffuse to the Mn$_4$CaO$_5$. In the same manner described by the CR$^3$O$_2$ model (see section 1.8.1.3 below). The recombination oxidizes the Mn cluster inducing Mn release. Once the Mn is released the mechanism of inactivation follows the donor side limitation model (see next section). Mechanism (iv) proposes an alternative to the direct absorption on the Mn, still dependent on excessive light. However, Song hypothesis does not match with the well-known fact that among all ROS, H$_2$O$_2$ and O$_2^-$ are the most stable ROS (Apel and Hirt, 2004). Mechanism (iv) has not received a lot of attention in the field and it has not been included in any of the reviews of photodamage (Tyystjärvi, 2008, Tyystjärvi, 2012, Vass, 2012) since its publication.

All acceptor side limitation mechanisms are not compatible with the linearity of $k_{pi}$ vs irradiance, since $k_{pi}$ vs irradiance should behave in a nonlinear way.

![Figure 1.8 Mechanism (iv) hypothesis. (Taken from Song et al. (2006))]
1.8.1.2 Donor side limitations

Excessive light energy hypotheses comprise the second group of hypotheses based on excitation pressures. Only two models are considered in this group and these mechanisms primarily depend on the absence or inactivation of the donor side.

Mechanisms (v) was the first mechanism to be proposed (Callahan and Cheniae, 1985, Callahan et al., 1986). It was demonstrated in BBY-particles where the Mn₄CaO₅ was impaired by TRIS treatments (Theg et al., 1986), and where artificial electron donors such as di-phenyl-carbazide (DPC) (Klimov et al., 1990) or NH₂OH (Blubaugh et al., 1991, Chen et al., 1992) protect the RC activity from damage. P680 is still functional in the absence of the Mn₄CaO₅. In such conditions, it can perform charge separation; however, this will induce the accumulation of highly oxidizing radicals such as P680⁺ and Y₂⁺ that can react with the surrounding environment, impairing the RC activity (Jegerschöld and Styring, 1996). This model is valid only if the Mn₄CaO₅ cluster is removed first; therefore, it lacks importance under natural conditions. However mechanisms (iv, vi, vii, viii) use it to explain the successive damage to the RC activity of PSII. This mechanism may have an impact in nature when photosynthetic organism are subject to temperature stress (above 40 °C) (Takahashi and Murata, 2008) or contamination by heavy metals (Pagliano et al., 2006, Pätsikkä et al., 2001).

Mechanism (vi) uses elements of mechanism (v) to translate it to in vivo systems (Anderson et al., 1998). It is known that during the S -state transitions, not all the OEC’s transition to next state under single-turnover flashes (Joliot and Kok, 1975, De Wijn and Van Gorkom, 2002). Since charge separation occurs even in the absence of transitions between S states, in a small percentage of PSII P680⁺ is accumulated (Christen et al., 1999). If P680⁺ lives long enough it oxidizes its environment via the mechanism (v). Tyystjärvi (2008) suggests that evidence for the stochastic donor-side inhibition would be saturation by light in the same fashion that light saturates photosynthesis; therefore this would not explain linear response of kₚᵣ vs irradiance. However, this is not necessarily true, since the miss-hits are a stochastic process, and charge separation is dependent on chlorophyll absorption. Both are not limited by irradiance; therefore, linearity is to be expected. However, the main obstacle for this model is the action spectrum of photodamage which seems to favour mostly the blue region of visible spectrum.

1.8.1.3 Charge recombination and ¹⁰⁰₂ mediated model (CR²O₂) revisited

Mechanism (vii) has been proposed by Vass and Cser (2009) and was termed the revised charge recombination and ¹⁰⁰₂ mediated mechanism (CR²O₂). This mechanism does not only
consider acceptor-donor side limitations but also the formation of the recombining charge pairs driven by photosynthetic pigments. For the CR$^1$O$_2$ mechanism several events of direct recombination occur as shown in Figure 1.9 and it is based on observations where photodamage is mediated by ROS produced via charge recombination. Charge stabilization by secondary electron transport plays an important role in this process, affecting the yield of different species at each step. Competing with the charge recombination, spin inversion may yield either $^3$Chl in the antenna or $^3$P680$^{+}$Pheo$^{-}$/ $^3$P680, thereby inducing $^1$O$_2$ formation. As these charge recombination events occur at all light intensities (Keren et al., 1995, Keren et al., 1997) and formation of $^1$O$_2$ is proportional with light intensity (Vass, 2012, Rehman et al., 2013), this is in agreement with the linear light intensity dependence of $k_{pi}$. This shows that not only the Mn-dependent mechanisms but also the pigment dependent mechanism can explain the linear light intensity dependence.

![Figure 1.9 Mechanism (vii) of charge recombination-mediated photodamage. Taken from Vass (2011)](image)

The accumulation of $^1$O$_2$ would damage the PSII proteins, pigments and surrounding lipids. Also, this mechanism takes into consideration the possibility that changes in the oxidation state on both acceptor and donor sides would affect PSII photodamage (Krieger-Liszkay and Rutherford, 1998, Fufezan et al., 2007). Even though this model explains various observations concerning photoprotection via non-radiative charge recombination pathways (Vass 2012), it does not explain the action spectrum of photodamage. However, Vass (2012) proposed that
perhaps photodamage is joint effect of mechanisms of Mn hypotheses and charge recombination operates during PSII damage.

1.8.2 Excitation pressure-independent hypotheses

In this category two subgroups can be found. The first one suggests that the event that triggers photodamage is the inactivation of the Mn\(_4\)CaO\(_5\) cluster. In the other group, exogenously generated-ROS are the cause of photodamage. Both ideas are independent of the excessive light energy but they remain the less accepted models in the field.

1.8.2.1 Direct absorption of light by the Mn (The Mn hypothesis)

**Mechanisms (viii)** reported by Hakala et al. (2005) suggests that the Mn atoms are capable of absorbing light and the Mn cluster becomes inactivated. This process is independent of excessive light energy absorbed by photosynthetic pigments and it only depends on the probability of a photon deactivating a Mn\(_4\)CaO\(_5\). This mechanism was based on the evidence that when a sample is exposed to light, the activity of the RC (measured by electron transport between DPC to di-chlorophenolindophenol or DCPIP) is affected less than the total activity of the PSII (measured by electron transport from H\(_2\)O to DCPIP). This result was especially true for wavelengths in the UV and blue region. By comparing the action spectrum of photoinhibition against the absorption spectrum of the Mn model compounds (Hakala et al., 2005, Hakala et al., 2006), it was assumed that the Mn cluster is affected by direct absorption of light. The absorption of light disrupts one of the Mn oxo bridges at the cluster, releasing one Mn into the lumen (Hakala et al., 2005). The Mn release is the cause of the OEC inactivation, which is followed by donor side limitation. In the original interpretation the authors suggested that this is the only model that can explain the action spectrum of photoinactivation of PSII and linearity of the k\(_{PI}\) vs irradiance. The proposed mechanism of inactivation is that photons are absorbed by the Mn, which induces an excitation of Mn-oxo bridges that breaks the coordination bond. This was later supported by a second action spectrum at higher resolution by Sarvikas et al. (2006) and the result that other Mn compounds and enzymes suffered the same type of inactivation (Hakala et al., 2006). Later Wei et al. (2011) published an independent study in which the compounds capable of water oxidation suffered inactivation by light. This photoinactivation of model compounds was accompanied by chemical modifications as observed in the absorption spectrum.

**Mechanisms (ix)** was reported by Ohnishi et al. (2005) independently of Hakala et al. (2005). Here the Mn also absorbs light and becomes inactivated, with the difference that they
describe a two-step damage. After UV and blue light had inhibited the OEC, light in other regions of the visible spectrum would inhibit the RC activity by donor side limitations. Another difference in this work is the experimental material, which consisted of thylakoid membranes of a thermophilic cyanobacterium. Another important feature is that, in the (Ohnishi et al., 2005) work, there is no distinction in damage between Mn cluster and RC beyond 530 nm, which does not explain what is occurring in samples illuminated with red light at high irradiances.

Even though mechanisms (viii) and (ix) are very similar, they differ in the action spectrum. While Hakala et al. (2005) propose that this is the universal trigger of PSII photodamage, Ohnishi et al. (2005) assume that this is only valid for wavelengths closer to Mn absorption peak in the UV. However, later reviews (Nishiyama et al., 2006, Murata et al., 2007) have acknowledged the Mn hypothesis as the main mechanism of photoinhibition. Furthermore, in Murata et al. (2007) they have reported that it is widely accepted. Nevertheless, there is no clear evidence of what is occurring at wavelength between 530 to 700 nm, where clearly photoinhibition occurs even if this is of lesser magnitude (Tyystjärvi, 2012, Vass, 2012, Hou and Hou, 2013, Schreiber and Klughammer, 2013).

The Mn hypotheses (viii and ix), if they are true, may explain the linearity and the action spectrum. However, they lack direct spectroscopic evidence that shows that the Mn atoms can absorb light more efficiently than the photosynthetic pigments (Zavafer et al., 2015a). Also, it has been ignored by some of the authors that the probability of light absorption by the Mn clusters is far smaller than the probability of absorption by antennas and RCs together (Berg et al., 2002). Mn compounds present extinction coefficients in the visible region lower than photosynthetic pigments; this makes it difficult for photons to excite the Mn complex efficiently (Zavafer et al., 2015a). Another limitation of the Mn model is that the energies of the red region are not high enough to disrupt an oxo bridge. In order to disrupt a Mn bond it is necessary at least to overcome the energy of the coordination bonds of the Mn to the PSII protein scaffold (Meyer and Caspar, 1985). This is clearly possible for UV wavelengths as demonstrated by multiple groups (Tyystjärvi, 2008, Wei et al., 2011, Vass, 2012, Tyystjärvi, 2012, Hou and Hou, 2013), but for a red photon that has less energy, it is highly unlikely.

Another strong argument against the Mn hypothesis has been made by Vass (2012), where he states that protection mechanisms against direct absorption of the Mn should be present as a response to environmental adaptation. This is clearly the case for UV protection mechanisms (Hakala-Yatkin et al., 2010) present in photosynthetic organisms, but according to Vass (2012) protection against direct damage to the Mn cluster by visible light has not been found.
The evidence that clearly shows that the redox potential of Qa affects photodamage suggests that the Mn hypothesis may not be the only photoinhibition mechanism (Idedan et al., 2011). It is possible that in regions close to the UV (400-410 nm) a small amount direct absorption by Mn may contribute to PSII damage, but this cannot occur in the peak region in the absorption of the chlorophylls (420 nm upwards).

1.8.2.2. Exogenously generated ROS

This mechanism has been reported by two independent groups. Both groups suggest that damage to the PSII could occur by production of ROS exogenously generated.

**Mechanism (x)** by Jung and Kim (1990) proposes that $^{1}\text{O}_2$ is produced in the iron-sulphur centres (Fe-S centres) and cytochromes of the thylakoids. This has been done by comparing the action spectrum of inhibition with action spectrum of $^{1}\text{O}_2$ production. The hypothesis is backed up by the fact that blue and UV-A-light-induced photoinhibition is enhanced in plants grown with excess iron, where there is increase of the non-heme iron content in the chloroplast. After the publication of mechanism (x), Chung and Jung (1995) published another paper stating that the main $^{1}\text{O}_2$ source in thylakoid membranes are the Fe-S centers of PSI. However, this is contested by data published by Hideg and Vass (1996) where they demonstrated that the $^{1}\text{O}_2$ producing capacity of PSI is negligible when compared to that of PSII.

**Mechanism (xi)** by Santabarbara et al. (Santabarbara et al., 2002, Santabarbara et al., 2001b, Santabarbara et al., 2007) also proposes that $^{1}\text{O}_2$ is produced outside PSII. It is based on the results that the red peak of the action spectrum of photoinhibition is blue-shifted by 2-4 nm compared to the absorption spectrum of PSII. A similar blue shift was found in thylakoid membranes isolated from the chlorophyll b-less mutant of barley. However, in the opinion of (Tyystjärvi, 2008) it also resembles the chlorophyll phosphorescence peak which suggests that triplet formation may be related to the PSII damage.

1.9 What is the action spectrum of PSII photodamage?

To date 12 action spectra of photodamage have been reported (Jones and Kok, 1966a, Jung and Kim, 1990, Santabarbara et al., 2001b, Santabarbara et al., 2002, Hakala et al., 2005, Ohnishi et al., 2005, Sarvikas et al., 2006, Takahashi et al., 2010, Schreiber and Klughammer, 2013, Karim et al., 2015, He et al., 2015). All of them coincide in the fact the strongest photodamaging effect is located in the UV. However, in the visible part of the action spectrum all results are rather different. The difference can be found not only in the spectral profile
(peak positions) but in the relative damage at each wavelength. If there were a single mechanism of photodamage the results in the visible parts of the action spectrum should be very similar (like what happens in the UV regions). In Figures 1.10 to 1.14 all reported spectra are compared: It can clearly be observed that all action spectra are different. A review in depth of all technical aspects of action spectra of PSII photodamage is available at (Zavafer et al., 2015a). For practical reasons, only a summary will be presented here.

In order to facilitate the direct comparison between spectra, the twelve action spectra are separated in Figures 1.10 to 1.14. All data are normalized to their own efficiency of photodamage (PDE) value between 425 to 430 nm. Figure 1.10 shows the action spectra of samples illuminated in suspension, samples that share the same type of light harvesting complexes (LHC-type). The first action spectra reported by Jones and Kok (1966a), was obtained in isolated chloroplast from spinach. By measuring O$_2$ evolution, photodamage was estimated by the quantum yield of inactivation. The spectrum of Jones and Kok (1966a) clearly shows some resemblance to the absorption of photosynthetic pigment specially between 420 to 700 nm; nevertheless, some authors have claim that this action spectrum does not follow the pigment absorption, suggesting that it follows more the Mn hypothesis (Ohnishi et al., 2005, Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Murata et al., 2012, Hakala et al., 2005, Hakala et al., 2006, Sarvikas et al., 2006, Tyystjärvi, 2008).

Figure 1.10 Photoinactivation action spectra of Jones & Kok, Jung & Kok, Hakala et al. and Klughammer & Schriber. Jones & Kok (1966) red triangles; Jung & Kim (1991) blue circles; Hakala et al. (2005) green squares; Schriber & Klughammer (2013) inverted black triangles. Normalised absorptance spectrum of spinach thylakoids, grey area. All action spectra adapted from respective references and
normalised around the 420 nm region. Inset: Action spectra plotted from 350 to 700 nm; arbitrary units on the vertical axes are the same as on the main axis.

The second action spectrum reported in the literature was published by Jung and Kim (1990), who used isolated chloroplast from spinach as an experimental model and Chl a fluorescence as a technique, and the photodamage was estimated by $k_{PI}$. Their action spectrum is rather dissimilar to that of Jones and Kok (1966a). The Jung and Kim (1990) spectrum (Figure 1.10) presents a clear band in the green region and a much smaller band around the 670 nm. The interpretation of Jung and Kim (1990) was that since the action spectrum did not follow the pigment absorption, the photodamage was caused by another photosensitiser. Furthermore, in their research they observed that the $^{3}$O$_{2}$ production spectra and the action spectra matched almost identically. Therefore, they suggested that the origin of PSII photodamage is not in the PSII itself (see mechanism xi).

In 2005, Hakala et al. (2005) published a series of low resolution action spectra as supportive material for the Mn hypothesis. Hakala et al. (2005) (Figure 1.10) used as an experimental model thylakoid membranes isolated from pumpkin. They used oxygen evolution measurements and the photodamage was estimated using $k_{PI}$ values. It can be observed that beyond 450 nm, very little difference can be observed between the different wavelengths. The result of Hakala et al. (2005) action spectrum would mean that beyond 450 nm (upwards), photodamage is only dependent on a photon reaching, with low efficiency, the photosensitiser, which in this case is the Mn atoms. Also in the Hakala et al. (2005) work three more action spectrum of photodamage are presented. The first one is to compare anoxygenic conditions, which resembles almost identically the control condition suggesting that the doubly reduced Q$_{A}$ mechanism was not in operation (mechanism i). Furthermore, they presented two other action spectra of the $k_{PI}$ for total PSII activity and RC activity, showing that while RC photoinactivation follows the pigment profile, the total PSII photoinactivation is independent of the light absorbed by pigments.

In contrast to the Hakala et al. (2005) and Jung and Kim (1990) action spectra, Schreiber and Klughammer (2013) published an action spectrum of photodamage where two clear peaks are present (Figure 1.10). In their approach, Schreiber and Klughammer (2013) used whole cells of Chlorella for their study and they estimated the photodamage as the relative decrease in $F_{v}/F_{M}$. While photoinactivation was only reported for five discrete wavelengths, the study took into account an important aspect highlighted by the authors: the action spectrum of PSII photoinactivation and the action spectrum of PSII turnover were measured under identical conditions, with same spectral resolution which facilitates direct, quantitative comparison
between the two. Two observed peaks were interpreted as two different photosensitisers: the Mn atoms primarily absorb in the blue region and the other sensitiser in the RC absorbs primarily in the red region of the visible spectrum. However, among all action spectra, this is the one with the lowest resolution and the two peaks are not well resolved.

The highest resolution action spectra ever reported corresponds to the works of Santabarbara et al. (Santabarbara et al., 2001b, Santabarbara et al., 2002), but they only cover the regions between 630 to 720 nm. The results presented in Figure 1.11 correspond to the so-called red band. The first action spectrum published by (Santabarbara et al., 2001b) showed a well resolved blue-shifted band (approx. 4 nm) from the absorption band at 680 nm. The experiments were done in isolated thylakoid membranes of the barley wild type (WT) & chlorina f2 mutant, using fluorescence measurements to estimate the photodamage based on $k_p$. Santabarbara's work showed that in chlorina f2 barley mutants the action spectrum changes substantially. This could mean that the action spectrum of photodamage is affected by chlorophyll absorption but not the chlorophyll absorption of the PSII. This is explained because the two action spectra do not match exactly with the 680 nm absorption band. Nevertheless, antenna absorption may be implicated in PSII photodamage. Later work from Santabarbara et al. (Santabarbara et al., 2001b, Santabarbara et al., 2002) suggests that a population of loosely coupled chlorophylls are related to the PSII damage, and not chlorophylls in PSII itself. However, the position of this peak is difficult to identify in other works due to their low resolution. For example, in the study of Jung and Kim (1990) the peak occurs at 665 nm, while in that of Jones and Kok (1966a) it occurs at 678 nm (see Figure 1.10). Experiments done by Santabarbara et al. (Santabarbara et al., 2001b, Santabarbara et al., 2002) are very important in order to resolve the action mechanism and the identity of a possible photosensitiser in the red region of visible spectra. It also has the highest resolution action spectrum ever reported.
In leaves of plants, three action spectra has been reported, two studies have been done on *Arabidopsis* by two independent groups (Sarvikas et al., 2006, Takahashi et al., 2010) and a third one has been done in spinach leaves (He et al., 2015). The first one published by Sarvikas et al. (2006) is a continuation of the work done by Hakala et al. (2005). They used a whole leaf of *Arabidopsis* treated with lincomycin to inhibit D1 protein synthesis, using oxygen evolution measurements after isolation of thylakoids post illumination as a technique to determine the photodamage by kPI. The observed action spectrum showed a strong feature below 450 nm; above 450 nm, the action spectra are relatively featureless. By comparison with excitation spectrum of Chl a fluorescence, the authors concluded that while there is a superficially good fit in the red region of the visible spectrum, other photosensitisers are strongly implicated due to poor fit at shorter wavelengths, in particular the UV region. Furthermore, the authors suggest that the Mn₄CaO₅ cluster is a photosensitiser based on comparison with absorption spectra of model compounds of the Mn₄CaO₅ cluster. In addition, in the study of Sarvikas et al. (2006) a comparison is presented between different NPQ mutants. The results clearly show that NPQ does not affect significantly the action spectrum of photoinhibition. The results also show that the NPQ deficient mutants are between 15-25% more susceptible towards photoinactivation in visible light.
The action spectrum reported by Takahashi et al. (2010) is in comparison flatter than that of Sarvikas et al. (2006) (Figure 1.12). Below 420 nm the photodamage increases compared to the portion between 420 to 700 nm. Furthermore, a broad positive band in the yellow-orange region of the visible spectrum is observed. This would mean that orange portion of the visible spectrum is more important than the red region to induce photodamage. However, some technical issues may affect the results in this work: (1) in comparison to other works, the photodamage was estimated only from two points (control and value of $F_v/F_m$ after damage), (2) the light intensity was extremely low at each wavelength (being between 1 to 6 µmol photons m$^{-2}$ s$^{-1}$), (3) the amount of damage measured was around only 5% of the total activity, and (4) light intensity and spectral characteristics changes across the day and on environmental conditions. If it is true that Takahashi’s action spectrum offers novel insights about the importance of each wavelength of the solar spectrum, the normalization used by Takahashi et al. (2010) to obtain the photodamage may not be ideal since it does not take in consideration equalization of electron transport or the use of the same light intensity. In other words the conditions were not the same.

The third action spectrum reported for leaves correspond to the work of He et al. (2015) who reported the action spectra of wild type and chloropyll b-less barley leaves (Figure 1.12).
Photoinactivation treatment was conducted on lincomycin treated leaf segments. PSII activity was assayed by measurement of redox kinetics of P700. The action spectra indicate a strong feature below 423 nm that is more than an order of magnitude higher compared to weak features at wavelength longer than 600 nm. The Chlorophyll b-less mutant did exhibit a slightly more pronounced feature in the red region compared to the wild type as expected based on the photosynthetic pigment paradigm. However, the overall action spectrum seems to indicate that photoinactivation of PSII proceeded mainly via the Mn₄CaO₅ cluster paradigm. The authors suggested that the apparent discrepancies between the action spectra of barley leaf and isolated thylakoids reported by Jones and Kok (1966) (Figure 1.10) may be due to extent to which electron transport is permitted.

The action spectrum of two strains of dinoflagellates from *Symbiodinium* has been obtained recently (Karim et al. 2015) (Figure 1.13). Both strains showed highest inhibition below 420 nm and then a minor increase in the $k_{PI}$ at 680 nm. It is interesting that Karim et al (2015) interpreted their data as two different mechanisms of photodamage. According to their interpretation, strain CP-164 presents only Mn-mediated damage exclusively but CCMP2459 presents a dual mechanism. Based on the comparison of Figure 1.13, the normalized action spectra of both strains are similar. Since they did not calculate the action spectrum itself or the $k_{PI}$, their interpretation was rather different to the one presented in this thesis. The only difference between these two strains may be in the degree of resilience to photodamage.
Figure 1.13 Photoinactivation action spectra of Symbiodinium. Photoinactivation action spectrum of Symbiodinium phylotypes CCMP2459 (red triangles); CS-164 (blue circles) and absorptance spectrum of phylotypes CCMP2459 (solid line); CS-164 (dotted line). Figure generated based on data of Karim et al. 2015; a single exponential fit is used to obtain $k_{PI}$ and normalised based on point around 425 nm. Inset: Action spectra plotted from 350 to 700 nm; arbitrary units on the vertical axes are the same as on the main axis.

Finally, Ohnishi et al. (2005) spectrum is presented separately in Figure 1.14; this is an action spectrum where the sample did not have LHC-type proteins but phycobilisomes. The Ohnishi et al. (2005) action spectrum was done by measuring the total PSII activity by electron transport from $\text{H}_2\text{O}$ to DCPIP in thylakoid membranes isolated from Themosynechococcus elongatus. The photodamage was estimated based on the $k_{PI}$ for different wavelengths. Similar to Hakala et al (2005), Ohnishi et al. (2005) observed a predominant effect in the blue region, followed by green and finally by red light. Also, they observed that the RC inactivation spectrum follows the pigments, while the inactivation of the total PSII activity did not.
At least five independent reports have identified a positive band in the red region, suggesting that the direct absorption of the photosynthetic pigments cannot be completely ignored in photodamage. As seen so far, the action spectra of PSII photodamage are very diverse and difficult to interpret. The differences between all action spectra may be due to growth conditions of the sample, techniques used to monitor photodamage, parameters to estimate the photodamage and temperature of the experiments. Another significant obstacle to interpreting the accuracy of the action spectrum is the wavelength resolution. Most action spectra consist of a few points across 300 nm of the visible spectrum. However, based on the technology available it is difficult to generate very narrow monochromatic light with high irradiance.

One should bear in mind that the action spectrum of PSII photodamage is a consequence of the primary site of damage and its mechanism. Since 2005 some action spectra of photodamage have been used as definitive proof of the Mn hypothesis by some groups (Ohnishi et al., 2005, Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Murata et al., 2012) by assuming all action spectrum are similar. However this generalization is a misconception as can be observed in a comparison of Figures 1.10 to 1.14. The reader will notice that each action spectrum is not well resolved and no consensus exists (Tyystjärvi, 2008, Vass, 2012, Hou and Hou, 2013).
According to Mn hypotheses supporters (Ohnishi et al., 2005, Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Murata et al., 2012, Hakala et al., 2005, Hakala et al., 2006, Sarvikas et al., 2006, Tyystjärvi, 2008) all action spectra are similar and since the action spectra do not match exactly the absorption of photosynthetic pigments, therefore excessive light energy does not explain photodamage. However, in most of these studies, UV and visible wavelengths are presented together in the same spectrum which eclipses the changes occurring in the visible region. If the spectra contain only visible wavelengths, it can be observed that all action spectra are rather different especially when compared between 420 nm and 700 nm. By comparing all actions spectra, it seems logical to believe that below 420 nm the predominant mechanism seems to be following the Mn hypothesis due to its similarity to the absorption spectra of Mn model compounds (Hakala et al., 2005, Hakala et al., 2006, Wei et al., 2011, Hou and Hou, 2013). However, for the rest of visible light the Mn hypothesis may not be the most suitable explanation.

It is clear, however, from all studies (Jones and Kok, 1966a, Jung and Kim, 1990, Hakala et al., 2005, Ohnishi et al., 2005, Sarvikas et al., 2006, Takahashi et al., 2010, Schreiber and Klughammer, 2013) that at the borderline between UV and visible photodamage there is an increase in magnitude of photodamage compared to the red region of the spectrum.

In nature, the contributions of UV damage to the PSII are rather minimal because of two factors: UV is less abundant in the solar spectrum that reaches the earth in comparison with visible and blue light is not as intense compared to green, yellow and red light (Takahashi et al., 2010). As presented by Takahashi et al. (2010), in nature the photodamage in the region between 490 to 700 nm of visible light is more important for photosynthetic organism than the photodamage in the blue region.

Based on current evidence, absorption of light by photosynthetic pigments as a cause of PSII damage cannot by fully discarded but neither can the role of the Mn hypothesis. Even though there have been claims from some groups (Ohnishi et al., 2005, Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008, Murata et al., 2012) that all action spectra are the same and they do not follow the photosynthetic pigment absorption, the fact that 8 of the 12 spectra show at least some degree of visible wavelength dependency, makes a strong argument for reviewing the role of damage. Indeed this has motivated some groups to propose hybrid theories.

In particular the experiments of (Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b) have shown strong evidence to support these claims by using light of different wavelengths.
This is very important especially in relation to studying photodamage in leaves (Oguchi et al., 2011a). Since light absorption in leaves is much more complicated than in solution samples, it is expected that the photodamage that occurs in the top, middle and bottom layer of the chloroplast would also be rather different.

1.10 ROS and photodamage

To conclude this Chapter, the role of ROS as a key factor of PSII photodamage is considered. As previously presented, for the acceptor-donor side limitations models, ROS play an important role as the chemical agents which cause damage to PSII. Studies in vitro clearly have shown that ROS can damage PSII (Knox and Dodge, 1985, Tjus et al., 2001) and that the presence of ROS scavengers slows down photodamage (Richter et al., 1990, Barényi and Krause, 1985, Tschiersch and Ohmann, 1993). By contrast, not all in vivo studies have been able to show the absolute importance of ROS during photodamage. The two studies of Nishiyama et al. (Nishiyama et al., 2001, Nishiyama et al., 2004) gave the strongest evidence that ROS is not important for PSII photodamage. Nishiyama et al. (2001) presented evidence that ROS play an important role only in inhibiting the photorepair mechanism; in particular, their interpretation was that the hydroxyl radical originating from H\textsubscript{2}O\textsubscript{2} may be the ROS responsible for inhibition of the de novo D1 synthesis. From the data of Nishiyama et al. (2001) it can be observed that increasing the concentration of superoxide and peroxide by addition of methyl viologen or exogenously supplied H\textsubscript{2}O\textsubscript{2} did not accelerate PSII photodamage. However, it is interesting that to determine oxygen evolution activity after photodamage the sample was exposed to 1 mM 1,4-benzoquinone which is a rather high concentration. It would be expected that cells would not require an artificial electron acceptor, as reported by (Price et al., 2008, Price and Badger, 1989), nevertheless the samples were supplied with one. Additionally, the exogenously supplied H\textsubscript{2}O\textsubscript{2} in complete cells may have several sites of action before it reaches the PSII.

In mechanisms dependent on excitation pressure \textsuperscript{1}O\textsubscript{2}, in theory, plays an important role in photodamage. The second work from Nishiyama et al. (2004) focuses on \textsuperscript{1}O\textsubscript{2} instead of the other ROS. Apparently, under their experimental conditions, \textsuperscript{1}O\textsubscript{2} did not accelerate photodamage when the \textsuperscript{1}O\textsubscript{2} was supplied exogenously to intact cells by rose Bengal photosenzitation. However, \textsuperscript{1}O\textsubscript{2} is an extremely reactive species, so it is likely to have interacted with other cellular structures before reaching PSII. Therefore, it is logical to think that if \textsuperscript{1}O\textsubscript{2} is produced outside the cell, the probability that it interacts with PSII is very much lower; hence no difference should be expected. In other words, the experiments of Nishiyama et al. (2004) only demonstrated that exogenous ROS did not accelerate photodamage.
The work of Nishiyama et al. is contested by a series of recent results which demonstrate the correlation of photodamage with the rate of $^{1}\text{O}_2$ production in different cyanobacterial mutants (Rehman et al., 2013, Bersanini et al., 2014, Hakkila et al., 2014, Sedoud et al., 2014). These findings support the important role of pigment driven ROS production in photodamage of PSII, and should be included in the discussion.

Ohnishi et al. (2005), Nishiyama et al. (2006), Murata et al. (2007) and Murata et al. (2012) have claimed that the evidence of Nishiyama et al (1) demonstrates that ROS does not cause photodamage and (2) supports the Mn hypothesis. However, authors like Tyystjärvi (2008), Tyystjärvi (2012) and Vass (2012) have found other explanations to this discrepancy. In the opinion of Tyystjärvi (2008), the apparent effect of ROS in vitro is due to concentration. While in in vivo systems the ROS concentration is maintained low in chloroplasts and cyanobacteria by the natural scavenger mechanisms, in vitro this concentration reaches higher values, therefore causing damage. By contrast, Vass (2012) argued that according to the extended model of CR$^{3}\text{O}_2$ model, $^{1}\text{O}_2$ can induce damage, especially if it is generated inside the PSII core, where no other scavenger may reach it. In the CR$^{3}\text{O}_2$ Vass and Cser (2009), Vass (2011) model the production of $^{1}\text{O}_2$ is dependent exclusively on closing of the RC which linearly relates with the light intensity.

This discrepancy highlights the difficulty in examining the role of ROS in PSII photoinactivation as quenching of ROS or excited state pigments has a significant effect on the net generation of ROS.

1.11 Objectives of the present thesis.

At this stage, the author hopes that the reader will appreciate the great complexity and controversy that surrounds the topic of PSII photodamage. Despite intensive research on this topic, the ultimate causes and mechanism(s) of PSII photodamage remains elusive. Up to date, the two main ideas to explain PSII photodamage, excessive energy and direct damage to the water splitting site, require further investigation and validation.

For that reason, the author has dedicated his efforts to scrutinize the current ideas about photodamage. The author has done a series of experiments using BBY-particles to answer three questions:

1.- Which one is the primary site of light induced damage in PSII?

2.- Is the Mn$_4$CaO$_5$ cluster affected by all visible light?
3.- What is/are the molecular mechanism(s) by which PSII gets inactivated?

Furthermore, the topic of PSII photodamage has important implications for the study of photoprotection mechanisms, in particular those associated with the dissipation of excessive excitation (NPQ). NPQ has been widely accepted as one of the main photoprotection mechanisms. However, this putative physiological role is only valid if PSII photodamage is caused by excessive excitation. For this reason, the author has also explored the kinetic properties of quenching of excitation in relation to PSII photodamage (in vivo and in vitro) to elucidate if quenching does or does not protect the PSII from photodamage.

In order to do so, the author has combined a diverse series of spectroscopic tools (optical spectrometry, electron paramagnetic resonance, chlorophyll fluorescence and picosecond time resolved fluorescence) and interpreted the obtained results in the context of the current paradigms that explain photodamage.
2.1 Introduction

As mentioned in the previous Chapter, two main hypotheses have been proposed to explain PSII photodamage. (1) The acceptor-donor side limitation model proposes that excessive light energy absorbed by photosynthetic pigments leads to damage of PSII; this limitation may occur either on the acceptor or the donor side (Vass et al., 1992). (2) The Mn model suggests that the loss of photosynthetic activity is caused by the direct absorption of light by the \( \text{Mn}_4\text{CaO}_5 \) cluster, which is independent of the excessive energy absorbed by photosynthetic pigments (Hakala et al., 2005, Ohnishi et al., 2005).

The Mn model has been accepted to happen if PSII samples are exposed to UV or blue regions of the visible spectrum (Hideg and Vass, 1996, Jansen et al., 1998, Hideg et al., 2002, Zsiros et al., 2006, Tyystjärvi, 2008). This model has been supported by the light absorption of Mn-oxo model compounds at high-energy wavelengths (Wei et al., 2011), proposing that the Mn absorbs light and gets damaged. In fact, there are reports showing that under UV illumination \( \text{O}_2 \) evolution stops and that Mn is released into the buffer (Zsiros et al., 2006). In the original hypothesis of Ohnishi et al. (2005) photodamage happens in two steps: first Mn is inactivated by blue light, then light absorbed by chlorophyll damages the RC. The RC damage is subsequently due to limitations on the donor side.

However the Hakala et al. (2005) and Ohnishi et al. (2005) models do not explain why photodamage occurs in red light as well. Some authors (Schreiber and Klughammer, 2013, Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b) have proposed a hybrid model where two possible sensitizers exist during photodamage. One of the sensitizers is the \( \text{Mn}_4\text{CaO}_5 \) cluster that gets inactivated by blue light, while the photodamage in the green to red regions of the visible spectrum is initiated by light absorption by Chl molecules exclusively.

Solid evidence to demonstrate whether there is a single predominant mechanism of PSII photodamage or two parallel ones is still missing. Also, there is a controversy in establishing where the primary site of photodamage is located (Tyystjärvi, 2012, Tyystjärvi, 2008, Vass and Aro, 2008) (Vass, 2012, Vass, 2011). Therefore, the interest of the present work is to explore the damage of (1) the total photosynthetic electron transport rate and (2) RC activity, after exposure to strong light in the entire visible spectrum. To do so, BBY-particles were subjected
to strong illumination in the presence or in the absence of an artificial electron donor and acceptor, in order to differentiate if limitation on the acceptor side or donor side plays an important role in photodamage. Also, the effect of different types of polychromatic light is studied in order to investigate what type of damage occurs in blue, green and red parts of the visible spectrum.

2.2 Materials and Methods

2.2.1 Sample preparation

O2 evolving BBY-particles were prepared from fresh market spinach as described by (Berthold et al., 1981). Then the sample was suspended in standard buffer, flash frozen in liquid nitrogen (LN2) and stored at −80 °C until used. Before light exposure, the sample was thawed and resuspended in a pH 6.5 buffer containing 40 mM Mes and 40 mM sucrose (Buffer A). Then it was centrifuged at 16000 × g for 5 minutes and resuspended again in Buffer A. The chlorophyll concentration was measured according to (Porra et al., 1989) and adjusted to 150 µg Chl mL−1 (this will be termed a BBY-particles stock suspension). The sample was kept in darkness and 4 °C at all times unless stated otherwise.

2.2.2 Photodamage assays

Before illumination, a BBY-particles stock suspension was transferred to plastic petri dishes. The total volume depended on the optical pathlength for irradiance treatment, which was 2.5 mm from top (surface of the liquid) to bottom. In the case of the experimental group involving an electron acceptor, phenyl-para-benzoquinone (PPBQ) was added at a concentration of 0.6 mM. As an electron donor, di-phenyl-carbazide (DPC) was added to give a final concentration of 10 µM. When a mixture of the acceptor/donor was required, both chemicals were added at same respective concentrations as above. Since PPBQ and DPC were dissolved in dimethyl-sulfoxide (DMSO), the control group was supplemented with DMSO at a concentration of 0.5% by volume.

For white light illumination, a halogen Benq MX503 XGA projector was used (for emission spectrum, see Figure 2.1). For illumination at different wavelengths a xenon lamp model MAX-303 (Asahi Spectra Co. Ltd, Japan) was used (emission spectrum in Figure1). Short and long pass filters were used to obtain the following wavelengths intervals: Blue 385-510 nm, Green 510-590 nm and Red 590-710 nm. All illuminations were done at 4 °C.
2.2.3 Measurement of oxygen evolution

Oxygen evolution was measured using a Clarke-type electrode (Hansatech) at 25 °C using continuous saturating white light (2400 photons m\(^{-2}\) s\(^{-1}\)) from a xenon lamp to excite the sample. The sample was adjusted to a concentration of 10 µg Chl mL\(^{-1}\).

2.2.4 Electron transport measurements

Total PSII activity was measured by monitoring the light-induced electron transport from H\(_2\)O to di-chloro-phenol-indophenol (DCPIP) and from DPC to DCPIP to estimate the RC activity. For the assay a mixture of 7.5 µg of chlorophyll of illuminated BBY-particles was used. For RC activity DPC (100 µM) and DCPIP (1 mM) were dissolved in Buffer A. The reduction of DCPIP was estimated spectrophotometrically by measuring the absorption changes at 600 nm with a UV-VIS spectrophotometer (Cary 300, Varian) at a temperature of 25 °C. The spectrophotometer was equipped with a custom made cuvette holder based on the TLC 42 cuvette holder (Quantum Northwest). The illumination was provided by a pair of blue 430 nm Luxeon type LED with an irradiance of 2400 photons m\(^{-2}\) s\(^{-1}\). The photomultiplier (PMT) detector was protected by a narrowband filter of 600 nm to ensure the PMT detected only the monochromatic light transmitted by the sample.
2.3 Results.

2.3.1 Addition of PPBQ does not slow down $O_2$ photoinactivation significantly

The loss of oxygen evolving capacity can be explained by either direct damage to the RC or to the Mn$_4$CaO$_5$ cluster. It has been proposed that the limitation on the acceptor side could affect the sequence of photodamage (Vass et al., 1992, Vass, 2012). Therefore, the effect of an excess of artificial electron acceptor was studied. Figure 2.2 shows the rates of oxygen evolution for the BBY-particles after 5 minutes of exposure to polychromatic white light at different irradiances (0, 250, 750 and 1000 µmol photons m$^{-2}$ s$^{-1}$). A comparison between the presence and absence of 600 µM PPBQ is presented. In general, the presence of the electron acceptor did not cause any significant effect (based on $p<0.05$) at any of the light intensities. This result indicates that the presence of PPBQ (electron acceptor) does not suppress PSII photodamage.

![Figure 2.2 Effect of an electron acceptor (600 µM PPBQ) on the extent of PSII photodamage of PSII particles.](figure)

The effect of photodamage was estimated by measuring the maximum rate of oxygen evolution with a Clarke-type electrode; the average value is presented ± SD. PSII particles were exposed to 0, 750, 1800 and 2400 µmol of photons m$^{-2}$ s$^{-1}$ of white light during 5 minutes at 4 °C in the presence of PPBQ (600 µM). Control samples were exposed to light in the buffer (sucrose MES) only. After illumination, samples were rescued, centrifuged at 20000 × g and resuspended in fresh buffer before the PSII activity was measured. All samples are normalized (rate of O$_2$ evolution) to the dark exposed group for Control or +PPBQ samples which correspond to 100%, the absolute values being 602 and 598 µmol O$_2$ mg$^{-1}$ Chl h$^{-1}$, respectively. The difference between groups and treatments was estimated by ANOVA $p < 0.05$. Data presented here was calculated from 6 technical repeats, each experiment repeated in triplicate at different days.
2.3.2 Acceptor-Donor side limitation model explain photoinactivation to the RC only

It has been proposed that limitations on the acceptor-donor side caused by the excessive light energy could lead to photodamage by limitation on both sides (acceptor and donor). To address if the presence of an electron donor and acceptor could avoid photodamage, BBY-particles were exposed for 5 minute to strong polychromatic white light and the rates of electron transport were measured by means of an absorption change of DCPIP at 600 nm. This assay was performed in two ways: to measure the total PSII activity (electron transport from H₂O to DCPIP) or to measure the RC activity (RC activity = electron transport from DPC to DCPIP). The results of this measurement are presented in Figure 2.3.

In the case of the control (Figure 2.3), both activities, total and RC, were affected by the strong illumination. However, the RC activity was higher than the total PSII activity by a small margin (14%); this result suggests that the RC activity was less affected than the total PSII activity. In the case of PPBQ treatment (Figure 2.3) the results for the fragments exposed to light in the presence of PPBQ; under these conditions there was an equal inhibition of the total and the RC activity (40% and 42% respectively). This result indicates that the presence of an electron acceptor (PPBQ) does not decrease the damage to either the total activity or the RC activity. In a similar fashion to the control, the presence of an electron donor during illumination did not alter the inhibition in the total PSII activity (Figure 2.3), and since damage to the RC is similar to the control it can be concluded that the electron donor does not prevent the damage to the RC. In contrast with the previous conditions, in the presence of both acceptor and donor (Figure 2.3), the damage to the RC was suppressed almost completely (the RC activity reaching 84% of the dark-treated sample). Meanwhile the total PSII activity dropped to 44%, a value similar to the previous conditions; this result indicates that in the combined presence of electron acceptor and donor the RC was protected. Nevertheless, the inhibition to the complete PSII activity was still occurring. This last result suggests that the RC is not the primary site of damage in PSII if the limitations in the donor side and acceptor side are alleviated by adding an artificial electron donor and acceptor together.
Figure 2.3 Effect in electron acceptor and donor on photodamage to PSII after 5 min exposure at 1000 µmole of photons m⁻² s⁻¹. The effect of photodamage was estimated by measuring the electron transport rate from H₂O to DCPIP (Total PSII activity) or DPC to DCPIP (reaction center activity); average values are presented ± SD. Total Act – represents the total PSII activity (electron transport between H₂O to DCPIP) and RC Act – represents the activity of the PSII Reaction Center (electron transport from DPC to DCPIP). Total white light illumination was 5 minutes. Control, buffer (sucrose + MES) only; PPBQ, 600 µM PPBQ; DPC, 100 µM DPC; and DPC+PPBQ, 100 µM DPC + 600 µM PPBQ. The 100% values of total PSII activity were 256, 262, 252 and 248 µmol reduced DCPIP (mg Chl)⁻¹ h⁻¹ for Control, PPBQ, DPC and DPC+PPBQ, respectively). For RC activity the 100% values corresponded to 275, 281, 222 and 268 µmol reduced DCPIP mg⁻¹ Chl h⁻¹ (Control, PPBQ, DPC and DPC+PPBQ, respectively). The difference between groups and treatments was estimated by ANOVA p < 0.05. Data presented here was calculated from 6 technical repeats, each experiment repeated in triplicate on different days.

2.3.3 Damage to the Mn₄CaO₅ cluster is independent of the wavelength and the Mn₄CaO₅ cluster is the primary site

In similar fashion as the experiment in Figure 2.3, the presence of electron donor and acceptor was tested at different wavelengths. BBY-particles were exposed in the same conditions as those in Figure 2.3 under 1000 µmol photons m⁻² s⁻¹ of blue (400 to 520 nm), green (520 to 590) or red light (590 to 720 nm). The total duration of illumination was determined by inactivation of the total PSII activity by 50%. As seen in previous studies blue light induced damage faster than red or green. The results are presented in Figure 2.4.
Figure 2.4. Effect of electron acceptor and donor on photodamage to PSII after 5 min exposure at 1000 µmol of photons m\(^{-2}\) s\(^{-1}\) of polychromatic light of different wavelengths in the presence of PPBQ (600 µM) or PPBQ + DPC (600 µM and 100 µM). In Top row (Total PSII activity) and bottom row (RC activity). The effect of photodamage was estimated by measuring the electron transport rate from H\(_2\)O to DCPIP (Total PSII activity) or from DCP to DCPIP (reaction center activity), with average values presented ± SD. Far left, a schematic of the reaction is presented. The presented data corresponds to illumination time 5 min for blue, 30 min for green and 15 min for red. All values of samples are normalized to the dark exposed group which correspond to 100% (300 µmol reduced DCPIP mg\(^{-1}\) Chl h\(^{-1}\)) used in Figure 2.3 and is listed as Fraction of electron transport. The difference between groups and treatments was estimated by ANOVA p < 0.05. 100% was estimated based on the values of dark treated sample in Figure 2.3. Data presented here was calculated from 6 technical repeats, each experiment repeated in triplicate on different days.

In Figure 2.4, it can be observed that when the Total PSII activity was measured in BBY-particles, Blue, Green and Red light induced the same response as white light (Figure 2.3), where the presence of the electron acceptor and the combination of donor and acceptor, during exposure at 1000 µmol of photons m\(^{-2}\) s\(^{-1}\), did not prevent photodamage to the total activity. This result indicates that even if the limitations of the acceptor and donor side are alleviated during exposure at 1000 µmol of photons m\(^{-2}\) s\(^{-1}\), PSII was photodamaged. By contrast, when the activity of the RC was measured after blue, green and red illuminations,
only in the case of exposure at 1000 µmol photons m$^{-2}$ s$^{-1}$ in the presence of both the donor and acceptor was the majority of the original RC activity obtained.

When the sample was illuminated with strong red light in the presence of artificial electron donor and acceptor, it was observed that although the damage to the RC was suppressed, damage to the total PSII activity was similar to that in blue or in green light. *This data shows that the Mn$_4$CaO$_5$ cluster could be affected by all wavelengths and that damage to RC activity can be slowed down by alleviating the acceptor- and donor-side limitations.*

2.4 Discussion

2.4.1 Photodamage occurs primarily in the Mn$_4$CaO$_5$ cluster

The results obtained from the electron transport rate demonstrate that the most affected part is the Mn$_4$CaO$_5$ cluster when the limitations on the donor and acceptor side are alleviated. When the sample was illuminated in the combined presence of donor and acceptor it was clear that RC, including during red light illumination (590 to 750nm), showed minor negative changes in the electron transport rate. The difference between the RC activity and the total PSII activity offers an estimate of the extent of the damage to the Mn$_4$CaO$_5$ cluster. In this manner, it is demonstrated that the only possible candidate is the Mn$_4$CaO$_5$ cluster as primary site of photodamage.

2.4.2 Alleviation of limitations on only one side (either acceptor or donor side) only partially avoided the photodamage to the RC

In all conditions the presence of the electron donor alone did not avoid the full extent of the damage to the RC. This behaviour could be explained because the limitations on the acceptor side also led to photodamage of the RC of PSII. By contrast, when both DPC and PPBQ were present, the damage to the reaction was suppressed, such that the RC activity was up to 80% compared with that of the control sample. The remaining 20% of damage could be explained by either direct damage to the RC, or direct limitations on the acceptor side after several minutes of light exposure. This shows that two types of photodamage are occurring in parallel, supporting the claims of (Oguchi et al., 2011a, Oguchi et al., 2011b, Oguchi et al., 2009).
2.4.3 Only the combined presence of electron donor and acceptor during light exposure enabled the isolation of the two types of photodamage

It is interesting that only in the presence of both electron donor and acceptor during exposure at 1000 µmol of photons m$^{-2}$ s$^{-1}$ was it possible to differentiate between damage to the RC and that to the Mn$_4$CaO$_5$ cluster. When only the donor or the acceptor was present, the extent of the loss of total PSII activity and RC activity was similar. In the control condition (without any electron acceptor or donor), the damage occurred in the total PSII activity, while the RC preserved 10% higher activity. This is a strong argument to support the two-step photodamage model (Ohnishi et al., 2005), in which the primary site of damage is located in the Mn cluster.

However, even if it is evident that limitations on the acceptor or donor side are the cause of damage to the RC, it is still not possible to determine if the two sites of damage are linked or they are independently damaged. In the original proposal of the two step model, only blue light induces damage to the Mn$_4$CaO$_5$ cluster, whereas red or green light affect the RC. However, here it is clear that even under red light the Mn$_4$CaO$_5$ cluster and RC were damaged. This can be explained by predictions (Vass, 2011, Vass, 2012) that in the absence of a functional donor side of the Mn$_4$CaO$_5$ cluster the accumulated free radical species such as P680$^+$ or Y$_z^+$ oxidize the surrounding amino acids, thereby damaging the RC.

2.4.4 Visible light in the red region was able to induce the same type of Mn$_4$CaO$_5$ cluster damage as blue or white light.

The present work demonstrated that that even in red light the preferential site of damage is the Mn$_4$CaO$_5$ cluster when the electron donor and acceptor are present during exposure at 1000 µmol of photons m$^{-2}$ s$^{-1}$. The photodamage to the Mn$_4$CaO$_5$ cluster has been demonstrated to occur specially under UV (Zsiros et al., 2006) and blue illuminations (Hakala et al., 2005) and this is generally accepted (Vass, 2012). The Mn$_4$CaO$_5$ cluster type of damage is explained by intrinsic light absorption of Mn-oxo compounds (in particular damage to the Mn-oxo coordination bonds) which occurs primarily in UV radiation and to a lesser extent in blue light (Wei et al., 2011). In the present work, if the red light illumination is compared with the blue light illumination, the type of photodamage is similar: the total PSII activity was lower than RC activity. This is the first report that the Mn$_4$CaO$_5$ cluster could also be affected by red light effectively. This data seems to contradict the notion that damage to the Mn$_4$CaO$_5$ cluster is only attributed to the blue region of the visible spectrum. However, it is possible to find in the literature some data supporting the present observations. Takahashi et al. (2010) demonstrated that not only blue region has the strongest photodamage peak but there is a
strong photoinhibition peak located at 625 nm in Arabidopsis leaves. Wei et al in 2011 (Wei et al., 2011) showed that a Mn-oxo-mixed-valance dimer model compound (which is capable of evolving oxygen) has an absorption in the green and red regions and its photoinhibition peak overlaps exactly to the absorption spectrum. Similar observations had been reported by Schreiber and Klughammer (2013) who reported a second peak of photodamage beyond 600 nm.

Generally the absorption of light in the red region has been associated with chlorophyll and damage to the RC. However, the present data indicates that the Mn$_4$CaO$_5$ cluster is the primary structure that suffers damage. Furthermore, Hakala et al. (2006) reported that photoinactivation under visible light could happen either in the model enzyme MnCat (which contains a Mn catalytic site) or pumpkin leaves under aerobic and anaerobic conditions. Both experimental models offer a similar inhibition mechanism that supports the Mn hypothesis. In their observations, the photosensitivity of Mn enzymes showed that Mn-mediated damage may occur despite the low absorption of visible light by Mn.

Data supporting the direct damage to Mn in previous studies have not been able to provide spectroscopic information to demonstrate that the cluster can absorb red light. Additional spectroscopic data like those published by Antal et al. (2009) and Wei et al. (2011) showed that the Mn model compounds have small absorption peak around the 600 nm region. In this manner, it is possible to hypothesize why red region of the spectra used in this work causes photodamage to the Mn$_4$CaO$_5$ cluster.

However, differences from the original two-step model hypothesis were observed in the present study that contradict the direct absorption of light by the Mn. Although a possible absorption may occur around 600 nm, the photodamage in red was higher than in green, in opposition to the action spectrum of Ohnishi et al. (2005) and Hakala et al. (2005). If the Mn hypothesis were the only possible mechanism operating during PSI photodamage, higher photoinhibition rates should have been observed in green. In contrast, the result presented here show that green light is less efficient to induce damage to BBY-particles. This last observation matches better with the action spectrum originally published by (Jones and Kok, 1966a), that was done in thylakoids extracted from spinach, where red light had a stronger effect than green. The explanation of this difference may be in the pigment profile of the sample: while Ohnishi et al. (2005) worked with cyanobacteria which clearly have different pigments, Hakala et al. (2005) used a different type of thylakoid membranes than spinach.
2.4.5 Is excessive light energy discarded as a possible explanation?

The results from oxygen evolution and electron transport rate presented in this work seem to support the arguments related to photodamage mechanisms independent of excessive light energy (Tyystjärvi, 2008, Tyystjärvi, 2012). The measurements of oxygen evolution demonstrated that in the presence of electron acceptor the magnitude of photodamage is similar to that in the absence of an electron acceptor, during exposure at 1000 μmol of photons m$^{-2}$ s$^{-1}$, regardless of the irradiance used (Figure 2.2). In addition, the experiments of electron transport showed that the presence of an electron acceptor alone does not affect the damage when compared to the control condition. However, caution is advised, since the acceptor (PPBQ) used in the present work at a high concentration, could be toxic due to quinone-mediated toxicities or accumulation of semiquinone radicals which are highly toxic (Zweig et al., 1972, Monks et al., 1992, Monks and Jones, 2002, Tukaj and Aksmann, 2007). In this case, further experiments need to be carried out using other electron acceptors such as ferricyanide (see Chapter 3).

2.5 Conclusions

The data presented in this work suggests the Mn$_4$CaO$_5$ cluster as the primary site of photodamage by all wavelengths of the visible spectrum. Even if the presence of an artificial electron donor and acceptor did not prevent the damage to the total PSII activity, the damage to the RC could be avoided. Perhaps this result could be explained by the direct light absorption of the Mn cluster but further spectroscopic data is required.
Chapter 3: PHOTODAMAGE AT THE Mn₄CaO₅ CLUSTER INDUCED BY EXCESSIVE EXCITATION

3.1 Introduction.

The results in the previous Chapter showed that the Mn₄CaO₅ is the primary site of PSII photodamage. However, the experimental evidence suggested that inhibition of the OEC is somehow dependent on the absorption of light by photosynthetic pigments. This result is contradictory to the observation that the artificial electron acceptor, PPBQ, did not slow down photodamage, an observation which seems to favour the Mn hypotheses. The only possible way to settle this discrepancy is to answer the three following questions. (1) What part of the OEC is inhibited? (2) What causes the inhibition of the OEC? (3) Is the damage to the Mn₄CaO₅ completely independent of excessive light energy?

The Mn hypothesis originally advanced by Hakala et al. (2005) proposed that the OEC gets inactivated by direct absorption of light by the Mn. Absorption of photons would induce an excited state of the electrons from the Mn-oxo coordination bonds, thereby disrupting it. The result of this damage is the release of one atom of the Mn₄CaO₅ cluster into the lumen. There have been no reports about Y₂ as the primary site of photodamage, but this site could be disregarded based on the experimental evidence presented in Chapter 2, since photodamaged PSII complexes are capable of accepting electrons from DPC via Y₂, which means that Y₂ is intact.

Concerning what may cause photoinhibition, Hakala et al. (2005) proposed that light could disrupt one of the Mn-oxo bonds. However, this hypothesis considers that UV or blue photons, because of their high energy, can disrupt the coordination bonds of the Mn₄CaO₅ cluster. However, this mechanism may not be applicable to photons of lower energy (green and red region of the visible spectrum), since the energy is not enough to induce an excited state necessary for photolysis of the coordination bonds. Also, the Hakala et al. (2005) theory does not explain why red light had a stronger photodamaging effect than green photons (which have higher energy) as discussed in the previous Chapter. On the other hand, this result is to be expected if the PSII photodamage in red region of the visible spectra is dependent on pigment absorption (Jones and Kok, 1966a, Jung and Kim, 1990, Schreiber and Klughammer, 2013).

Song et al. (2006) proposed that the production of all possible ROS may disrupt the Mn₄CaO₅ cluster in the OEC by oxidizing the Mn. Interestingly, their data relates the ROS production to the Mn release, by comparing the behaviour of BBY-particles treated with ROS artificially added with photodamaging treatments. This results is in accord with the work of Jung and Kim.
showing that the action spectrum of PSII photodamage overlaps with the action spectrum of $^{18}$O$_2$ production in BBY-particles.

The hypotheses of Song et al. (2006) and Jung and Kim (1990) could explain the results reported by proposers of hybrid theories (Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b) (Schreiber and Klughammer, 2013). In these hybrid theories two sites of damage can be found and each could be induced by a differential type of sensitizer: the PSII RC or the Mn$_4$CaO$_5$ cluster. The damage of the OEC has been explained by chemical damage (Song et al., 2006) or a light-induced damage by two photosensitisers (Schreiber and Klughammer, 2013, Hakala et al., 2006). The only inconsistency with this argument is the role of excessive light energy, as questioned by experimental data obtained by Tyystjarvi and Aro (1996) and as repeated by other groups (Baroli and Melis, 1996).

The present work scrutinises the mechanisms that have been proposed to explain the inactivation of the Mn$_4$CaO$_5$ (Vass et al., 1992, Hakala et al., 2005, Ohnishi et al., 2005, Oguchi et al., 2009, Schreiber and Klughammer, 2013) and the role of excessive light energy (Tyystjarvi and Aro, 1996). To do so, photodamage was studied in BBY-particles exposed to light of two different wavelength bands, and the fate of the Mn$_4$CaO$_5$ cluster and the RC was monitored using EPR and Chl a fluorescence. The results present conclusive evidence for the damage to PSII occurring in a two-step manner, which is independent of wavelength but dependent on excessive light energy.

3.2 Materials and Methods

3.2.1 Plant Material and sample preparation

O$_2$ evolving BBY-particles were prepared from fresh market spinach as described by (Berthold et al., 1981). The chlorophyll concentration was adjusted to 5 mg of Chl mL$^{-1}$ (this will be addressed as a BBY-particles stock suspension). The sample was kept in darkness and 4 °C at all times otherwise stated. Then the sample was solubilized in a standard buffer (400 mM sucrose, 25 mM MES-NaOH, 15 mM NaCl, 5 mM MgCl$_2$, pH 6.5), flash frozen in liquid nitrogen (LN2) and stored at –80 °C until used.

3.2.2 Light irradiation of photosystem II.

Before use, PSII membranes were thawed in darkness and kept at 4 °C at all times in Eppendorf tubes (1.5 mL). When required, artificial electron acceptors (150 µM PPBQ + 2 mM ferricyanide) or an artificial electron donor (300 µM DPC) or a mixture of the three chemicals
were added to the samples. A suspension (75 µL) of PSII membranes was transferred into three custom-made quartz cuvettes; the liquid was kept in a flat region of the cuvette which had an optical thickness of 100 µm (Figure 3.1). The end part of each quartz cuvette was sealed with Teflon caps and reinforced with Parafilm (Figure 3.1 E). Then the cuvettes were placed inside custom made LED light boxes (photoinhibition boxes) made of aluminium (Figure 3.1 A and D). The illumination was set to 1300 µmol of photons m$^{-2}$ s$^{-1}$ of monochromatic light (460±10 nm and 660±10 nm see Figure 3.2). Light was provided by arrays of 16 units of 3 W Luxeon-type LEDs that were bolted into the lids of the aluminium boxes (Figure 3.1 A and C). In order to dissipate the heat generated by the LED array, the box lid was attached to a cooling block (Figure 3.1 D and G). Water at 4 °C passed through the cooling block; this not only removed possible heat effects but also ensured stability of the LED sources (Figure 3.1 A and G). The temperature of the LED panel was 12 °C. The sample was kept at 4 °C by contact with water (Figure 3.1 G). To enhance effective light absorption, a visible light mirror was placed under the cuvettes (Figure 3.1 G). The cuvettes were constantly flipped from one side to the other to ensure homogeneous illumination. The light dose (illumination time of exposure) depended on the type of experiment (see figure description). The light intensity was measured using a spectroradiometer (Comet-black, Stellar net, Inc). The spectral profile and irradiance of the LED is presented in Figure 2.1.

![Figure 3.1](image_url)

**Figure 3.1 Experimental setup for photodamage experiments.** (a) Diagram of the LED light source. (b) Diagram of the water cooling at the sample compartment. (c) Diagram of the box ensemble. (d) Diagram of the water cooling system for the LED. (e) Flat quartz cuvette for the EPR measurements. (f) Diagram of the cuvette samples. (g) Lateral view of the experimental setup during illumination.
3.2.3 Determination of Mn²⁺ content by Continuous Wave Electron Paramagnetic Resonance (CW-EPR).

Mn²⁺ release was evaluated by the EPR signal of Mn²⁺. The EPR measurements were performed using a Bruker ESP 300E ESR spectrometer with a cavity type Bruker standard (ER4102ST). All measurements were performed at 5 °C and the temperature was controlled by a custom made gas flow system using nitrogen gas. The samples with a total volume of 50 µL were transferred into custom made capillary tubes (same as the ones described in photodamage experiments). The measurement conditions were: microwave frequency 9.60, the microwave power 64 mW, modulation frequency 100 kHz and modulation amplitude 8 G.

3.2.4 PS II activity measurements

PSII activity was evaluated by the maximum photochemical efficiency of PSII (Fv/Fm) using Chl a fluorescence measured at room temperature by the fast rise of the Chl a fluorescence using a M-PEA fluorometer (Multichannel-Plant Efficiency Analyser 2, Hansatech Instruments (Strasser et al., 2010)). After EPR measurement in darkness, fluorescence was measured in the same cell after more than 15 min dark adaptation. Three regions of the quartz cell were measured using the standard leaf clip provided by the manufacturer, the distance between two adjacent spots being at least 3 cm. The cell was illuminated with a 660 nm red saturating illumination (3000 µmol photons m⁻² s⁻¹) for 30 s. After the measurements, the sample was placed back into the
LED photoinhibition box. All samples were manipulated under dim green LED light with an irradiance at the sample of less than 1 µmol photons m$^{-2}$ s$^{-1}$.

3.2.5 Recovery of the PSII activity rescue assays in the presence of an artificial electron acceptor.

PS II membranes, exposed in the exact same conditions as described above, were illuminated for 30 min. Then the sample was transferred from the cuvette to an Eppendorf tube. The F$_{V}$/F$_{M}$ was measured after 15 min dark adaptation, by transferring 5 µl of the suspension onto paper filter (Watman 2). Then the sample was incubated with 300 µM DPC for 15 min in darkness. The activity was measured afterwards using the same method as described above.

3.2.6 Data analysis

Data was analysed statistically using OriginPro software (v 9.1) and Igor Pro. All EPR spectra analysis was done in Igor using the multicomponent component approach, where the Tyrosine D (Y$_{D}$) and Chl signals were subtracted from the observed signals. The 100% Mn$^{2+}$ content was evaluated by the amount of the Mn$^{2+}$ signal of heat treated sample and the 100% of the Y$_{D}$ Chl spectrum was estimated from a pre-illuminated sample. A representative series of EPR spectra are presented in Figure 3.3. At both wavelengths of 460 and 660 nm the release of Mn$^{2+}$ was observed, indicating that MnCa$_{4}$O$_{5}$ was losing a portion of the Mn$^{2+}$ atoms from the cluster upon illumination.
3.3 Results.

3.3.1 *Loss of PSII efficiency is faster than release of Mn$^{2+}$ ions.*

Figure 3.4 shows the time course of the Mn$^{2+}$ EPR signal and $F_v/F_M$ ratio detected by fluorescence of samples under 460 (panel a) and 660 nm (panel b) illumination.

*Figure 3.3 EPR spectra for the Mn$^{2+}$ signal of BBY-particles after illumination.* 100% of Mn$^{2+}$ content was estimated as those in heated PS II. Only a representative set of data is presented (660 nm). $Y_o$ signals were removed by functional processing. Each experiment was repeated in twice for 460 or 660 nm.
Figure 3.4 Comparison between FV/FM ratio and the Mn$^{2+}$ EPR signal after illumination with 460 and 660 nm. (a) 460 nm and (b) 660 nm. The black axis and line (dotted) represent the normalised signal intensities of released Mn$^{2+}$. The magenta axis and line (continuous) represent the signal intensities of FV/FM. The inset in each panel is a magnification of the first 120 min of illumination. Data presented here was calculated from 3 technical repeats, each experiment was repeated twice at different days.

The increase of Mn$^{2+}$ EPR signals are best fitted with biphasic exponential functions, and decay of FV/FM are fitted with monophasic exponential function (with an offset, $y_0$) and the derived half-life times ($T_{50}$) are presented in Table 3.1.

Notably, there was no significant difference in the rate of release of Mn$^{2+}$ between the two wavelengths which suggests that Mn$^{2+}$ release is independent of wavelength. Similarly, there was no significant difference in the rate of loss of FV/FM for samples under 460 and 660 nm illumination. Moreover, the $T_{50}$ for decay of FV/FM was significantly lower compared to the $T_{50}$
of Mn$^{2+}$ release indicating that release of Mn$^{2+}$ occurs after loss of $F_v/F_M$. This observation suggests that the release of Mn$^{2+}$ and loss of $F_v/F_M$ are two separate events in photodamage of PSII. When PSII was fully inactivated ($F_v/F_M < 0.2$), the Mn$^{2+}$ released was about 25% of the total numbers (see insets in Figures 3.4 a and b). This could be interpreted as, on average, one Mn atom released per PSII as a consequence of photodamage.

Table 3.1 $T_{50}$ values (min) for Mn$^{2+}$ release and loss of $F_v/F_M$ during 1200 min light exposures. Each $T_{50}$ is presented ± standard error. Mn$^{2+}$ release was fitted to a biphasic curve and the $T_{50}$ for the slow and fast phase are presented individually ± std error. The $y_0$ represents the offset used for the fitting ± std error. Amplitudes are presented in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>460 nm</th>
<th>660 nm</th>
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<tr>
<td>Mn$^{2+}$ release</td>
<td>$^* T_{fast}$ 38.5 ±10.3 (0.18)</td>
<td>25.6 ± 14.8 (0.18)</td>
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<tr>
<td></td>
<td>$^* T_{slow}$ 495 ± 117 (0.73)</td>
<td>459.07 ± 176 (0.77)</td>
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<td></td>
<td>$y_0$ 0.91 ± 0.07</td>
<td>0.94 ± 0.12</td>
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<tr>
<td>$F_v/F_M$ decay</td>
<td>$^* T_{50}$ 15.4 ± 1.2</td>
<td>15.4 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>$y_0$ 0.17 ± 0.05</td>
<td>0.17 ± 0.05</td>
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3.3.2 Mn$^{2+}$ release only occurs during illumination.

When the sample was kept in darkness after 120 min of illumination, there was no significant Mn$^{2+}$ release nor uptake (Figure 3.5). Release of Mn$^{2+}$ was resumed when the samples were further illuminated after 900 min. This clearly shows that Mn$^{2+}$ release, after the diminution of $F_v/F_M$, was caused by illumination.
Figure 3.5 Mn$^{2+}$ release occurs only in the light. A representative series of data is presented for the Mn$^{2+}$ release (660 nm). The red frames represent illumination of the sample. The middle area represents the period when the sample was kept in darkness. Data presented here was calculated from 1 technical repeats.

3.3.3 Mn$^{2+}$ release and photodamage are caused by limitations on the acceptor side.

The two-step model implies that in the presence of an excess of artificial electron donor (ED) and/or electron acceptor (EA) would not alter the rate of photoinactivation as it is independent of excessive excitation. In order to test this hypothesis, the first two hours of illumination at 460 and 660 nm were monitored in the absence and the presence of an ED and/or EA (Figure 3.6). To facilitate direct comparison of rates, the data presented in Figure 3.6 can be satisfactorily fitted to a monophasic exponential equation (with an offset).

At both wavelengths, the Mn$^{2+}$ release was significantly slower in the presence of PPBQ + ferricyanide (EA) relative to the control sample (approx. 6 times see Figure 3.7). This indicates that if limitations on the acceptor side are alleviated by an artificial EA, Mn$^{2+}$ release is suppressed. Furthermore, when PSII membranes were exposed to light in the presence of both ED and EA, the rate of Mn$^{2+}$ release also was slower (approx. 5 to 7 times see Figure 3.7). By contrast, the presence of ED did not have any effect on the Mn release during 460 nm illumination; at 660 nm, the Mn$^{2+}$ release was slightly increased (approx. 1.2 times see Figure 3.7). These results suggest that limitations on the acceptor side are the main factor in the Mn release for wavelengths 460 to 660 nm.
Figure 3.6 Comparison between PSII photodamage and Mn$^{2+}$ release in the different chemical treatments. (a) Mn$^{2+}$ release during illumination at 460 nm. (b) Mn$^{2+}$ release during illumination at 660 nm. In this figure, for simplicity, a single exponential fitting is used. (c) PSII photodamage at 460 nm. (d) PSII photodamage at 660 nm. Average results are presented ± std error in each case with its respective curve fitting. Data presented here was calculated from 3 technical repeats, each experiment repeated in twice on different days.

Figure 3.6 also shows the corresponding changes in $F_v/F_m$ ratios for samples under identical conditions as the EPR experiments. For the 460 nm illumination (Figure 3.6 c), in the presence of the EA, the $T_{50}$ for loss of $F_v/F_m$ increased compared to the control (approx. 4 times, Figure 3.7 a). Notably, there is a smaller increase in the $T_{50}$ of Mn$^{2+}$ release in the ED treatment at 660 nm (Figure 3.7 b) (approx. 1.8 times). In the presence of EA and ED, the $T_{50}$ was also increased relative to the control (approx. 4 times Figure 3.7 b). These results suggest that under blue light there are two types of limitations, on the acceptor side and the donor side. The effect of acceptor side limitation is more pronounced than the donor side limitation (the latter being due to direct damage by light to the Mn). The responses obtained at 660 nm illumination are similar to those obtained at 460 nm. Notably, there is a smaller increase in $T_{50}$ in the ED
treatment at 660 nm (Figure 3.7b), indicating that limitation on the donor side at 660 nm has a lower impact on photoinactivation.

![Graph showing the ratio of T50 of loss of Fv/FM and Mn2+ release after illumination with 460 nm and 660 nm.](image)

**Figure 3.7** A comparison between the ratio of T50 of loss of Fv/FM and Mn2+ release after illumination with 460 nm and 660 nm. The ratio of T50 of (a) loss of Fv/FM and (b) Mn2+ release after illumination with 460 nm (blue) and 660 nm (magenta) illuminations. Each plot contains the ratio of the half lifetime of each treatment ($T_{50}^{\text{treat}}$) to the value of the control ($T_{50}^{\text{Cont}}$) ± std error. Data presented here was calculated from 3 technical repeats, each experiment repeated in twice on different days.

Figure 3.7 shows that inactivation of PSII (loss of Fv/FM) and Mn2+ release are strongly correlated, but they are separate processes. Nevertheless, at both wavelengths the acceptor side limitation had a significant impact on photoinactivation and loss of Mn2+. These results show that excessive light energy does play an important role in the mechanism of PSII photodamage and the subsequent Mn2+ release.

3.3.4. **PSII efficiency is recovered if the sample is incubated with an artificial electron acceptor after photodamage.**

In order to test the effect on the donor side, the Fv/FM ratio was measured after further addition of DPC to the illuminated samples. After illumination for 30 min and the measurement of Fv/FM, the sample was incubated for 15 min in the dark in the presence of DPC and the Fv/FM was re-measured. Figure 3.8 shows the changes in Fv/FM ratios of the samples illuminated with 460 nm and 660 nm light, before and after addition of the DPC. In all treatments, the Fv/FM ratio was recovered (~80% relative to dark control for both wavelengths) after DPC incubation. This indicates that the QA reducing capacity (RC activity) was much less affected compared with the Mn4CaO5 cluster, in agreement with a previous
Moreover, the extent of recovery was greatest in the EA treated sample, further confirming the role of acceptor side limitations in photoinactivation of PSII. Additionally, this result shows that limitations on the acceptor side are also the cause of photoinactivation of the RC as a full recovery after incubation with DPC was not observed in all chemical treatments and at the two wavelengths.

Figure 3.8 Comparison between the different treatments in the rescue experiments PSII activity in 460 nm experiments and PSII activity in the 660 nm experiments. (a) PSII activity in 460 nm experiments. (b) PSII activity in the 660 nm experiments. Average values ± std deviation. Data presented here was calculated from 3 technical repeats, each experiment was repeated in twice at different days.

3.4 Discussion.

3.4.1 Photodamage occurs at two sites but due to limitations on the acceptor side.

In this work, Mn²⁺ release was detected for illumination with 460 and 660 nm wavelengths and the Fₐ/Fₘ ratio was recovered when the sample was incubated with an electron donor (ED). The results are consistent with a recent report (Zavafer et al 2015b), where the inactivation of the Mn₄CaO₅ cluster occurred even in the red regions of the visible spectrum. However, they are not consistent with the some reports (Hakala et al., 2006, Ohnishi et al., 2005, Tyystjärvi, 2008), where the damage to the Mn₄CaO₅ cluster is caused by direct absorption of light by the cluster. They (Hakala et al., 2006, Ohnishi et al., 2005, Tyystjärvi, 2008) proposed that (1) Mn₄CaO₅ cluster inactivation is independent of limitations on the acceptor side, and (2) light of 660 nm causes damage to the Mn₄CaO₅ cluster, which is 100 nm away from the predicted
blue-green absorption region of the Mn-oxo compounds. (Hakala et al., 2005, Hakala et al., 2006, Hou and Hou, 2013, Schreiber and Klughammer, 2013). The possibility that the damage to Mn₄CaO₅ cluster at 660 nm (Hou, 2013, Hou and Hou, 2013) is excluded, because the acceptor side limitation has a much stronger influence since the addition of electron acceptors slowed down the loss of PSII activity. Instead, the paradigm of excessive light energy absorbed by the photosynthetic pigments seems a more reasonable explanation for PSII photodamage. Santabarbara et al. demonstrated that PSII photodamage in isolated thylakoids follows the pigment absorption in the red region (Santabarbara et al., 2007, Santabarbara et al., 2001a, Santabarbara et al., 2002, Santabarbara et al., 2001b).

The present results show that photodamage occurs in two steps, supporting partially the claims of previous works of Ohnishi et al. (2005) and Zavafer et al. (2015b). However, this result shows that the Mn₄CaO₅ damage and the Mn²⁺ release are caused by not direct light absorption of Mn²⁺ cluster, but limitation on the acceptor side. This does not exclude the possibility that regions close to the UV like 400-420 nm inactivates preferentially the Mn₄CaO₅ cluster by direct absorption of light by the Mn atoms (Hakala et al., 2005, Hakala et al., 2006, Hou and Hou, 2013, Schreiber and Klughammer, 2013).

3.4.2 What causes Mn²⁺ release?

PSII photodamage with light absorption by photosynthetic pigments is usually ascribed to singlet oxygen, a form of ROS. (Macpherson et al., 1993, Rinalducci et al., 2004) (Ivanov et al., 2008, Vass and Cser, 2009, Vass, 2011, Rehman et al., 2013). The traditional primary site of light induced ROS damage is the QA reducing site (Vass, 2012). The results presented here are consistent with the excessive light energy paradigm and the acceptor side limitations. However, the results also show that the Mn₄CaO₅ cluster is the primary site of damage instead of QA. This is evident by the recovery of Fv/FM ratio after photoinactivated samples were incubated with an exogenous ED indicating a high level of functionality of the RC and QA site. It follows that the most likely agent(s) for inactivating the Mn₄CaO₅ cluster are ROS. Indeed, Song (Song et al., 2006) showed a positive correlation between ROS and Mn release in-vitro.

The role of ROS during PSII photodamage is contentious as different reports support or reject their importance. Nishiyama et al (Nishiyama et al., 2001, Nishiyama et al., 2004, Nishiyama et al., 2005, Nishiyama et al., 2006) suggest that ROS are not relevant for PSII photodamage, but their results have been contested by more recent data (Hideg et al., 2007, Rehman et al., 2013, Bersanini et al., 2014, Hakkila et al., 2014, Sedoud et al., 2014). Tyystjärvi (2008) suggested that the different roles of ROS during photodamage are due to the differences in
concentrations of ROS. While for \textit{in vitro} systems the concentrations of ROS are expected to be higher, \textit{in vivo} systems usually keep the ROS at lower concentrations. However, it has been reported that the action spectra of photoinhibition and of singlet oxygen production in thylakoid membranes are very similar (Jung and Kim, 1990) which supports the role of ROS as a driving force of PSII photodamage. The ROS for PSII photodamage may be produced in close proximity to the Mn$_4$CaO$_5$ (Macpherson et al., 1993, Telfer et al., 1994); therefore, added ROS scavengers may not have access to slow down PSII photodamage. This also would explain why the OEC is the preferential site of damage and it is affected before everything else suffers oxidation.

3.4.3 \textit{Mn}^{2+} \textit{release is not the cause of loss of PSII activity, but a later consequence.}

In the two step mechanism (Hakala et al., 2005), it has been proposed that light induces the release of one atom Mn from the Mn$_4$CaO$_5$ cluster, consequently impairing O$_2$ evolving activity and PSII is inactivated. Comparison of the T$_{50}$ for loss of Mn$^{2+}$ and loss of F$_{V}$/F$_{M}$ shows that the rate of loss of Mn$^{2+}$ is significantly slower compared to rate of F$_{V}$/F$_{M}$. Also, the addition of EA slowed down the Mn$^{2+}$ release, which shows that the release of Mn$^{2+}$ can be explained by limitation on the acceptor side.

Previous reports (Cleland et al., 1986), Idedan et al. (2011) based on results obtained by different spectroscopic methods have proposed inhibition at the Q$_A$ site. The data presented here, however, confirms that the other PSII components (Y$_{p}$, P680, Pheo and the Q$_A$) retain function as the incubation with ED the F$_{V}$/F$_{M}$ recovered for during the first light exposure, which discards the possibility that the primary site of damage is located in other PSII components. The discrepancies between previous results and ours can be explained by the fact that in previous works (Cleland et al., 1986), Idedan et al. (2011) the PSII activity was not measured in the presence of DPC after photodamage.

The double exponential kinetics observed during loss of Mn$^{2+}$ suggests a possible mechanism for Mn$^{2+}$ release. The fast phase occurs during the first 2 hours of illumination and is driven by the limitation on the acceptor side (as the addition of EA slowed down the release). It is well documented that under such conditions, ROS accumulation is enhanced. At longer times, electron transfer to Q$_A$ is inhibited as all available Q$_A$ is fully reduced. As the acceptor side in PSII is fully reduced, formation of P680$^+$Pheo$^-$ pair still occurs and P680 triplet will be formed but at a lower rate. This results in a slow phase in which the rest of the Mn atoms from the cluster are released. Another possibility is that the ROS produced due to triplet Chl at the antenna diffuse more slowly to the PSII inner core and these ROS contribute to the Mn$^{2+}$
release, because triplet formation at the chlorophylls in the antenna, by intersystem crossing, remains even if the PSII is inactivated.

3.4.4 Excessive light energy inactivates the OEC.

There has been debate concerning whether excessive light energy causes photodamage (Tyystjarvi and Aro, 1996, Melis, 1999, Tyystjärvi, 2008, Tyystjärvi, 2012, Vass, 2012). The linear dependence of the rate coefficient of PSII photodamage on irradiance ((Tyystjarvi and Aro, 1996)) has been used to support the idea that photodamage is independent of the excessive light energy (Hakala et al., 2005, Hakala et al., 2006, Sarvikas et al., 2006, Takahashi and Murata, 2005, Murata et al., 2007, Hakala-Yatkin et al., 2010, Takahashi et al., 2010). It has been further supported by the experiment in vivo that showed that inhibition of the Calvin cycle does not accelerate PSII photoinactivation (Takahashi and Murata, 2005, Takahashi and Murata, 2006). Also, the recent reports that Mn₄CaO₅ is the primary site of damage in red, green and blue of the visible spectrum (Zavafer et al. 2015b) supports the role of excessive light energy in photodamage. However, in all those works (mentioned above with the exception of Hakala et al. 2005) direct Mn²⁺ release has not been measured. In addition, such linearity has been also explained in the terms of the excessive energy by Vass et al (Vass and Cser, 2009, Vass, 2011, Vass, 2012), based on the role of the charge recombination and singlet oxygen model. Furthermore, production of singlet oxygen in the antennas also followed a linear relation to the irradiance (Ballottari et al., 2013). Since chlorophyll excitation follows a linear relation versus irradiance, and there is proportionality between excitation and triplet accumulation, the observed linearity between PSII photodamage and irradiance can be explained.

It is noted that the present results are not fully consistent with the results of the presented in Chapter 2 where exogenous electron acceptor (PPBQ) did not slowed down photodamage. A reason why in previous studies in BBY-particles did not slow down photodamage to the Mn₄CaO₅ (Zavafer et al (2015b) could be that the sample was not incubated with DPC before the PSII activity and only PPBQ was used as an electron donor, while here PPBQ and ferricyanide were used. Furthermore, it is interesting that the authors (Zavafer et al., 2015b) observed that red light had a stronger effect than green light, which does not support the original hypothesis of the two step model (since it would be expected that damage in green light would be higher). Finally, the concentrations of electron acceptors were significantly lower in Zavafer et al (2015b) than the ones used in the present work.
3.4.4 Future measurements with electron acceptor inhibitors.

A simple experiment that it is considered in the perspectives of this work is the use of inhibitors of the electron transport during photodamage. This experiments would require the use of two inhibitors at the level of QA: 3’-(3,4-dichlorophenylenyl)-1,1’-dimethyl urea (DCMU) and bromoxynil. DCMU induces a decrease in the redox potential of QA, which in turn has a protective effect against photodamage (Ideaidan et al., 2012). In turn, if bromoxynil is used an increase in the redox potential of QA would enhance photodamage of PSII. If my hypothesis about the damage to the Mn cluster due to excessive excitation is correct, the use of bromoxynil during photodamage would enhance the Mn2+ release, while DCMU would decrease the resale and the PSII damage.

3.5 Conclusions

In this work, BBY-particles were exposed to monochromatic light (460 or 660 nm) at high irradiances. The results were put in the context of the three different hypotheses for PSII photodamage. Damage to the PSII is initially localised at the Mn₄CaO₅, in agreement with the two step model. However, the primary cause of photoinactivation is attributed to limitations of the acceptor side, as per the excessive light energy paradigm. The extent of damage at 460 nm is slight higher relative to 660 nm; this may be attributed to direct absorption of light by the Mn. In this manner, here the author proposes a two-site damage model, where the main site of inactivation is the Mn₄CaO₅ and its inactivation is mainly dependent on the limitations of the acceptor side. This result does support the statements of the two-step model concerning the primary site of damage, but disagrees on the role of limitations on the acceptor side and by consequence excessive energy absorption. Also a minor contribution by what seems to be direct light absorption by the Mn²⁺ was observed at 460 nm. These results explain why previous works have ascribed the Mn₄CaO₅ cluster to the primary site of damage, why the Mn₄CaO₅ cluster damage is enhanced at shorter wavelengths, and why at longer wavelengths the Mn₄CaO₅ cluster gets inactivated. The present work is in agreement with the claims of the hybrid model of PSII photodamage. Figure 3.9 shows the new model for PS II inactivation. By illuminating PS II in the initial state (Figure 3.9 a), the acceptor side is fully reduced by repetitive turnovers (Figure 3.9 b). By the increasing P680 triplet via the recombination of P680⁺Ph⁻ and/or formation of radical on the acceptor side, the resulting ROS attack the Mn²⁺ cluster and release one Mn³⁺ ion (Figure 3.9 c). Accumulation of ROS occurs at several parts of PSII and they diffuse to several sites (Figure 3.9 d). Mn₄CaO₅ cluster is inactivated by ROS (with no release of Mn²⁺) (Figure 3.9 e). Once the Mn₄CaO₅ cluster is inactive, the Mn³⁺ ion is released (Figure 3.9 f). In parallel or sequentially to the inactivation to the Mn₄CaO₅ cluster,
ROS may deactivate the acceptor side activity on a gradual way but lower rate ROS (Figure 3.9 g). As a consequence of the oxidative stress the 3 remaining Mn\(^{2+}\) ions are released; however, at this step, PSII is completely inactive. The model is also consistent with previous observations that excessive light energy absorbed by photosynthetic pigments plays an important role in PSII photodamage. In order words, the results of this work reconcile the differences between previous reports, stating that all in a sense are correct. Regardless of whether this mechanism also operates in vivo, it has been demonstrated that limitations in the acceptor side can explain the inactivation of the Mn\(_4\)CaO\(_5\).

**Figure 3.9 Proposed mechanism of PSII photodamage in high light.** (a) Active PSII absorbs photons. (b) The pool of acceptors is fully reduced. (c) Repetitive turnover P680\(^{+}\)Q\(_a\)^{−} induces formation of P680 triplet or side reactions as described by Vass (2012). (d) Accumulation of ROS occurs at several parts of PSII and they diffuse to several sites. (e) Mn\(_4\)CaO\(_5\) cluster is inactivated by ROS. (f) Once the Mn\(_4\)CaO\(_5\) cluster is inactive, the Mn\(^{2+}\) ion is released. (g) In parallel to inactivation to the Mn\(_4\)CaO\(_5\) cluster, ROS may deactivate the acceptor side activity on a gradual way but lower rate ROS. (h) As a consequence of the oxidative stress the 3 remaining Mn\(^{2+}\) ions are released; however, at this step, PSII is completely inactive.
Chapter 4 : A NOVEL QUENCHING MECHANISM NOT RELATED TO NPQ AND ITS ROLE IN PHOTOPROTECTION OF PHOTOSYSTEM II

Note that this Chapter has been done in collaboration with a fellow PhD student, Ievgeniia Iermak, from Wageningen University. Both us have contributed in equal manner to this Chapter.

4.1 Introduction.

As discussed in Chapter 1 photosynthetic organisms defend themselves against photoinhibition by a diverse set of strategies. One of the most studied photoprotection mechanisms is the NPQ. In sensu lato, NPQ is the energy dissipation not related to primary photochemistry that quenches the excitation of the chlorophylls. The main characteristic of NPQ is the quenching of the chlorophyll \( a \) fluorescence, since fluorescence is a side effect of chlorophyll excitation. Even though it is widely acknowledged that the quenching of excitation plays an important role as a photoprotective mechanism, the recent rise of paradigms that advocate that PSII photodamage is independent of NPQ (Hakala et al., 2005, Ohnishi et al., 2005) has questioned the relevance of quenching of excitation to PSII photoprotection.

The nature and the mechanism(s) of PSII photodamage also have important implications for the required photoprotection mechanisms, as most of the proposed mechanisms only consider excessive light energy as the cause of photodamage. It is widely accepted that PSII photodamage is alleviated by protective mechanisms and that this dissipation of the excessive excitations prevents PSII photodamage (Horton and Ruban, 2005, Ruban et al., 2012). For supporters of the Mn photoinactivation paradigm, the physiological role of NPQ is not to avoid PSII damage but to protect PSII photorepair only (Nishiyama et al., 2006, Murata et al., 2007, Takahashi and Murata, 2008). The main experimental evidence supporting the role of NPQ in photoprotection (Li et al., 2002) was obtained in the presence of repair mechanism(s), and therefore PSII photodamage per se was not directly measured. Even though most reports about NPQ acknowledge its role as a mechanism of PSII photoprotection (Demmig-Adams and Adams III, 1992, Chow, 1994, Niyogi, 1999, Lee et al., 2001, Cruz et al., 2005, Horton and Ruban, 2005, Ruban et al., 2012) there is also experimental evidence that NPQ has only a low efficiency in protecting against photodamage (Tyystjarvi et al., 1999, Santabarbara et al., 2001a, Nishiyama et al., 2006, Sarvikas et al., 2006).

Interestingly, Matsubara and Chow (2004) have proposed that even in the absence of all NPQ mechanisms PSII photoprotection occurs in vivo, because inactive PSII RCs protect active PSII
from photodamage by dissipating the excessive light energy. Two results were used to support this proposal: (1) the time course of PSII photodamage was described by a double exponential decay of PSII activity; (2) a characteristic decrease was found for the average fluorescence lifetime ($\tau_{AV}$) of the sample. The double exponential decay found for the PSII efficiency might suggest that there are two PSII populations that get photodamaged at different rates, one that decays faster (a PSII population that is inactivated quickly) and a slower one (the remnant PSII that is protected by the inactive PSII). In other words, a fraction of PSII becomes inactive and then quenches excitations and protects the active PSII, which would explain the decrease in fluorescence lifetime. However, this hypothesis has been questioned by Sarvikas et al. (2010), who provided evidence that the time course of PSII photodamage follows first-order kinetics, subsequently corroborated by Kou et al. (2012).

In order to determine whether the quencher formed during photodamage protects PSII, we investigated the nature of the decrease of $\tau_{AV}$ to provide insight into its role in photoprotection. To do so, the changes in $\tau_{AV}$ were evaluated during the time course of PSII photodamage. Two experimental model systems were compared: photorepair-deficient spinach leaves (in vivo system) and BBY-particles (in vitro system), in order to see if the decrease of $\tau_{AV}$ only occurs in leaves or is intrinsic to PSII. Since the decrease of $\tau_{AV}$ might be related to damage of the Mn$_4$CaO$_5$ cluster or might be induced by photosynthetic pigments, we photodamaged the two experimental model systems using blue and red light (460 and 660 nm) to explore if there is any wavelength dependency. The time-dependent changes of the fluorescence lifetime, which is quenching dependent, are compared to the time course of PSII photoinactivation.

4.2 Materials and Methods

4.2.1 Plant material and sample preparation

Fresh and intact spinach leaves were purchased at local markets (Wageningen, NL). The leaves were stored at 4°C in a cold room with the petiole submerged in tap water until use. Only plants with high PSII efficiency, determined by measuring the PSII yield ($F_V/F_M$) were selected for all experiments (see Chl $\alpha$ fluorescence for PSII efficiency measurements). BBY-particles were prepared from fresh market spinach as described in (Berthold et al., 1981). Then the sample was solubilized in a standard buffer (400 mM sucrose, 25 mM MES-NaOH, 15 mM NaCl, 5 mM MgCl$_2$, pH 6.5), flash frozen in liquid nitrogen (LN2) and stored at −80°C until use. Before light exposure, the sample was thawed and re-suspended in the standard buffer without sucrose (Buffer B). Then it was centrifuged at 16,000 x g for 5 min and re-suspended in Buffer
B. The chlorophyll content was measured according to (Porra et al., 1989) and adjusted to 150 µg Chl mL$^{-1}$ (this will be referred to as BBY-particles stock suspension). The sample was kept in darkness at 4°C at all times unless otherwise stated.

4.2.2 Photodamage in vivo

Before illumination, to inhibit photorepair, leaves were infiltrated with 5 mM lincomycin by passive petiole infiltration for 12 hours in order to ensure its uptake by the leaf tissue (Aro et al., 1993a, Matsubara and Chow, 2004). Samples were kept at 4°C at very low light intensity (<5 µmol photons m$^{-2}$ s$^{-1}$) before they were used. PSII activity was measured before illumination using the $F_v/F_M$ Chl a fluorescence parameter (Tyystjärvi and Aro, 1996). Leaves were transferred to plastic Petri dishes with a 1 mm layer of water to ensure moistening (Figure 4.1). Each leaf was facing the light on the adaxial side. Then the Petri dishes were placed inside custom-made LED light boxes (photoinhibition boxes) made of aluminium (Figure 4.1 AD). The irradiance at the surface of the leaf upon illumination was 1300 µmol photons m$^{-2}$ s$^{-1}$ of monochromatic light (460±10 nm or 660±10 nm). The sample was illuminated for 30, 60, 120, 180 and 300 min. The control group was kept in identical conditions in darkness inside the photoinhibition boxes. Light was provided by 16 LED’s of 3 W each bolted onto the lid of the aluminium boxes (Figure 4.1 a and d). In order to suppress the heat irradiated from the LED array, the box lid was attached to a cooling block (Figure 4.1 a and d). Water at 4°C passed through the cooling block, and in this way not only was the heat removed but also the stability of the LED sources was ensured (Figure 4.1 f). The temperature of the box lid was 6°C at the plate and 12°C at the LED. The sample was kept floating in contact with 4°C water inside the box (Figure 4.1 h).
4.2.3 Photodamage in vitro

All sample handling for BBY-particles was done at all times at 4°C. The BBY-particles stock suspension was diluted with a standard buffer and the concentration of chlorophyll was adjusted to 150 µg mL⁻¹. Then the sample was transferred to sterile plastic Petri dishes. The optical path length was 2.5 mm. To ensure that the volume remained the same during the whole illumination period, Petri dishes were closed and sealed with Parafilm. Each Petri dish was transferred to the LED photoinhibition boxes. The Petri dish was kept in contact with water at 4°C. The irradiance at the surface of the liquid was 1300 µmol photons m⁻² s⁻¹. Samples were illuminated with either 460 or 660 nm light for 1, 2.5, 5, 20 or 60 min. BBY-particles were then centrifuged at 20,000 x g, after which they were re-solubilized in standard buffer for cryogenic storage. The sample then was flash frozen in liquid nitrogen (LN2) and stored at −80 °C until use.

4.2.4 PSII activity measurements

The Petri dishes that contained the leaves were removed from the light and were dark adapted for at least 15 min at room temperature. Each leaf was measured from the base to the tip in three different regions. After the measurements, the leaves were transferred back to the LED photoinhibition boxes. The PSII efficiency was measured at room temperature with a Multichannel-Plant efficiency Analyzer 2 (M-PEA, Hansatech Instruments for the full description of the instrument see Strasser et al. 2010). The wavelength of the excitation,
saturating and actinic light was 660 nm with an intensity of 3000 µmol of photons m$^{-2}$ s$^{-1}$ for 10 s. All sample manipulation occurred under low light of less than 1 µmol photons m$^{-2}$ s$^{-1}$.

To measure the PSII efficiency of BBY-particleless, the sample was defrosted at 4°C and then diluted with Buffer B and centrifuged at 20,000 x $g$. Then the concentration of the BBY-particles was adjusted to 150 µg Chl mL$^{-1}$. PSII efficiency was addressed by measuring Chl $\alpha$ fluorescence in the same way as described above. 30 µL of the sample was transferred to wet filter paper before measurement. All sample manipulation occurred under green light with an intensity of 1 µmol photons m$^{-2}$ s$^{-1}$ or in complete darkness.

4.2.5 Time Correlated Single Photon Counting (TCSPC)

Excitation was carried out by 0.2 ps excitation pulses (412 nm) at a repetition rate of 3.8 MHz. The excitation power was reduced with neutral density filters and the detection rate was kept below 30,000 photons s$^{-1}$. The diameter of the excitation spot was 2 mm, and the excitation laser power was kept at 1-5 µW to keep PSII reaction centers (RC) in the open state. The sample was kept in a flow cuvette (flowing speed $\sim$2.5 ml s$^{-1}$) connected to a sample reservoir (7.5 mL), kept at 20°C. The optical path length of the cuvette was 3 mm and the optical density of the sample was 0.1 per cm. Fluorescence was collected at right angle to the excitation beam, under magic angle (54.7°) polarization with a 680 nm interference filter (15 nm bandwidth), as described previously (Somsen et al., 2005).

The instrument response function (IRF) was obtained from the 6 ps decay of pinacyanol iodide in methanol (van Oort et al., 2008) (the full width at half maximum of the IRF was 35 ps and a resolution of 2 ps per channel was used). Data analysis was performed with a home-built program (Novikov et al., 1999, Digris et al., 1999). For the global analysis the decay lifetimes were kept equal for each run of measurements on the sample exposed to a certain illumination time. The fit quality was judged from the residuals of the fit. Fluorescence decay curves were fitted to a sum of exponentials with the amplitudes $a_i$ and fluorescence decay times $\tau_i$, convoluted with the IRF (Digris et al., 1999). Three components were used to fit the average lifetime and TCSPC measurement took 3 min.

Average lifetimes were calculated as:

$$\tau_{AV} = \sum_{i=1}^{N} a_i \times \tau_i$$

Equation 4.1

where $\sum_{i=1}^{N} a_i = 1$ and $\tau_i$ is the lifetime of the $i$-th component.
4.2.6 Two-photon Fluorescence lifetime Imaging Microscopy (FLIM)

Imaging was performed on a multiphoton Biorad Radiance 2100 MP system, coupled to a Nikon TE300 inverted microscope. A tunable Ti-Sapphire laser Coherent Mira was used as an excitation source. It was pumped with a 5 W Coherent Verdi laser. Excitation pulses of 860 nm wavelength were 250 fs long and a repetition rate of 76 MHz was used. The laser beam was focused by a Nikon 60x water immersion Apochromat objective (NA 1.2). The fluorescence was detected by non-descanned direct detectors via a Hamamatsu R3809U photomultiplier, operated at 3.1 kV. Fluorescence was selected using a 680 nm interference filter (13 nm bandwidth) and a 770 nm cut-off filter was used to prevent detection of the excitation beam. A neutral density filter was used in the excitation path to reduce the excitation light intensity. The output of the detector was coupled to a Becker&Hickl single-photon counting module (SPC 830) (Becker and Bergmann, 2003). The time window was set to 256 channels and fluorescence was recorded for 5 min at a count rate of 10,000 counts s\(^{-1}\). The instrument response function (IRF) of 25 ps was obtained from the 6 ps decay of pinacyanol iodide in methanol.

SPCImage software from Becker&Hickl was used to process FLIM images. Fluorescence decay curves were fitted to a sum of \(N\) exponentials, convoluted with the IRF. Average lifetimes were calculated using equation 4.1. Three components were used to fit the average lifetime and FLIM measurement took 5 min.

4.2.7 Statistical analysis

All measured \(F_v/F_m\) signals and obtained changes in the fluorescence lifetimes were processed and fitted using OriginPro9.1 software. Changes in PSII activity were fitted, assuming first-order reaction kinetics, to a single-exponential decay function, using Equation 4.2:

\[
A_{PSII}(t) = A_0 e^{-k_{PI} t} + y_0 \quad \text{Equation 4.2}
\]

where \(A_{PSII}(t)\) is the PSII efficiency at a given time \(t\), \(t\) is the time in h or min, \(A_0\) is the activity before illumination and \(k_{PI}\) the rate constant of photoinhibition. Finally, \(y_0\) is the residual of the PSII activity.

The changes in the average fluorescence lifetimes for leaves and BBY-particles during photodamage were fitted according to Equation 4.3:

\[
\tau_{AV}(t) = \tau_{AV0} \times \sum_{i=1}^{n} a_i \times e^{-k{T_i}^* t} + y_0 \quad \text{Equation 4.3}
\]
where $\tau_{AV}(t)$ is the average fluorescence lifetime after a given illumination time (see Table A.1 and A.2 at the Appendix section), $\tau_{AV0}$ is the average lifetime of a non-damaged sample, $n$ is the number of decay components ($n > 1$), $t$ is the illumination time (duration of illumination), $a_i$ is the amplitude of the $i$-th component, $k_{Ti}$ is the corresponding rate constant that describes the change in lifetime of this component, and $y_0$ is the residual of the $\tau_{AV}$.

4.3 Results.

4.3.1 PSII photodamage is accompanied by a shortening of $\tau_{AV}$

From (Tyystjarvi and Aro, 1996) it is known that the loss of $F_v/F_m$ reflects the magnitude of PSII photodamage if the repair is absent. In order to test if a decrease of $\tau_{AV}$ also occurs in the $F_o$ state during PSII photodamage, photosynthetic samples (leaves and BBY-particles) were illuminated with 1300 µmol photons m$^{-2}$ s$^{-1}$ and the time course of PSII photodamage was determined by measuring the changes in PSII efficiency ($F_v/F_m$) in parallel with the changes in $\tau_{AV}$ (Figure 4.2). It can be observed that for both illumination wavelengths, 460 and 660 nm, the efficiency of PSII decreased. The decrease of the efficiency was fitted using Equation 4.2 and the fit parameters are presented in Table 4.1. Data for Figures 4.2 a c were fitted to a single exponential, whereas changes in the $\tau_{AV}$ in panel d were fitted to a double exponential.
Figure 4.2 Changes in PSII efficiency and average fluorescence lifetimes after illumination with 460 nm (blue data points) and 660 nm (purple data points) light. Effect of photodamage on PSII efficiency ($F_{v}/F_{m}$) in (a) spinach leaves, (b) BBY-particles. Changes in average fluorescence lifetimes upon illumination, measured on (c) spinach leaves, (d) BBY-particles. Fitting results using equations 4.2 and 4.3 are represented by continuous lines. Data presented here was calculated from 3-8 technical repeats, each experiment was repeated in three times at different days.

In order to facilitate the comparison between different treatments, the time point where 50% of the activity remains ($T_{50} = \ln 2/k_P$) was compared for both experimental models. It can be observed that for 460 nm illumination $T_{50}$ is smaller than for 660 nm (see Table 4.1). These data are in good agreement with the previously reported action spectra of photodamage for different species (Jones and Kok, 1966b, Jung and Kim, 1990, Hakala et al., 2005, Ohnishi et al., 2005, Sarvikas et al., 2006, Takahashi and Murata, 2008) where the blue region of the visible spectrum induced a higher degree of damage than the red region. In a similar manner PSII efficiency of BBY-particles (Figure 4.2 b) can be described best with a first-order reaction equation. As was observed for leaves, BBY-particles showed shorter $T_{50}$ values for 460 nm than for 660 nm illumination, and the ratio between the two $T_{50}$ values for both wavelengths is similar to the one observed for leaves (see Table 4.1). In both systems no repair is present, and it is concluded from Figures 4.2 a and b that photodamage occurs in both cases and more effectively with 460 nm blue light.
The photodamage was accompanied by a clear shortening of $\tau_{AV}$ in both leaves and BBY-particles, as presented in Figures 4.2 c and d. In the case of leaves (Figure 2 c) illumination with 460 nm light induced a faster shortening of $\tau_{AV}$ than illumination with 660 nm light. This shortening of $\tau_{AV}$ follows a single-exponential decay and the fitting parameters are presented in Table A.1 (Appendix 1).

**Table 4.1. Values of half-lifetime ($T_{50}$) obtained for changes in PSII efficiency ($F_v/F_m$) and $\tau_{AV}$ for 460 and 660 nm illumination in leaves and BBY-particles.** The first ratio 660/460 reflects the ratio of $T_{50}$ values obtained for $F_v/F_m$ at 660 and 460 nm. The second ratio 660/460 reflects the ratio of $T_{50}$ values obtained for $\tau_{AV}$ at 660 and 460 nm. The amplitudes for each curve are presented in parenthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\lambda$ (nm)</th>
<th>$T_{50}$ ($F_v/F_m$)</th>
<th>Ratio 660/460</th>
<th>$T_{50}$ ($\tau_{AV}$)</th>
<th>Ratio 660/460</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td>460</td>
<td>1.8±0.3 (0.6)</td>
<td>1.81</td>
<td>0.9±0.2 (242)</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>3.3±1.0 (0.6)</td>
<td></td>
<td>1.55±0.6 (197)</td>
<td></td>
</tr>
<tr>
<td><strong>BBY particles</strong></td>
<td>460</td>
<td>15.4±5.0 (0.5)</td>
<td>1.93</td>
<td>4.5±0.3 (22 + 27)</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>660</td>
<td>29.8±9.0 (0.5)</td>
<td></td>
<td>4.9±0.3 (25 + 31)</td>
<td></td>
</tr>
</tbody>
</table>

* Units in hours for $T_{50}$ ** Units in minutes for $T_{50}$

BBY-particles also show a difference in $\tau_{AV}$ for 460 nm and 660 nm light, but the difference is not statistically significant (Table 4.1). The observed kinetics does not follow a simple first-order rate law; at least two components are needed (Equation 4.3). This means that there were (at least) two distinct populations of BBY-particles. It is remarkable that for both wavelengths most of the changes in $\tau_{AV}$ occur during the first minutes of illumination.

It has been hypothesized that PSII photodamage will increase the level of fluorescence in the $F_o$ state (Björkman, 1987, Tyystjärvi and Aro, 1996). An increase of $F_o$ corresponds to an increase of the average fluorescence lifetime of PSII. In Matsubara and Chow (2004) it was reported that even in the absence of NPQ, $\tau_{AV}$ was decreasing, but the decrease was observed for closed PSII RCs ($F_m$ state). In the dark adapted state, that is the state in which samples were measured, the fluorescence lifetime is the shortest since all RCs are completely open (Holzwarth et al., 2009, Vasil'ev and Bruce, 1998, Miloslavina et al., 2009). Energy cannot be used faster by the RCs than in the dark adapted state. Therefore we interpret the observed shortening of $\tau_{AV}$ as an energy dissipation event which is different from primary photochemistry.

4.3.2 *The change in fluorescence lifetime precedes PSII photodamage*
If the changes in $\tau_{AV}$ are a consequence of the same photodamage that leads to the decrease of $F_{v}/F_{m}$, they should occur more slowly or at most as fast as changes in PSII efficiency, which apparently is not the case. In order to look at this in more detail we replotted the data in double Y axis graphs in Figure 4.3.

![Figure 4.3](image.png)

**Figure 4.3** Changes in the average fluorescence lifetimes (blue line for 460 nm illumination and purple for 660 nm), and changes in PSII efficiency $F_{v}/F_{m}$ (black line) for (a) leaves and (b) BBY-particles for 460 nm illumination; and (c) leaves and (d) BBY-particles for 660 nm illumination. Data presented here was calculated from 3-8 technical repeats, each experiment was repeated in three times at different days.

In Figure 4.3 a and c the data for leaves are presented, showing that the changes in $\tau_{AV}$ occur faster than the changes in PSII efficiency for both wavelengths (the quencher accumulates around 2 times faster than the inactivated PSII, see Table 4.1). This implies that the change in fluorescence lifetime is not a direct consequence of PSII inactivation. In a similar fashion, BBY-particles (Figure 4.3b and d) showed a faster change in $\tau_{AV}$ than in PSII efficiency: $T_{50}(\tau_{AV})$ is 3.4 times shorter than $T_{50}(F_{v}/F_{m})$ in case of 460 nm excitation, while for 660 nm it is even 6.1 times shorter. Despite the differences in complexity (BBY-particles do not have an unlimited source of electron acceptors, PSI is absent, other molecular pathways are not present, etc.), both experimental model systems show strikingly similar behaviour in this respect.
It is interesting that both experimental model systems showed: (1) single exponential photodamage; (2) faster photodamage induced by 460 nm than by 660 nm light; (3) shortening of $\tau_{AV}$; (4) a faster decrease of $\tau_{AV}$ than of PSII activity. All these observations support the idea that the mechanism(s) of photodamage and quenching in vitro and in vivo is/are similar, although some authors have hypothesized that the mechanism of photodamage is different in vivo and in vitro (Kirilovsky et al., 1994, Melis, 1999, Tyystjärvi, 2008, Vass, 2011).

4.3.3. Two-photon FLIM shows that there is more than one chloroplast population during photodamage.

Whereas the photodamage behaviour seems to be similar for leaves and BBY-particles, it is evident that the ratio $T_{50}(FV/FM)/T_{50}(\tau_{AV})$ is different for both systems. Moreover, BBY-particles show a double exponential decay for $\tau_{AV}$ upon illumination, whereas the changes in leaves fit well to a single exponential decay. So, where is this difference coming from?
Figure 4.4 The change of the average fluorescence lifetimes of chloroplasts in leaves illuminated with 460 nm light (1300 µmol photons m$^{-2}$ s$^{-1}$). Representative FLIM micrographs are shown for each time point (0 h, 1 h, 2 h, 3 h and 6 h of illumination). Each histogram, which corresponds to the sum of individual histograms for 3-5 replicas, is shown on the right. Scale bars = 20 µm. Images were recorded with a 680 nm bandpass filter with a bandwidth of 13 nm. The dotted line represents the mean average lifetime calculated from individual micrographs. Note that the mean average lifetime is not located in the centre of the distribution and does not coincide with the median or the mode which would be expected for a single Gaussian population, indicating that there is more than one population.
In agreement with previous experiments (Tyystjarvi and Aro, 1996, Sarvikas et al., 2010, Nixon et al., 2005, Nishiyama et al., 2005, Six et al., 2007, Tyystjärvi et al., 1994, Loebl et al., 2010, Six et al., 2009, Key et al., 2010, Kou et al., 2012), we found that photodamage follows a single-exponential kinetics, which has been interpreted as an irreversible process that occurs in one single population of PSII. However, even if changes in PSII efficiency \( (F_{V}/F_{M}) \) follow single exponential kinetics, changes in other parameters, such as fluorescence lifetimes, still can follow double exponential kinetics. It has been argued by Sarvikas et al. (2006, 2010) that because PSII efficiency decays according to single-exponential kinetics: (1) all PSII complexes “die” in an equal manner during photodamage (photodamage occurs in single exponential manner) and (2) the formation of quencher does not protect from photodamage (all NPQ mutants had virtually the same action spectrum of photodamage). In order to explore if heterogeneity exists in the PSII pool in leaves during PSII photodamage, which would reflect if all PSII is affected in the same fashion or not, fluorescence lifetime images were analysed during the time course of photodamage. The results are presented in Figures 4.4 and 4.5.

In Figure 4.4 the changes in \( \tau_{AV} \) of leaves after 460 nm illumination are shown. On the left side of the figure the \( \tau_{AV} \) values are presented in false colours. Colours correspond to values of \( \tau_{AV} \) ranging from 0 ps (red) to 700 ps (blue). The various images taken at different time points show that \( \tau_{AV} \) shortens during the illumination experiment. Also, it can be concluded from the distributions of \( \tau_{AV} \) that there is not a single population distribution, because the histograms (even before illumination) are not symmetric and the average value of \( \tau_{AV} \) does not match with the median or the mode of the distribution. As the light treatment advanced, \( \tau_{AV} \) shifted to shorter values and the asymmetry remained throughout the treatment. Since the FLIM measurements were performed on the same (upper) layer of cells, we can discard uneven illumination and monitoring different cell layers as an explanation for this difference. However, the microenvironment of each chloroplast is different and the effect of shading by other chloroplasts due to chloroplast movement (Chow et al., 1988) is possible. Nevertheless, there is clear evidence that within one cell there is a light gradient which may alter the antenna size in chloroplasts located at different depths, and therefore a different amount of photodamage is expected for chloroplasts present in the same cell (Oguchi et al., 2011a) (Vogelmann and Evans, 2002).

For 660 nm illumination (Figure 5) a similar trend as for 460 nm can be observed. The \( \tau_{AV} \) values change from long (blue) to short (red). There is an asymmetry present in each of the histograms (presented the right).
Figure 4.5 The change of the average fluorescence lifetimes of chloroplasts in leaves illuminated with 660 nm light (1300 µmol photons m$^{-2}$ s$^{-1}$). Representative FLIM micrographs are shown for each time point (0 h, 1 h, 2 h, 3 h and 5 h of illumination), and the histogram which corresponds to the sum of the individual histograms obtained for several replicas is shown on the right. Scale bars = 20 µm. Conditions were the same as Figure 4.6. The dotted line represents the mean average lifetime calculated from individual micrographs.

The asymmetry in the $\tau_{av}$ histograms observed in this work suggests that more than one population of chloroplasts might exist, indicating that even if the time course of PSII
photodamage follows first order kinetics, there could still be several PSII populations, perhaps present in different chloroplasts.

4.4 Discussion

4.4.1 What is the origin of the decreased value of $\tau_{AV}$?

In this work we address the issue that photodamage is accompanied by a decrease in fluorescence and in $\tau_{AV}$. We start by listing 5 scenarios that might in principle explain a shortening of $\tau_{AV}$ and then discuss which of them can also be responsible for the decrease in $\tau_{AV}$, that we observed.

(1) quenching as a consequence of PSII photodamage (Kyle et al., 1984, Kirilovsky et al., 1994);


(3) reorganization of the thylakoid membrane (Iwai et al., 2010, Ünlü et al., 2014);

(4) dissipation of excessive light energy by inactivated PSII RCs (Matsubara and Chow (2004); and

(5) accumulation of a photoproduct, not related to PSII photodamage itself (Barzda et al., 2000);

**Scenario 1.** This scenario would imply that the observed quenching effect is a consequence of photodamage, that inactivates PSII, but this is not the case, since the change in $\tau_{AV}$ occurs with a faster rate than the accumulation of PSII photodamage. Several previous studies about photodamage of isolated PSII particles have reported a clear decrease in the lifetimes of different samples (Vass et al., 1993, Renger et al., 1995, Gilmore et al., 1996, Richter et al., 1999). The quenching of fluorescence is commonly interpreted as a consequence of a detrimental process caused by inactivation of the acceptor side or the RC itself (Kyle et al., 1984, Kirilovsky et al., 1994). However, most studies focused on the decrease of the variable fluorescence only. In those studies only isolated particles were used and it was assumed that those particles lack any kind of protective mechanisms. However, the present work demonstrates that changes in $\tau_{AV}$ develop faster than photodamage in vitro and in vivo.

**Scenario 2.** NPQ is a collective term for various photoprotection mechanisms that are switched on in high light and lead to the formation of fast dissipative channels, which shorten the
excited-state lifetimes and concomitantly the observed fluorescence lifetimes. Most NPQ subprocesses like qE, qZ, and qT are rapidly reversible (Müller et al., 2001, Niyogi, 1999, Ruban et al., 2004) and cannot explain the shortening of the fluorescence lifetime for leaves because the measurements were performed many hours after illumination. Also, NPQ cannot explain the lifetime shortening for BBY-particles, since no proton gradient, xanthophyll cycle, or persistent levels of zeaxanthin are present in these preparations.

The only NPQ subprocess that is slow enough to be a candidate to explain the quenching is qI, since it relaxes over many hours (Krause and Jahns, 2004, Schansker et al., 2006). One of the main components of qI, D1 turnover, cannot operate in lincomycin treated leaves (Aro et al., 1993a). Whereas in normal leaves qI is reversible because of D1 repair, here it must be irreversible because of the presence of lincomycin. Other molecular mechanisms underlying qI, such as a long lasting ΔpH in the dark, can also be ruled out as an explanation of the observed mechanism, because it has been demonstrated that in conditions where ΔpH has been removed by chemical treatments fluorescence quenching still occurs (Matsubara and Chow (2004)).

Therefore, the observed quenching effect cannot be directly related to the previously reported NPQ mechanisms qE, qZ, and qT, but it might be related to qI.

**Scenario 3.** In principle, a change in lifetime could also be explained on the basis of conformational changes of the thylakoid ultrastructure, which occur for instance during state transitions. However, state transition themselves should be reversible, and the samples should go back to the unquenched state when the samples are dark-adapted again. It is believed that during the reorganization of the thylakoid membrane in state transitions, PQ reduction leads to LHCII phosphorylation, which disrupts the highly stacked membrane, which can lead to fluorescence quenching, for instance due to increased spillover from PSII to PSI (Zer et al., 1999), or due to aggregation of antenna complexes (Iwai et al., 2010, Ünlü et al., 2014). Matsubara & Chow (2004) have demonstrated that fluorescence quenching during photodamage occurs as well in leaves treated with DTT and nigericin, which prohibits the energization of the thylakoid membrane by disrupting the ΔpH (Matsubara and Chow, 2004). In such conditions, the thylakoid reorganization should not occur. Additionally, in the case of BBY-particles no reorganization takes place in the membranes since they are truncated.

**Scenario 4** has been proposed by Matsubara and Chow (2004), where PSII photodamage causes the formation of a quencher (the reaction center of photodamaged PSII) that has the ability of protecting active PSII by dissipating excessive excitations. This scenario is dependent
on PSII photodamage, as the quenchers are the inactivated PSII. It is possible that photoinactivated PSII contributes to the formation of the quencher. However, this scenario does not explain why the accumulation of the quencher is faster than photodamage.

**Scenario 5** would imply that in high light a photoproduct is generated that acts as a quencher of PSII fluorescence, but is not related to PSII photodamage. For example, evidence from Barzda et al. (2000) suggests that long-lived quenchers can be formed in LHCII complexes, thereby explaining shortening of $\tau_{AV}$, and since both candidates are common to both experimental models, scenario 5 offers a plausible explanation for the experimental observations.

### 4.4.2 Does the quencher protect PSII from photodamage?

If a quencher (or quenchers) is (are) formed, it must be long lived, since its effect can be observed even after 45 min of dark adaptation. This implies that the quencher is a stable species and it accumulates faster than the PSII efficiency decreases; in other words the accumulation of the quencher precedes the occurrence of photodamage. The faster formation of the quencher as compared to the accumulation of photodamaged PSII has two implications: (1) it is a protective mechanism and (2) quencher formation depends only on illumination and not on photodamaged PSII accumulation because the two kinetics (photodamage and change in average lifetime) do not resemble each other. In the context of the excessive energy (Vass et al., 1993, Vass and Cser, 2009, Vass, 2011, Vass, 2012) and the hybrid paradigms (Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b, Schreiber and Klughammer, 2013) quencher formation can operate as a photoprotective mechanism, by helping to dissipate excess excitation energy. In contrast, for the paradigm of direct damage to the Mn cluster quencher formation does not alleviate photodamage, because excessive excitation is not the cause of inactivation (Hakala et al., 2005, Ohnishi et al., 2005, Tyystjärvi, 2008, Sarvikas et al., 2010). As demonstrated in Chapter 3, excessive light absorption does cause PSII photodamage and for that reason it would be expected that decrease in the excitation pressure can protect from photodamage. Perhaps, the previously reported quenchers tested (Tyystjarvi et al., 1999, Santabarbara et al., 2001a, Nishiyama et al., 2006, Sarvikas et al., 2006) showed low efficiency in protection against PSII photodamage as they do not act directly in PSII or because they are long term adaptations to light exposure.

It is controversial whether quenching of excited states protects PSII from photodamage (Tyystjarvi et al., 1999, Santabarbara et al., 2001a, Nishiyama et al., 2006, Sarvikas et al., 2006). Experiments done by Sarvikas showed that the action spectrum of photodamage is the same
for NPQ-less mutants and the wild-type Arabidopsis plant, which has been interpreted by the authors as evidence that NPQ does not prevent PSII photodamage (Sarvikas et al., 2006). Meanwhile artificial quenchers (Tyystjarvi et al., 1999, Santabarbara et al., 2001a) did not slow down photodamage significantly. Nevertheless, those quenching mechanisms cannot be compared to the mechanism presented in this work. We have seen in this study that at wavelengths where photodamage (indicated by a loss of PSII efficiency) was greater, also more quencher was formed (indicated by a shorter lifetime). This indicates that quencher formation was accompanying photodamage. The observation that the average Chl fluorescence lifetime declined ahead of that of PSII efficiency could be due to photoprotection of remaining active PSII complexes by the dissipation of excess excitation energy by the quenchers. That is, in this scenario, the quenchers photoprotect PSII by dissipation of the energy absorbed by photosynthetic pigments. If only light absorption by the Mn cluster could lead to photodamage, then the quencher would not exert a protective role.

The shortening of $\tau_{AV}$ is a shared event \textit{in vivo} and \textit{in vitro} and supports the idea that it is intrinsic to the PSII complexes. However, the present findings do not support the location of the quencher in inactive PSII because then the change in the lifetime would happen simultaneously with the change in PSII efficiency, but the change of $\tau_{AV}$ actually happens on a 2-6 times shorter time scale. Based on the difference of $T_{50}$ for photodamage and the change in $\tau_{AV}$, it is known that when 50% of the inactive centres are formed, the quencher is already maximally accumulated.

One could argue that the change in $\tau_{AV}$ may not be directly proportional to the concentration of quencher and even a small population of inactive PSII may be enough to quench excessive excitation, thereby protecting from photodamage; however, for this to happen this small population should be distributed homogenously across the thylakoid membrane near all active PSII, which is a possibility that cannot be discarded by the present data.

4.4.3 Wavelength dependence and the quenching of fluorescence.

The blue region of the visible light causes higher rates of PSII photodamage than green-red light (Jones and Kok, 1966a, Jung and Kim, 1990, Santabarbara et al., 2001b, Santabarbara et al., 2002, Hakala et al., 2005, Ohnishi et al., 2005, Sarvikas et al., 2006, Santabarbara et al., 2007, Takahashi et al., 2010, Schreiber and Klughammer, 2013, He et al., 2015, Karim et al., 2015). This has been explained before by \textit{direct damage to the Mn cluster} (Hakala et al., 2005, Ohnishi et al., 2005), since it has been hypothesized that the Mn cluster absorbs photons in the UV-blue region, whereas absorption in the green-red region is absent, or at least strongly
diminished (Nishiyama et al., 2006, Murata et al., 2007, Nishiyama et al., 2007, Takahashi and Murata, 2008, Tyystjärvi, 2008, Takahashi and Badger, 2011, Tyystjärvi, 2012, Tyystjarvi, 2013, Nishiyama and Murata, 2014, Hakala et al., 2005, Schreiber and Klughammer, 2013, Hakala et al., 2006, Hou and Hou, 2013). Also in this work we have observed for both experimental model systems that 460 nm light is more efficient in inducing damage to PSII than 660 nm light. However, only for leaves have we observed that the rate of change of $\tau_{AV}$ upon 460 nm illumination is higher. According to the direct damage to the Mn cluster paradigm quenching of excitations does not protect from PSII photodamage (Tyystjärvi, 2008, Takahashi and Badger, 2011, Tyystjärvi, 2012, Tyystjarvi, 2013, Nishiyama and Murata, 2014). If that argument is true and damage to the Mn cluster is the only cause of PSII photodamage, the formation of a quencher before photodamage cannot act as a protective mechanism because photodamage is independent of excessive excitation. In such case the fluorescence quencher might not be formed faster upon illumination with blue light. Perhaps, the fact that 460 nm illumination is more efficient to induce damage is due to a combination of two mechanisms, excessive energy and damage to the Mn complex (Ohnishi et al., 2005, Oguchi et al., 2011a, Oguchi et al., 2011b, Oguchi et al., 2013, Schreiber and Klughammer, 2013), and the quencher observed in this work only alleviates the damage induced by excessive light energy. Furthermore, the fact that the $T_{50}$ ($\tau_{AV}$) is not significantly different in BBY-particles, for both wavelengths, further supports the role of excessive light energy during photodamage.

4.4.5 Multiple PSII populations during photodamage?

Even though there are reports that at least two PSII populations are formed during PSII photodamage (Matsubara and Chow, 2004), Sarvikas et al. (2010) have proposed that since the time course of PSII photodamage follows first-order kinetics, there cannot be two PSII populations in terms of photodamage. As there is only one population of PSII, Sarvikas et al, suggest that the inactivated portion of the PSII does not protect the active portion. The change in $\tau_{AV}$ as obtained from the TCSPC measurements on BBY-particles can be described by double-exponential kinetics, which suggests that at least two populations with characteristic $T_{50}$ exist during photodamage. In the case of the FLIM data, we have observed asymmetry in the $\tau_{AV}$ histograms, which suggests that there are several PSII populations. As a consequence, our TCSPC and FLIM data do not support the claims of Sarvikas et al. of the existence of one single PSII population during photodamage. Those populations are characterized by different lifetimes, which reflects heterogeneity in the antenna size of PSII pool. Assuming that the mechanism proposed in Chapter 3 is also present in vivo, heterogeneity in the formation of the quenchers must have influence the photodamage process. In that case, one should expect a
deviation of the photodamage kinetics from a first order rate law, which was not the case of the present work. Perhaps, the observed heterogeneity is a result of chloroplast movement and shading that can only be observed using microscopy. Because Fv/Fm was measured across a large portion of the tissue, the heterogeneity of single chloroplast is not observed. For this reason it is important to obtain information of photosynthetic activity at the microscopic level to check the existence of heterogeneity during photodamage.

Our findings agree with those described by (Matsubara and Chow, 2004) since the decrease in the lifetimes accompanies photodamage. Whether multiple PSII populations originate from inactive and active PSII or from intrinsic heterogeneity in the PSII pool cannot be answered by the present results.

4.4.6 Quenching cannot be fully explained by PSII photodamage

We cannot fully discard the possibility that a small component of the observed quenching phenomenon is due to direct damage of PSII. However, if PSII is photodamaged, no photochemistry should occur. As a consequence the fluorescence lifetime should become longer as it happens in non-Q\textsubscript{A} reducing centers or isolated antennas which usually have average lifetimes of nanoseconds (Barzda et al., 2001, Miloslavina et al., 2011). This would lead to an overall increase of the lifetimes (Broess et al., 2009), but in the present work we have seen that the lifetimes became shorter as the illumination time increased. For that reason, photodamage cannot be considered as the main cause of the observed quenching phenomenon.

4.5 Conclusions

In the present work, it is shown that the fluorescence quenching that accompanies PSII photodamage is a long-lived phenomenon; the quenching occurs even when PSII has been dark adapted for a long time. The observed formation of the quencher cannot be ascribed to the classical NPQ mechanisms. During PSII photodamage, the fluorescence quencher accumulates faster than light induced loss of PSII efficiency both in vivo and in vitro. For this reason we propose that the quenching is expected to act as a photoprotection mechanism. Preventive dissipation of the excessive energy absorbed by photosynthetic pigments is consistent with the role of excessive energy and/or hybrid paradigms to explain PSII photodamage.
Chapter 5: DISCUSSIONS AND FINAL REMARKS

5.1 Chapter summary.

In this thesis several aspects of PSII photodamage and photoprotection were studied. Chapter 2 focused on damage of the Mn$_4$CaO$_5$ cluster by light of different wavelengths. It presents evidence that the Mn$_4$CaO$_5$ cluster is inactivated in red, green and blue regions of the visible spectrum. Also, it was possible to separate the damage to the RC and the damage of the Mn$_4$CaO$_5$ cluster. In this manner, it is reported that the damage to the Mn$_4$CaO$_5$ cluster occurs faster than the damage to the RC, even in red illumination. This presents for the first time evidence that the Mn$_4$CaO$_5$ cluster is also inactivated by red light, questioning the previous assumptions that the damage to the Mn$_4$CaO$_5$ cluster only occurs in the UV-Blue region of the light spectrum (Vass, 2012, Ohnishi and Murata, 2003, Hakala et al., 2005, Tyystjärvi, 2008). However, new questions raised based on the obtained results are: (1) What is the mechanism of photodamage of the OEC? (2) Does excessive light energy play an important role of photoinhibition? and (3) Is direct absorption of light by the Mn happening?

These questions were answered in Chapter 3, when the Mn$^{2+}$ release data and PSII activity were compared in terms of the three available theories (1) direct absorption of light by the Mn (Hakala et al., 2005, Ohnishi et al., 2005, Tyystjärvi, 2008, Tyystjärvi, 2012), (2) acceptor side limitations (Vass et al., 1992, Vass and Cser, 2009, Vass, 2012) and (3) dual (hybrid) mechanism of photodamage (Oguchi et al., 2009, Oguchi et al., 2011a, Oguchi et al., 2011b, Schreiber and Klughammer, 2013). It was confirmed that photodamage primarily occurs at the Mn$_4$CaO$_5$ cluster at 460 and 660 nm. However, it was also found that the inactivation of the Mn$_4$CaO$_5$ cluster is ruled by limitations in the acceptor-side. This data explains why in Chapter 2 a clear dependence of PSII photodamage on the wavelength was observed, where red induced more damage than green (as it is driven by photosynthetic pigments). This rules out the possibility that direct absorption of light by Mn for wavelengths between 460 to 660 nm is the main cause of photodamage. Furthermore, it was shown that that the release of Mn ions is not the cause of inactivation of the Mn$_4$CaO$_5$ cluster but a much later consequence. All these suggest that another agent is the cause of the inactivation of the Mn cluster, and this agent is dependent on the limitations on the acceptor side. As a final point, a new mechanism is presented, where the photodamage occurs at two sites due to limitations on the acceptor side: (1) damage to the OEC and (2) damage to the RC. This new mechanism reconciles the differences of the three hypotheses since it explains that there are two sites of photodamage, there is Mn release and it is not dependent on the wavelength.
Concerning the identity of the agents that induce damage to the Mn₄CaO₅ cluster, in Chapter 3, it is hypothesized that the agents are ROS, in particular \(^1\text{O}_2\), or other oxygen-containing radical species. Based on the evidence provided in Chapters 2 and 3 and in the literature provided by some authors (Jung and Kim, 1990, Song et al., 2006, Ballottari et al., 2013) an explanation of the full phenomena is presented. It is known, from the work of (Jung and Kim, 1990), that singlet oxygen production is greater in the blue regions of the visible spectrum than in the red regions. The action spectrum for production of singlet oxygen and photodamage matches almost exactly. Based on the results in Chapter 3, one knows that limitations of the acceptor side play an important role in the Mn₄CaO₅ inactivation. Most probably, as presented by several authors (Macpherson et al., 1993, Hideg et al., 1994, Telfer et al., 1994, Hideg et al., 1998, Rinalducci et al., 2004, Hideg et al., 2007, Triantaphylidès et al., 2008), limitations on the acceptor side induce either the accumulation of ROS or other radical species. These radical species somehow disrupt the Mn₄CaO₅ cluster, inactivating it. However, this hypothesis does not rule out the possibility of direct absorption of the Mn by shorter wavelengths (<460 nm). In the absence of the Mn cluster, limitations on the donor side occur, which will damage the reaction center.

Chapter 4 revisited another controversial topic, the quenching of fluorescence and its protective mechanism of PSII. Based on Chapter 3, it can be accepted that excessive light energy does cause PSII photodamage in vitro. Then, any quenching of excitation presumably would protect PSII from photodamage. However, why do previously reported quenchers have such low efficiency in photoprotecting PSII? (Tyystjarvi et al., 1999, Santabarbara et al., 2001a, Nishiyama et al., 2006, Sarvikas et al., 2006). Matsubara and Chow (2004) proposed that the inactive PSII that could protect the active ones via quenching of excitation. (Sarvikas et al., 2010) questioned this hypothesis on the premises that, as PSII followed a single exponential kinetic, there are not two populations of PSII. By comparing the kinetic properties of PSII photodamage in leaves and BBY-particles, it was shown in Chapter 4 the existence of a quencher that is formed regardless of the wavelength even if the time course of photoinhibition follows a first order kinetic. Also, it was presented that the quencher is formed faster than PSII photodamage in both experimental models. If dissipation of the energy precedes photodamage, this would mean that less energy is reaching the reaction center. Since limitations on the acceptor side play an important role in the deactivation of the Mn₄CaO₅ cluster, then by reducing the amount of energy that reaches the reaction center the photodamage slowed down.
Further analysis of the FLIM micrographs showed that, intrinsically, there are different populations of PSII with characteristic lifetimes. These populations may suffer photoinhibition and response to it in different ways. Perhaps, the few reports where double exponential kinetics were observed have been affected by changes in the ratio between this two populations. Therefore, further experiments are required to be done to establish the nature of the two populations (see Section 5.3). Another novel feature of this quenching mechanism is that its longevity. Compared to regular forms of NPQ that are rapidly reversible in darkness (Li et al., 2002, Amarnath et al., 2012), the quencher effects found in Chapter 4 are extremely long lived: even in BBY-particles dark adapted for an hour it was still possible to observe the quencher. Even though the identity and location of the quencher remain unknown, it is likely that this quencher is associated with the previous reports of Barzda et al. (2000) or Matsubara and Chow (2004) based on some similarities between their work and the data presented here and the inability to associate it with previously reported NPQ mechanisms.

5.2 Answers to the initial questions.

At the end of Chapter 1 three questions were raised (1) What is the primary site of light induced damage in PSII? (2) Is the Mn$_4$CaO$_5$ cluster affected by all visible light? (3) What is/are the molecular mechanism(s) by which PSII gets inactivated? Based on the summary given above (see section 5.1) these three questions can be answered. The answer to (1) is that the Mn$_4$CaO$_5$ cluster is the primary site of damage in all conditions (see Chapter 2 & 3). The answer to (2) is yes, all visible light affects the Mn$_4$CaO$_5$ (see Chapter 2); however the causes that prevail at wavelengths longer or shorter than 460 nm may be different. It was shown in Chapter 3 that photodamage at 460 and 660 nm mainly occurs due to limitations on the acceptor side. However it was also observed that at 460 nm a small contribution could be explained by the direct absorption of light by the Mn$_4$CaO$_5$ cluster. It is possible that effects at wavelengths shorter than 460 nm, where the Mn light absorption is stronger, would be mostly driven by the direct absorption of light by the Mn$_4$CaO$_5$ cluster. This could explain, for example, why polychromatic blue light (400 to 470 nm) used in Chapter 2 induced greater PSII photodamage than green and red light. Finally, question (3) can be answered by the results in Chapter 3, in particular Figure 3.9 where acceptor side limitations due to excessive light energy are the main cause of PSII photodamage. It is important to note here that the identity of the exact agent ($1^O_2$, other ROS or radical species) is not fully known and further characterization would be required (See Section 5.3).
5.3 Future perspectives.

The author would like to finish with some perspectives about his own work. First, more characterization is required to understand the chemical agents of PSII photodamage. In this work, ROS have been hypothesized to be that agent based on the large body of evidence reported in the literature (Rehman et al., 2013, Bersanini et al., 2014, Hakkila et al., 2014, Sedoud et al., 2014). For this reason measurements of EPR using spin traps to detect radical species (Pospíšil, 2009) compared to the photodamage rate will be necessary. Even though the mechanism presented in Chapter 3 has been demonstrated to occur in BBY-particles, demonstration of this mechanism needs to be validated in vivo. Measurements in vivo are much more challenging than the in vitro measurements as the more variables intervene during photodamage.

In author’s opinion, the main evidence in vivo to support the direct damage of the Mn$_4$CaO$_5$ cluster is the similarity of spectra of PSII photodamage and the Mn-oxo model compounds (see Section 1.9). Yet, the action spectra are circumstantial evidence and their publication will only contribute to the field if high spectral resolution is obtained with high resolution monochromators (Zavafer et al 2015a). For sure, the increase of diversity of high power LED and the use of high throughput-put methods will improve many of the technical aspects for the measurements in the near future.

Also, it is clearly necessary to move from the so-called white light experiments and focus only in studies of monochromatic light. Exploration of the photodamage properties similar to the ones performed in Chapter 3 and 4 are required for wavelengths between 400 to 460 nm, as this region seems to be the borderline between the damage to the Mn$_4$CaO$_5$ cluster and the excitation dependent mechanism (Zavafer 2015a).

Concerning the identity of the quenchers found in Chapter 4, further analysis is required in order to determine whether the quencher comes from the reaction center or the antennas. To do so, comparisons between the average lifetime and photodamage in PSII cores (which lack antennas) would be required. It is expected that if the quenchers are located in the antenna, PSII cores would not present the quenching and the lifetimes should become longer. If the quencher is indeed located at inactive PSII the observed quencher will also be observed in PSII cores. Another important experiment is to perform fluorescence lifetime measurements in BBY-particles and leaves excited at 485 nm where the antennas are preferentially excited. In case of leaves this would require the use of single photon FLIM (Eckert et al., 2006). It is expected that if the quencher is located in the antenna, the average lifetime would be even
shorter at 485 nm. Alternatively, future experiments done in *chlorina* mutants (Ryrie, 1983) or other type of mutants such as NPQ mutants (Niyogi et al., 1998, Li et al., 2002) would be useful to understand if the quenching effects are present, or if they also precede photodamage like it has been demonstrated in spinach and BBY-particles in the present work. Until the identity of the quenchers is established, it would not be possible to determine the true magnitude of the novel photoprotection mechanism found in the present work.
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## Supplementary Table A1. Parameters for the observed kinetics for the time course of photodamage.

<table>
<thead>
<tr>
<th></th>
<th>PSII photodamage ($F_v/F_m$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>460 nm</td>
<td>660 nm</td>
</tr>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{50}$</td>
<td>1.82±0.25</td>
<td>3.30±1.04</td>
<td></td>
</tr>
<tr>
<td>$k_{PI}$</td>
<td>3.8E-01±0.5E-01</td>
<td>2.1E-01±0.7E-01</td>
<td></td>
</tr>
<tr>
<td><strong>Ratio $T_{50}$</strong></td>
<td></td>
<td></td>
<td>1.81</td>
</tr>
<tr>
<td>660/460</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td><strong>BBY-particles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{50}$</td>
<td>15.43±4.97</td>
<td>29.75±8.92</td>
<td></td>
</tr>
<tr>
<td>$k_{PI}$</td>
<td>4.5E-02±1.4E-02</td>
<td>2.3E-02±0.7E-02</td>
<td></td>
</tr>
<tr>
<td><strong>Ratio $T_{50}$</strong></td>
<td></td>
<td></td>
<td>1.93</td>
</tr>
<tr>
<td>660/460</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Units for $k_{PI}$ and $T_{50}$ in hours

** Units for $k_{PI}$ and $T_{50}$ in minutes
Supplementary Table A2. Parameters for the observed kinetics for the change in the $\tau_{AV}$ during photodamage.

<table>
<thead>
<tr>
<th></th>
<th>Change in the $\tau_{AV}$</th>
<th>460 nm</th>
<th>660 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Leaves ***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_{50}$</td>
<td>0.90±0.19</td>
<td>1.55±0.58</td>
</tr>
<tr>
<td></td>
<td>$k$</td>
<td>7.7E-01±1.6E-01</td>
<td>4.5E-01±1.7E-01</td>
</tr>
<tr>
<td></td>
<td>$A$</td>
<td>242</td>
<td>197</td>
</tr>
<tr>
<td><strong>Ratio $T_{50}$</strong></td>
<td></td>
<td></td>
<td>1.72</td>
</tr>
<tr>
<td><strong>BBY-particles</strong> **</td>
<td>$T_{50}^1$</td>
<td>0.30±0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$k_{t1}$</td>
<td>2.30±1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_1$</td>
<td>0.45±0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_2$</td>
<td>0.55±0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total change in $\tau_{AV}$</td>
<td>49.00±0.07</td>
<td>56.00±0.07</td>
</tr>
<tr>
<td></td>
<td>$T_{50}$</td>
<td>4.49</td>
<td>4.88</td>
</tr>
<tr>
<td><strong>Ratio $T_{50}$</strong></td>
<td><strong>660/460</strong></td>
<td></td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Units for $k$ and $T_{50}$ in hours

** Units for $k$ and $T_{50}$ in minutes

The partial amplitudes ($A_1$ and $A_2$) are normalized to the value of total amplitude ($A_T$ in ps).
Supplementary Figure A1: Comparison of PSII activity measured by FV/FM (Chapter 4) and ETR by DCPIP (Chapter 2) in two experimental buffers: Standard Buffer A (see Chapter 2 and 3) and Buffer B (see Chapter 4).