# DETECTION OF SINGLET OXYGEN PRODUCTION AND ITS RELATION TO PHOTOSYSTEM II PHOTODAMAGE IN CYANOBACTERIA AND MICROALGAE

**PhD Thesis**


Presented by

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# PREFACE

This thesis is based on a research project whose aim is to develop a method for the detection of singlet oxygen and to understand the mechanisms of photodamage and photoprotection of Photosystem II in cyanobacteria and microalgae. The project was carried out in the laboratory of Dr. Imre Vass, Director of Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary. The thesis work is the part of Seventh Framework Program, EU Marie Curie Initial Training Network HARVEST project, and also supported by the Hungarian Granting Agency OTKA. In addition, this thesis work also includes collaborative work with Dr. Yagut Allahverdiyeva, Prof. Eva-Mari Aro, Dr. Taina Tyystjärvi and Dr. Kaisa Hakkila (Turku, Finland), Dr. Diana Kirilovsky (Saclay, France), Dr. Anthony Larkum, Dr. Peter J. Ralph and Dr. Ross Hill (Sydney, Australia), Dr. Christiane Funk And Dr. Tania Tibiletti (Umea, Sweden). I am obliged to the EU Marie Curie Initial Training Network program and Hungarian Granting Agency OTKA which allowed me to take part in a cutting edge research project.

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## ABBREVIATIONS

$^1O_2$ - singlet oxygen

$^3O_2$ - molecular oxygen

ASG - Arhus sensor green

ATP - Adenosine-5'-triphosphate

BChl - bacteriochlorophyll

CAP - chloramphenicol

Car - carotenoid

Chl - chlorophyll

Cyt-$b_6f$ - cytochrome $b_6$f complex

DanePy - 3-[N-(b-diethylaminoethyl)-N-dansyl] aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea

DMBQ - 2,5-dimethyl-p-benzoquinone

EPR- electron paramagnetic resonance

FDPs- flavodiiron proteins

FeCN - ferricyanide

Fm - maximum fluorescence in dark

Fo - minimum fluorescence

Fv/Fm - maximum quantum yield

Fv′/Fm′ - PSII efficiency based on single turnover flash excitation

GA- glycolaldehyde

His - histidine

KCN - Potassium cyanide

LHCI - light-harvesting complex I

LHCII - light-harvesting complex II

$NaN_3$ - sodium azide

NDH - NAD(P)H dehydrogenase

NPQ - non-photochemical quenching

OCP - orange carotenoid protein

P680 - reaction center chlorophyll of PSII

P700 - reaction center chlorophyll of PSI

PB - phycobilisome

pBQ - p-benzoquinone

Phe - pheophytin

PQ - plastoquinone

PQH2 - plastoquinol

PSI - photosystem I

PSII - photosystem II

$Q_A$, $Q_B$ - primary and secondary quinone molecules

RNO - p-nitrosodimethylaniline

ROS - reactive oxygen species

SOSG - singlet oxygen sensor green

TEMP - 2,2,6,6-tetramethylpiperidine

TEMPD - 2,2,6,6-tetramethyl-4-piperidone

TEMPD-HCl - 2,2,6,6-tetramethyl-4-piperidone hydrochloride

# 1. INTRODUCTION

## 1.1. Photosynthetic processes

Photosynthesis is a process in which plants, green algae and cyanobacteria utilize energy of sunlight to produce carbohydrates from carbon dioxide and water. It is the main source of energy for all plants to drive their metabolic process. It encompasses the harvest of solar energy, transfer of excitation energy, energy conversion, electron transfer from water to $NADP^+$, ATP generation and enzymatic reactions that assimilate carbon dioxide and synthesize carbohydrates. This process is called oxygenic photosynthesis. The light reactions take place in the thylakoid membrane while the dark reactions take place in the stroma. The photosynthetic apparatus contains four protein complexes embedded in the thylakoid membrane, namely photosystem I (PSI), photosystem II (PSII), cytochrome $b_6f$ and the ATP synthase. Cyanobacteria do not have chloroplasts, but they have scattered thylakoid membranes throughout the cell, where the electron transport chains of photosynthesis and respiration are located. On the other hand, plants do have highly structured chloroplast in which the thylakoids are enclosed.

Thylakoid membranes contain different kinds of pigments including chlorophylls (Chl), carotenoids and bilin pigments (in cyanobacteria), which absorb light in almost all part of the light spectrum. Chlorophylls absorb light mainly in the red and blue parts of the spectrum. When a Chl molecule absorbs a photon, it is converted from the ground state to the excited state (Chl*). The exited Chl returns to its ground state by either re-emitting a photon or by dissipating its excitation energy as heat, or it drives photosynthetic electron transport (Demmig-Adams *et al.*, 1996; Havaux *et al.*, 2000; Vogelmann & Han, 2000; Hendrickson *et al.*, 2004; Hendrickson *et al.*, 2005); for details see also Fig. 1.1. The primary photosynthetic reactions occur in PSI and PSII. These photosystems are embedded in the thylakoid membrane containing peripheral light-harvesting complex, pigments and redox-active cofactors that mediate electron transport reactions. These light harvesting complexes funnel the light energy into reaction centers, which use the energy to initiate the primary charge separation reaction (Fig. 1.1). In this process there is the possibility of excitation energy transfer via the chlorophyll triplet state ($^3$Chl*), which yields reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$). Different kinds of ROS are produced by the photosynthetic machinery during electron transport (Roth, 2014) see Fig. 1.1). Linear electron flow proceeds from PSII through cytochrome $b_6f$ (Cyt-$b_6f$) to PSI. PSII oxidizes

two molecules of water into one molecule of $O_2$, 4 protons and 4 electrons. The electrons extracted from the water molecules are transferred by the electron transport chain via Cyt-$b_6f$ and PSI to $NADP^+$. The formed proton gradient drives ATP synthesis via ATPase, which together with NADPH is formed in the linear electron transport and drives the reduction of $CO_2$ to carbohydrates (Forti *et al.*, 2003; Renger, 2010; van Grondelle & Zuber, 1992).

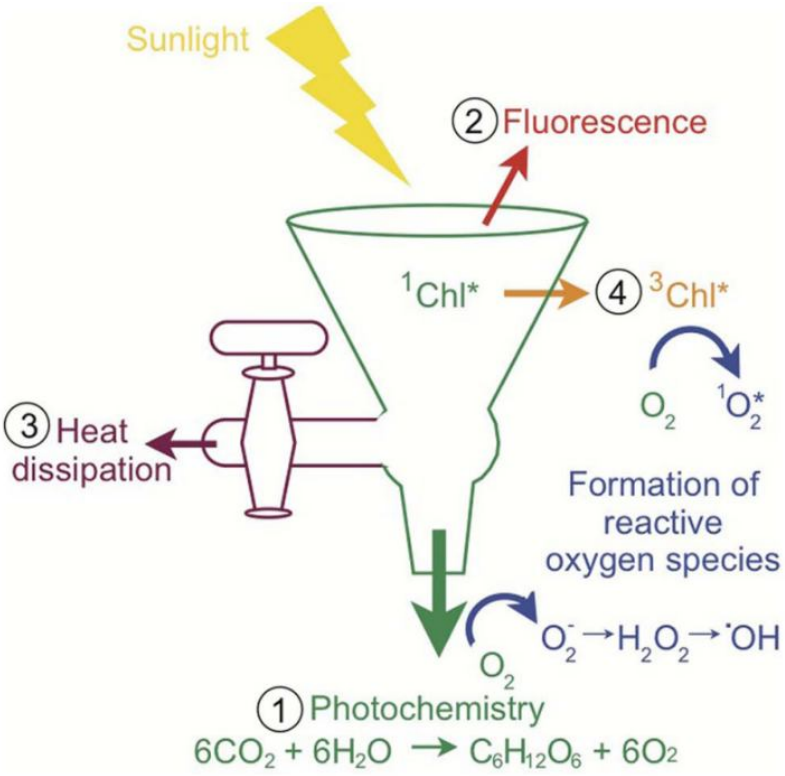


*Fig.1.1.The schematic description of the fate of light absorbed in photosynthetic apparatus (Roth, 2014).*

### 1.2. Structure and function of the photosynthetic apparatus

The photosynthetic apparatus is a highly conserved molecular machinery; however, the antenna systems vary substantially among different classes of photosynthetic organisms. In eukaryotes there is a pigment-protein antenna complex associated with PSII, which is called light harvesting complex II (LHCII), and an antenna complex associated with PSI called light harvesting complex I (LHCI). In addition to the outer antenna, PSII of plants and cyanobacteria contains two Chl *a* containing core antennas: CP43 and CP47 (Mori & Yamamoto, 1992; Alfonso *et al.*, 1994).

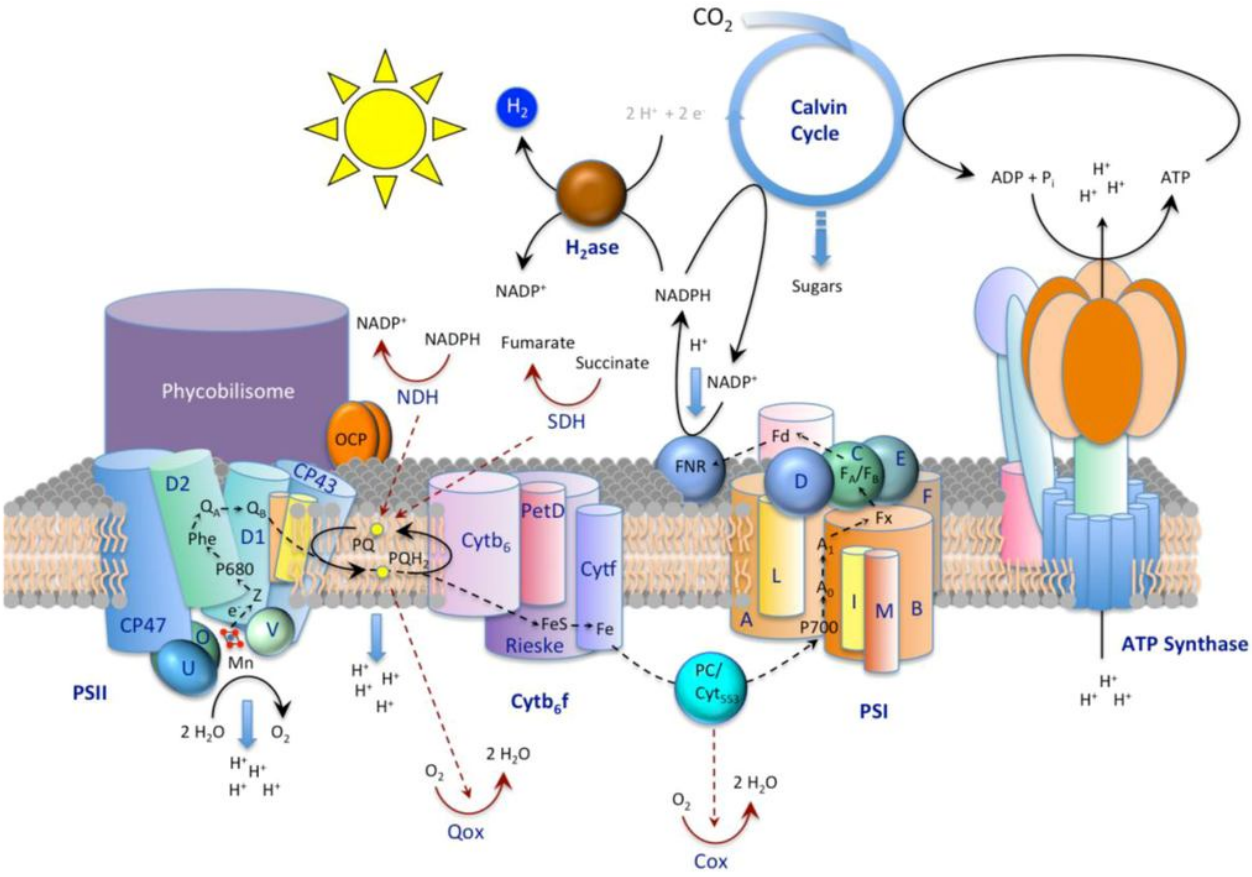


*Fig.1.2. Photosynthetic apparatus of cyanobacteria, the diagram shows photosynthetic electron transport and ATP synthesis in cyanobacterial thylakoid membranes (Kamennaya et al., 2012).*

LHCII and LHCI consist of a complex of several hydrophobic membrane-intrinsic polypeptides non-covalently binding Chl *a* and *b*, and carotenoid molecules. However, in cyanobacteria the principal light-harvesting complexes are phycobilisomes (PBs), which are peripheral to the thylakoid membranes (Kamennaya *et al.*, 2012), rather than the integral membrane Chl-*a/b* binding proteins which capture light in plants. Cyanobacteria do not have Chl-containing LHC but they contain bilin proteins (Miskiewicz *et al.*, 2002; Tandeau de Marsac, 2003) for details see also Fig. 1.2). In cyanobacteria light is captured by phycobilisomes and funneled to the reaction centers. PBs are located at the outer surface of thylakoid membranes (Fig. 1.2). They contain phycobiliproteins which covalently bind bilins pigments, and linker

peptides which are required for the organization of the phycobilisome (Tandeau de Marsac, 2003; Liu *et al.*, 2005).

The PBs comprises of allophycocyanin core rods (Shen *et al.*, 1993; Lea-Smith *et al.*, 2014). The subunits of rod segments are phycocyanin, phycoerythrin and phycoerythrocyanin. In freshwater cyanobacteria the rods contain only phycocyanin, while marine cyanobacteria are usually rich in phycoerythrin (Wingard *et al.*, 1997; Steglich *et al.*, 2001; Hirose *et al.*, 2010). These rods are associated with the allophycocyanin core, which in turn is connected to a linker protein. Linker proteins connect the PBs with the thylakoid membranes and the captured light energy is transferred to the reaction center of PSII and PSI. Primary photochemistry, carbon reduction and electron transport in cyanobacteria are functionally similar to those of plant chloroplasts. Cyanobacteria have a more simple genetic system than plants and algae and can be genetically engineered easily. Due to the genetic simplicity of cyanobacteria different photosynthetic mechanisms can be easily studied (Blankenship & Hartman, 1998; Blankenship, 2001; Kauny & Setif, 2014; Hays & Ducat, 2015; Cogdell & Gardiner, 2015; Ughy *et al.*, 2015; Lea-Smith *et al.*, 2016).

### 1.3. Photoinhibition

Photosynthesis is driven by light, which is a highly energetic and potentially dangerous element that can damage the photosynthetic apparatus (Vass *et al.*, 2007; Vass & Aro, 2008; Allen, 1995; Tyystjarvi, 2013; Zivcak *et al.*, 2015; Giovagnetti & Ruban, 2015). The photosystems are composed of reaction centers where charge separation occurs, and light-harvesting antennae that collect light energy and provide the reactions centers with excitation energy. Too much light reaching the photosynthetic apparatus can cause photodamage and ultimately can lead to the death of a cell. The light-induced decline of photosynthetic activity is broadly termed as photoinhibition, and this important phenomenon has been a topic of intense research in the last 30 years. The major site of photoinhibition is the PSII complex whose electron transport is inhibited and protein structure is damaged as a consequence of light exposure (Aro *et al.*, 1993b; Dewez *et al.*, 2009; Kato *et al.*, 2015) for details see also Fig. 1.3).

The main target of photodamage in PSII is the D1 protein. This whole process is reversible in intact systems, which are capable of protein synthesis. This means that when

D1 is damaged by high light (Nishiyama & Murata, 2014; Kusama *et al.*, 2015) or by environmental stress (Murata *et al.*, 2007), or by copper stress (Yruela *et al.*, 1996) it will be removed from the PSII complex and replaced by newly synthesized D1 protein, which results in the recovery of electron transport activity. Therefore, damaged PSII can be repaired via the synthesis of new D1 protein subunit. The rate of photoinhibition is directly proportional to the light intensity (Tyystjarvi & Aro, 1996).

Light stress to PSII becomes a problem for photosynthetic capacity when the rate of photodamage exceeds the capacity of repair process (Rintamäki *et al.*, 1995; Vass *et al.*, 1999b). The importance of photodamage is also emphasized by the large array of mechanisms that protect plants against the detrimental effects of light. These include: (i) dissipation of excess light energy in the antenna (Ruban *et al.*, 2007; Wilson *et al.*, 2006) or the PSII reaction center (Ivanov *et al.*, 2008) before photosynthetic electron transport occurs; (ii) quenching of dangerous Chl triplets and singlet oxygen by carotenoids (Frank & Cogdell, 1996; Telfer *et al.*, 2003; Telfer, 2005), and (iii) repair of the damaged PSII complexes via *de novo* synthesis of the D1 reaction center protein (Komenda *et al.*, 2007b).

### 1.4. Monitoring photodamage in the presence of protein synthesis inhibitors

Photodamage can be monitored in the presence of an inhibitor of protein synthesis, for example lincomycin or chloramphenicol (CAP), which blocks repair of PSII by inhibiting protein synthesis *de novo* whereas repair can be monitored in terms of the recovery of PSII activity after transfer of the photosynthetic organism from strong to weak light. Photoinhibition does not lead to loss of PSII activity unless the rate of damage is higher than the rate of repair. The rate of photodamage can only be measured in the absence of the concomitant repair. Therefore, it is important to monitor separately the rates of photodamage and of the protein synthesis dependent repair (Nishiyama *et al.*, 2004; Kato *et al.*, 2015).

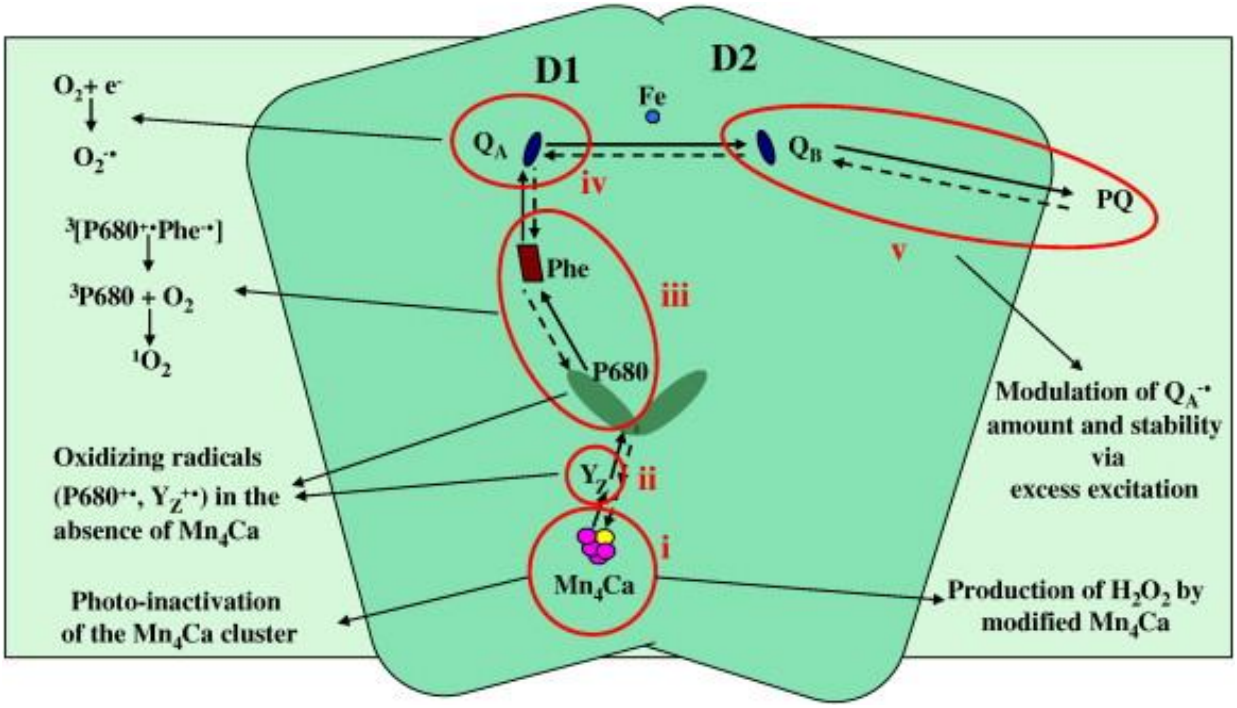


*Fig.1.3. Light-induced photodamage in the PSII complex of photosynthetic apparatus (Vass, 2012).*

### 1.5. Generation of reactive oxygen species (ROS) in the photosynthetic apparatus

The production of ROS is important in cellular processes like defense against infection, cellular signaling and it is associated with damage to cellular components such as proteins, lipids and nucleic acids. Protein complexes involved in the electron transport chain react with molecular oxygen producing ROS. In plant cells the major source of ROS production are the chloroplast and mitochondria (Apel & Hirt, 2004; Laloi *et al.*, 2004). ROS includes singlet excited oxygen, free radicals (which are superoxide and hydroxyl ions) and peroxides. These species are produced when cells are subjected to stress conditions (Asada, 2006). In chloroplast, the reaction centers of PSI and PSII are the major site of ROSs of generation. Previously Mehler (1951) described the mechanism of photoreduction of $O_2$ to hydrogen peroxide ($H_2O_2$) in PSI (Mehler, 1951). The reduction of $O_2$ results in superoxide anion ($O_2^-$) formation and its disproportionation produces $H_2O_2$ and $O_2$ upon dismutation by superoxide dismutase (Jakob & Heber, 1996a; Asada, 1999). On the other hand, $^1O_2$ is formed via interaction of ground state $O_2$

with Chl triplets that are produced either in the reaction center of the PSII complex, or in the light harvesting antenna (Krieger-Liszkay *et al.*, 2008; Krieger-Liszkay, 2005; Asada, 2006) see also Fig. 1.3 and Fig. 1.4).

The production of reduced and excited species of ROS is largely affected by physiological and environmental conditions. Under excess light the ROS production rate is enhanced and rapid scavenging of the ROS, which are produced in thylakoids prior to its diffusion to surrounding compartments from its generation site, is very important to protect the thylakoid membrane.

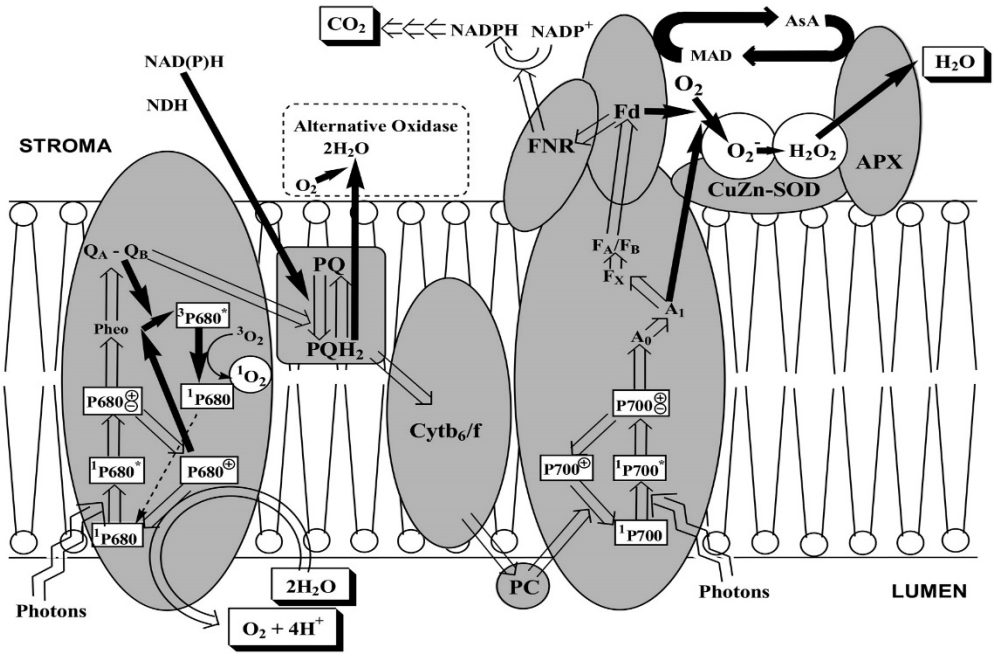


*Fig.1.4. The scheme shows production of $^1O_2$ in PSII and of superoxide in PSI in plant thylakoids (Asada, 2006).*

### 1.6. Production of $^1O_2$ in the photosynthetic apparatus

Singlet oxygen ($^1O_2$) is an excited state of O2 which is highly reactive (Skovsen *et al.*, 2005; Ogilby, 2010). It damages proteins, lipids and nucleic acids, so it is an important ROS in biological systems. It is less stable than triplet oxygen ($^3O_2$), and may be formed in a number of ways; however, the most common way is by excitation energy transfer from the triplet state of a photosensitized pigment or dye molecule to triplet oxygen (Telfer *et al.*, 1994b; Telfer *et al.*,

1999). $^1O_2$ is produced primarily inside chloroplasts during the process of photosynthesis. Light absorption by light harvesting Chls in the light harvesting complex (LHCII) and in the PSII reaction center leads to excitation of the P680 Chl. The charge separation between the excited reaction center Chl assembly (P680*) and the pheophytin (Phe) molecule, i.e. the formation of the primary radical pair/charge separated state ($P680^{+\bullet}Phe^{-\bullet}$), is a primary event during PSII electron transfer, which is followed by rapid charge stabilization processes. The primary radical pair is stabilized by reduction of $Q_A$ by $Phe^-$ and oxidation of Tyr-Z by $P680^{+\bullet}$ to prevent its rapid recombination.

The $P680^{+\bullet}Phe^{-\bullet}$ state is initially formed in the singlet spin configuration ($^1[P680^{+\bullet}Phe^{-\bullet}]$), which recombines to $^1$P680* (Phe). However, spin conversion can convert $^1[P680^{+\bullet}Phe^{-\bullet}]$ to the triplet $^3[P680^{+\bullet}Phe^{-\bullet}]$ state, whose recombination results in excited $^3$P680. Rapid scavenging of triplet $^3$P680 take place at its creation spot before $^1O_2$ formation (Ogilby, 2010). Under light stress conditions, the interaction of excited triplet $^3$P680 with ground state triplet oxygen leads to the formation of $^1O_2$ (Vass, 2011; Vass, 2012) Studies showed that the increased production of $^1O_2$ seems to occur primarily under high light stress conditions, which will ultimately result in the inactivation of PSII and photoinhibition. Under high light conditions the plastoquinone pool may be in the reduced state, especially under limited $CO_2$ availability, which enhances charge recombination reactions in PSII (Yang *et al.*, 2001; Kruk & Szymanska, 2012; Gerken *et al.*, 1989; Satoh *et al.*, 1990).

### 1.7. $^1O_2$ dependent mechanism of photodamage

As explained in the previous section, utilization of absorbed light during photosynthetic electron transport and the following process of excitation energy transfer in the light harvesting antenna leads to the production of $^1O_2$ in photosynthetic organisms (Hideg *et al.*, 1994a; Hideg *et al.*, 2000; Vass & Aro, 2008). A number of studies show that $^1O_2$ is formed under photoinhibitory conditions (Vass, 2011; Vass & Aro, 2008; Vass, 2012; Krieger-Liszkay, 2005; Hakala-Yatkin & Tyystjärvi, 2011; Mattila *et al.*, 2015; Tyystjärvi, 2013) and also that this is the most destructive ROS in plant cells (Krieger-Liszkay *et al.*, 2008), cyanobacteria (Hakkila *et al.*, 2014; Tomo *et al.*, 2012) and algae (Shao *et al.*, 2013; Trebst *et al.*, 2002).

Previous studies have shown that $^1O_2$ can be directly involved in damage of repair cycle and cleavage of the D1 and D2 reaction center subunits (Okada *et al.*, 1996). Other studies showed indirect damaging effect of $^1O_2$ in *Synechocystis* cells via inhibiting the repair cycle of PSII without damaging directly the PSII complex (Nishiyama *et al.*, 2001; Nishiyama *et al.*, 2004; Nishiyama *et al.*, 2006). In contrast to these studies, direct damage of PSII has also been demonstrated in intact tobacco leaves, which were infiltrated with Rose Bengal (Hideg *et al.*, 2007) and also many other group support the direct damage of PSII by $^1O_2$ disproportionation (Hakkila *et al.*, 2014; Tyystjärvi, 2013; Allahverdiyeva *et al.*, 2013; Aro *et al.*, 1993c; Aro *et al.*, 1994; Aro *et al.*, 1990; Vass & Aro, 2008; Komenda *et al.*, 2007a; Nixon *et al.*, 2010; Kerfeld & Kirilovsky, 2013; Kirilovsky *et al.*, 1994).

Asada (2006) reported enhancement of $^1O_2$ production under low O2 conditions and this increase in $^1O_2$ was quenched when ferricyanide was supplied externally (Asada, 2006). The reduction of PQ is enhanced under low oxygen condition, and also the reduction of PSII acceptors, which are involved in charge separation and charge recombination process to produce $^3$P680, is enhanced. Thus, Asada (2006) supported the idea of $^1O_2$ mediated direct photodamage by over-reduction of the PQ-pool and electron acceptor limitation (Asada, 2006). However, another model of charge recombination dependent and $^1O_2$-mediated model of photodamage was also proposed (Vass, 2011; Vass, 2012), which explains the correlation between the linear light intensity dependent increase in photodamage of PSII and subsequent production of $^1O_2$. In this model, the reduced $Q_A$ accumulates with increasing low light to high light intensities. The repulsion between the reduced $Q_A$ and $Phe^{-\bullet}$ slows down the rate of charge separation process. Therefore, due to the lack of forward electron transport toward $Q_A$, the lifetime of the produced $^1[P680^{+\bullet}\ Phe^{-\bullet}]$ enhances, which supports spin conversion into the $^3[P680^{+\bullet}\ Phe^{-\bullet}]$ state (Vass, 2011). The rate of primary charge separation is light intensity dependent (Tyystjarvi & Aro, 1996), the produced amount of $^3[P680^{+\bullet}\ Phe^{-\bullet}]$, generated $^1O_2$ and as well as the induced photodamage is likely to increase linearly with increasing light intensity even after reaching saturation of photosynthetic electron transfer (Vass, 2011; Vass, 2012).

### 1.8. Quenching of $^1O_2$ by carotenoids and other pigments

Carotenoids are found in all photosynthetic organisms. These pigments absorb in the range of 400 to 500 nm region of visible spectrum and they are associated with both antenna and

reaction center pigment proteins of thylakoid membranes (Griffiths *et al.*, 1995). In photosynthetic organisms the most abundant carotenoids are the carotenes and xanthophylls, which act as accessory light harvesting complex (LHCs) pigments (Frank & Cogdell, 1996; Cerullo *et al.*, 2002; Zhu *et al.*, 2010). The light harvesting complexes of PSII and PSI bind xanthophyll derivatives in higher plants (Sarry *et al.*, 1994; Demmig-Adams & Adams III, 1996; Ruban *et al.*, 1999) and cyanobacteria (Zhu *et al.*, 2010). Hence, carotenoids are incorporated in the structure of the thylakoid membrane (Ramel *et al.*, 2012a; Bilger & Bjorkman, 1990; Havaux *et al.*, 1996; Gilmore *et al.*, 1998), assembly of protein complexes, and serve as antioxidants to protect chloroplasts from ROS that are generated under excess light conditions (Stahl & Sies, 2003). Specifically, carotenoids are involved in scavenging of $^1O_2$ and establish the first line of defense against $^1O_2$ (Terao *et al.*, 2011; Ramel *et al.*, 2012a). Cyanobacterial carotenoids comprise of xanthophyll derivatives and β-carotene. The main xanthophylls in *Synechocystis* are zeaxanthin and myxoxanthophyll (Masamoto *et al.*, 1999; Kusama *et al.*, 2015).

The carotenoids which are embedded in thylakoid or attached to the pigment proteins have also role in light harvesting, quenching excess light energy and scavenging of $^1O_2$ in photosynthetic organisms (Green & Durnford, 1996; Frank & Cogdell, 1996). They are also capable of quenching triplet Chls directly at the site of its production which is the major source of $^1O_2$. The orange carotenoid protein (OCP) is a soluble cyanobacterial protein that binds the keto-carotenoid 39-hydroxyechinenone (Wilson *et al.*, 2008; Wilson *et al.*, 2012) and is present in most cyanobacterial species. It is well documented that OCP serves as a quencher of extra excitation energy (Krinsky, 1968; Wilson *et al.*, 2006; Kirilovsky, 2007; Boulay *et al.*, 2008; Kirilovsky & Kerfeld, 2013; Gupta *et al.*, 2015).

There are two main mechanisms, which are involved in quenching of $^1O_2$. Physical and chemical quenching: the physical quenching mechanism involves the interaction of quencher and $^1O_2$, which leads to the production of an excited complex followed by energy transfer and thermal deactivation. The carotenoids deactivate $^1O_2$ to the triplet unreactive ground state by energy transfer (Triantaphylides *et al.*, 2008; Triantaphylides & Havaux, 2009). They also function by physically quenching the triplet excited state of Chl directly before the formation of $^1O_2$ (Fraser *et al.*, 2001) see also Fig. 1.5). The chemical quenching mechanism of carotenoids involves a chemical reaction of $^1O_2$ and the quencher; as a result the quencher is oxidized and

$^1O_2$ is quenched. In plants, carotenoids are also involved in chemical quenching of $^1O_2$ (Ramel *et al.*, 2012a; Ramel *et al.*, 2012b). However, this quenching effect is less effective than physical quenching (Tanielian & Wolf, 1988). Although *in vitro* studies have shown that carotenoids can be oxidized by $^1O_2$ in solvent and confirmed the existence of a chemical quenching capacity of carotenoids, the existence of chemical quenching mechanism *in vivo* under high light stress is not well established (Edge *et al.*, 1997).

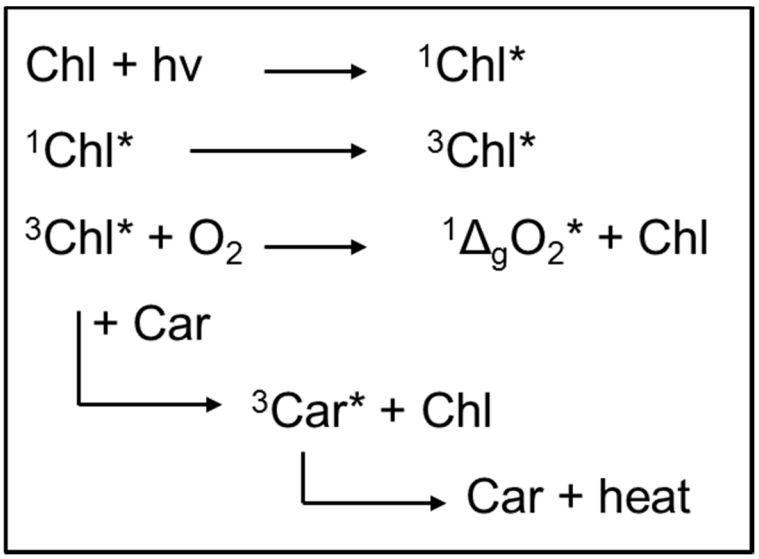


*Fig.1.5. Carotenoid mediated physical quenching of singlet oxygen, Figure modified from (Fraser et al., 2001).*

### 1.9. Direct and indirect detection methods of $^1O_2$

Production of $^1O_2$ has been demonstrated earlier in isolated PSII reaction center complexes by histidine- or imidazole-mediated chemical trapping (Telfer *et al.*, 1994a). This method is based on the interaction of the generated $^1O_2$ with the aromatic side chain of histidine or imidazole, leading to the formation of an oxidation product of histidine (Méndez-Hurtado *et al.*, 2012) that decreases the concentration of dissolved O2 in the suspension. The amount of produced ROS was expected to be proportional to the amount of $O_2$ uptake from the medium, which was monitored by an oxygen electrode (Fig. 1.6a and b). Previously a dye bleaching technique was also established for the detection of $^1O_2$ in isolated PSII reaction center complexes (Telfer *et al.*, 1994a). This technique was based on the bleaching of p-

nitrosodimethylaniline (RNO) by the intermediary endo-peroxide of histidine. This RNO bleaching can be measured photometrically at 440 nm, which results from the reaction between $^1O_2$ and histidine. However, this method seems to be inconvenient from the perspective of Chl-rich samples.

Another direct method which can be used for the detection of $^1O_2$ is assessing time resolved $^1O_2$ luminescence around 1270 nm (Macpherson *et al.*, 1993). This detection method can only be employed for isolated PSII cores because of the low efficiency of $^1O_2$ phosphorescence and the interaction with Chls. It has to be taken into account that water has luminescence in the range of 1260 nm; therefore the hydration artifact must be carefully separated from the original $^1O_2$ signals.

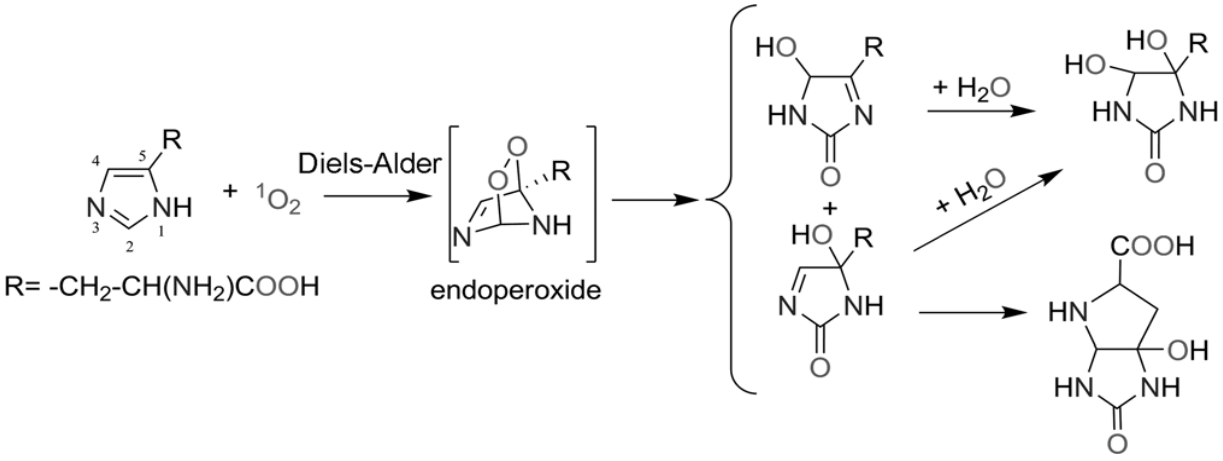


*Fig.1.6a. The chemical reaction of His with singlet oxygen (Méndez-Hurtado et al., 2012).*

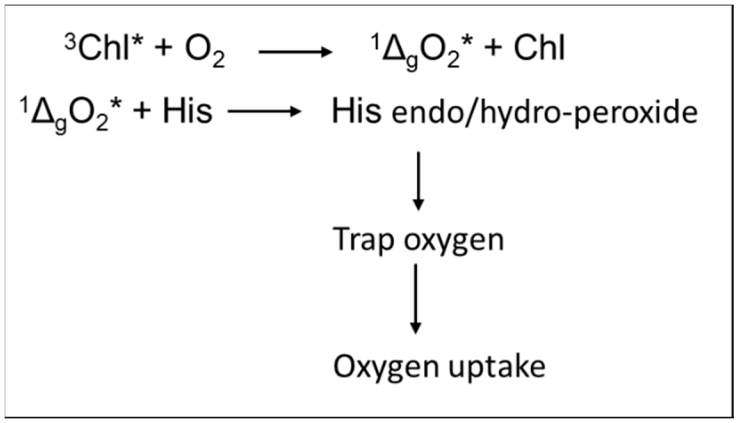


*Fig.1.6b. The scheme of His-mediated oxygen uptake in Synechocystis cell suspension (Rehman et al., 2013).*

EPR spin trapping is another useful technique for $^1O_2$ detection, which was previously employed in thylakoid membrane particles by using 2, 2, 6, 6-tetramethylpiperidine (TEMP, (Hideg *et al.*, 1994c; Hideg *et al.*, 1994b). This method is based on the reaction of $^1O_2$ with TEMP and TEMP derivatives (Lion *et al.*, 1980). As a result, paramagnetic nitroxide radical is formed, which is thus easily detectable by EPR spectroscopy. However, EPR measurement of nitroxide radical from biological samples is laborious. There are other probes like TEMPD-HCl (Fischer *et al.*, 2006a) and TEMPD (2,2,6,6-tetramethyl-4-piperidone) (Hideg *et al.*, 2011), which are more hydrophilic and could also be used for EPR spin trapping . However, TEMPD-HCl is preferred on TEMPD, because the hydrochloride form is more stable in storage. Until now this technique is used successfully only in isolated functional photosynthetic membranes, as it is less sensitive for *in vivo* studies. The main limitation is that these probes do not penetrate into intact cyanobacterial and algal cells.

Fluorescent probes are also useful for the detection of $^1O_2$ in intact plant systems, and the changes in the fluorescence of these specific $^1O_2$ probes can be measured by fluorescence imaging techniques. Earlier DanePy (3-(N-diethylaminoethyl)-N-dansyl) aminomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1H-pyrrole) has been applied for the detection of $^1O_2$ both in isolated thylakoid membrane particles and in intact leaves (Hideg *et al.*, 1998; Hideg *et al.*, 2001; Hideg *et al.*, 2007). DanePy undergoes fluorescence quenching upon reaction with $^1O_2$. This probe, however, has major disadvantages since it requires UV range excitation (Kalai *et al.*, 1997), which probably inhibits metabolic activity of cells. The other limitation of DanePy is the partial $^1O_2$ fluorescence quenching, which can create artifacts (Hideg, 2008). Therefore, this probe is only useful in detecting high concentrations of $^1O_2$.

Fluorescence Sensor Green (SOSG) probe has also been applied previously to detect $^1O_2$ in intact plant systems (Fischer *et al.*, 2013). Reaction of SOSG with $^1O_2$ produces endoperoxide of SOSG. Prior to the reaction with $^1O_2$, internal electron transfer quenches the fluorescence from the light-emitting fluorescein chromophore. Reaction of SOSG with $^1O_2$ leads to the formation of endoperoxide, and therefore electron transfer is blocked, and fluorescence is

observed which, can be scanned by conventional fluorescence imaging techniques (Gollmer *et al.*, 2011); for detailed mechanism see Fig. 1.7.

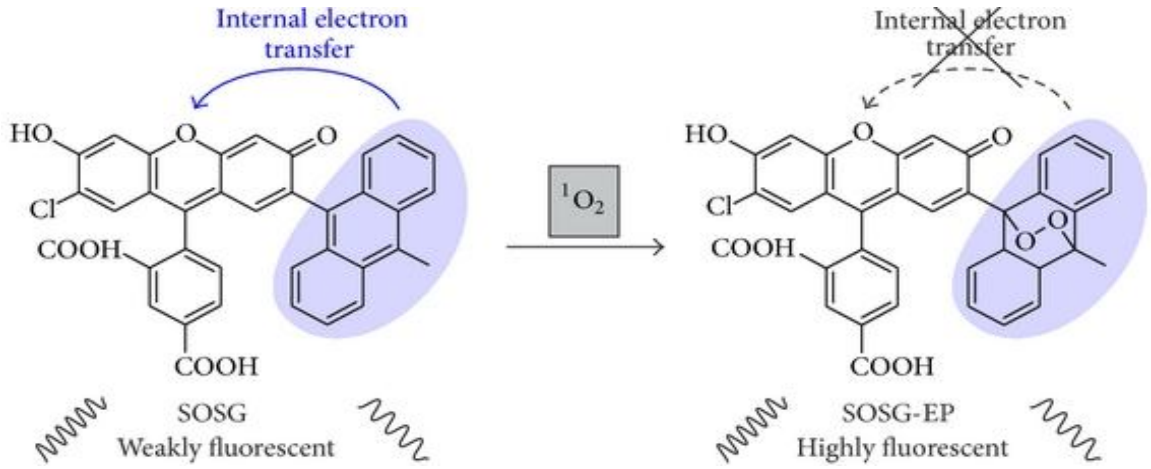


*Fig.1.7. The chemical structure of SOSG and its mechanism of reaction with singlet oxygen (Gollmer et al., 2011).*

Recent studied showed that SOSG and its intermediate reaction product endoperoxide are capable of forming the production of $^1O_2$ itself (Hideg *et al.*, 2007). Actually these endoperoxides photosensitize the production of $^1O_2$. In addition to this artifact, SOSG does not penetrate in intact cells of cyanobacteria and algae; however SOSG may be used for detection of $^1O_2$ in *in vitro* assays. It is of note that there has been an attempt to use SOSG in intact *Synechocystis* cells (Sinha *et al.*, 2012). In this study the cells were illuminated for a long time under photonhibitory conditions (3 h at 1000 μmole photons $m^{-2}s^{-1}$) for the induction of the SOSG fluorescence signal (Sinha *et al.*, 2012). Illumination of SOSG for a longer time itself induces an artifactual fluorescence increase, in the absence of exogenous $^1O_2$ source in *Synechocystis* (Ragás *et al.*, 2009).

Very recently Aarhus Sensor Green (ASG) has been synthesized (Pedersen *et al.*, 2014) and proposed that it overcomes several limitations which are related to the use of SOSG. The basic working principle of ASG and SOSG is similar, as the formation of highly fluorescent

endoperoxide occurs upon reaction with $^1O_2$. However, ASG and its intermediate product do not themselves photosensitize the production of $^1O_2$ which is a very useful characteristic for the detection of $^1O_2$ *in vivo* and *in vitro*. Therefore, ASG is claimed to be better and convincing probe for the detection of $^1O_2$ (Pedersen *et al.*, 2014) see also Fig. 1.8). However, ASG is still not commercially available probe for the detection of $^1O_2$.

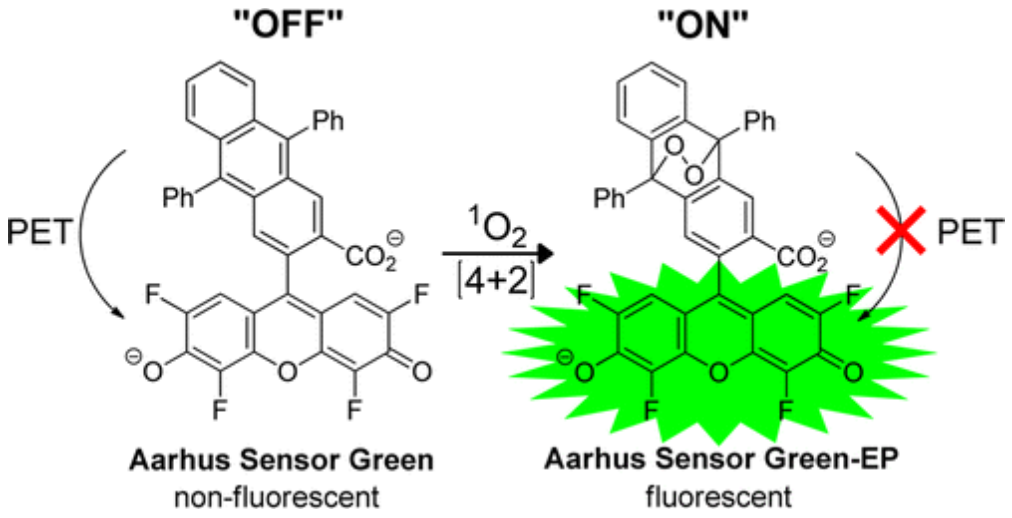


*Fig.1.8. The structure of fluorescent probe ASG and its reaction with $^1O_2$ (Pedersen et al., 2014)*

## 1.10. Production of $^1O_2$ in different cyanobacterial strains, as well as by a cyanobacterial metabolite M22

*Synechocystis* wild type, Q130E and Q130L mutants were used in the present study in which the D1-130 residue was replaced from glutamine to either glutamic acid (which is referred to as Q130E) or Leucine (which is referred to as Q130L). It has been reported that the mutation of the D1-Gln130 residue by Glu and Leu shifts Em(Phe/Phe$^-$) by about + 35and - 55 mV, respectively (Nixon *et al.*, 1992). It consequently influences the charge recombination reactions in PSII. It has been shown that the overall charge recombination from the $S_2Q_A^-$ state is accelerated in the Q130E mutant, and the contribution of the non-radiative recombination pathway via the $P680^{+\bullet}Phe^{-\bullet}$ primary radical pair is enhanced. However, in the Q130L mutant strong stabilization of charge recombination from $S_2Q_A^-$ and decreased contribution of the non-radiative recombination pathway was observed relative to the WT (Cser & Vass, 2007).

Photodamage of PSII was decreased in Q130E and the opposite effect was observed in Q130L compare to the WT (Cser & Vass, 2007). Therefore the mutation from glutamine to glutamic acid appears to be a vital mechanism of regulating phototolerance in many cyanobacteria during light adaptation conditions (Cser & Vass, 2008).

The *ΔsigCDE* mutant of *Synechocystis* was also used in the present study. The sigma (σ) factor mutant *ΔsigCDE* contains the multifunctional, stress responsive SigB as its only functional group 2 σ factor. *ΔsigCDE* acclimates slightly better to high salt than the control strain (CS) due to upregulation of the compatible solute producing enzyme glucosylglycerol phosphate synthase, heat shock protein HspA and carotenoids, all of which are known to increase high salt tolerance (Tyystjärvi *et al.*, 2013).

*Synechocystis* strains which either lack overexpress the Orange Carotenoid Protein (OCP) were also used in the present study. The OCP is a soluble protein that binds the keto-carotenoid 39-hydroxyechinenone (hECN) (Kerfeld *et al.*, 2003). OCP is essential for a photoprotective mechanism, which dissipates excess excitation energy in *Synechocystis* (Wilson *et al.*, 2006).

The role of flavodiron proteins (FDPs) in singlet oxygen production was also studied in the present work. Flavodiiron proteins (FDPs) are specific electron transfer enzymes present mainly in anaerobic bacteria, and they are found also in cyanobacteria. Cyanobacterial FDPs constitute a specific group that evolved differently to cooperate with oxygenic photosynthesis. In *Synechocystis* there are four FDPs, Flv1-Flv4. Two of them, Flv2 and Flv4, are encoded by an operon together with an Sll0218 protein (Zhang *et al.*, 2009). Several recent studies have suggested that the FDPs have an important role in photoprotection of the photosynthetic machinery in cyanobacteria (Zhang *et al.*, 2012; Allahverdiyeva *et al.*, 2013).

We also studied the role of the small Chl binding proteins (SCPs) using the PSI-less *Synechocystis* backround. Small CAB-like proteins (SCPs) are single-helix light-harvesting-like proteins found in all organisms performing oxygenic photosynthesis. SCPs are involved in chlorophyll biosynthesis (Xu *et al.*, 2004) and have role in photooxidation (Sandström *et al.*, 2002).

A novel secondary metabolite (which is denoted as M22) was purified from N2-fixing filamentous cyanobacterium *Nostoc* XPORK14A isolated from the Baltic Sea. M22 has a non-peptide structure and containing several subunits of total molecular mass of 1626 Da (Shunmugam *et al.*, 2014). The M22 compound absorbs within the UV-range and blue region of visible spectrum. It influenced the cell growth and cause inhibition of photosynthesis in *Synechocystis* cells, both in light and in darkness.

### 1.11. Production of superoxide

Superoxide is formed in the thylakoids of photosynthetic organisms by photoreduction of O2 at the acceptor site of PSI (Asada & Takahashi, 1987; Asada, 1994; Asada, 2006; Asada, 1999) see also Fig. 1.4). Superoxide can act as either oxidant or reductant; it can oxidize sulfur, ascorbic acid or NADPH; it can reduce cytochrome c and metal ions. Superoxide has high reactivity with thiol groups and iron-sulphur clusters causing inactivation of reaction centers of very important enzymes and release of iron (Flint *et al.*, 1993). Superoxide radicals have high reactivity; therefore, it is expected that locally generated superoxide will have effect mostly in the vicinity of its production site. The main site which has been reported for the production of superoxide is the water-water cycle which helps the plants to dissipate excess light energy by increasing the rate of electron transport (Asada, 1999). However, in cyanobacteria this pathway is missing, and instead of the superoxide-producing Mehler reaction, the flavodiiron proteins provide a dissipation pathway (Allahverdiyeva *et al.*, 2013).

PSII is also involved in the generation of superoxide through the reduction of $O_2$ by low redox-potential electron acceptors. The study by Pospísil and coworkers has suggested that the main sources for superoxide generation in PSII are reduced primary electron acceptor, $Phe^{-\cdot}$, both primary and secondary quinone electron acceptors, $Q_A^-$and $Q_B^-$, respectively (Pospísil, 2009; Pospísil, 2012). There is also evidence showing that electrons can be transferred to $O_2$ from the plastoquinone pool and cytochrome b559 to produce superoxide (Kruk *et al.*, 2003). In the presence of superoxide dismutase (SOD) superoxide is quickly dismutated to stable $H_2O_2$ (Fridovich, 1997).

Hydrogen peroxide ($H_2O_2$) formation at the acceptor side of PSII was shown previously by a chemiluminescence method with the use of a luminol-peroxidase assay in thylakoid

membranes. However, studies have also shown that $H_2O_2$ could also be formed on the donor side of PSII due to the oxidation of water (Klimov *et al.*, 1993). Hydroxyl radical can be the result of the reduction of $H_2O_2$ by low-valent transition metals (Klimov *et al.*, 1993). It is of note that oxygen is one of the important electron carriers in the electron transport chain. Its role in electron transport is flexible, depending on light intensity as well as on other factors, which affect the electron flow from PSII to PSI (Khorobrykh & Ivanov, 2002; Mubarakshina *et al.*, 2006).

### 1.12. Involvement of ROS in the breakdown of coral symbiosis

Reef-building corals, which belong to the class Anthozoa (Cnidaria), form endosymbiosis with dinoflagellate algae from the genus *Symbiodinium* (also known as zooxanthellae), which reside within the endodermal tissue of the anthozoan host (Wooldridge, 2010); see Fig. 1.9). In this symbiotic relationship the algae provide organic carbon in the form of photosynthates to their host to support metabolic energy requirements while they are given protection and receive inorganic compounds such as $CO_2$ and nitrogen compounds from the host, which creates a sustainable environment for coral physiology (Yellowless *et al.*, 2008).

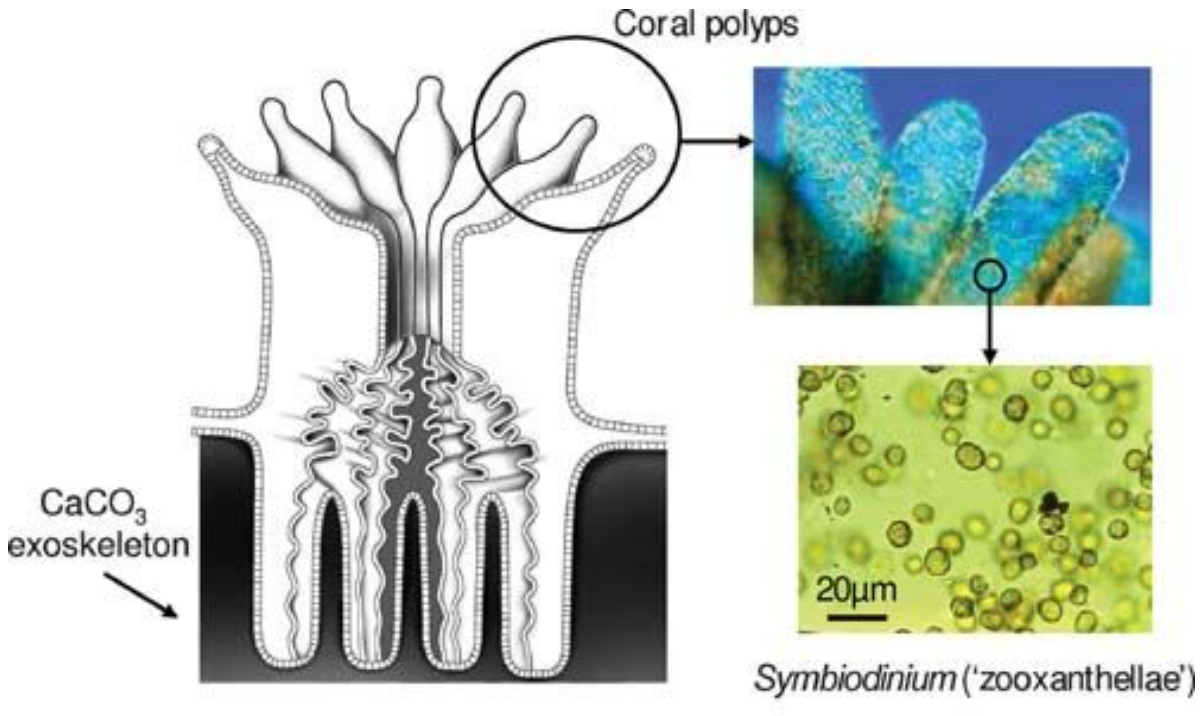


*Fig.1.9. Symbiotic association between the host and dinoflagellate algae (Wooldridge, 2010)*

As a result of environmental stress factors, the symbiosis between the coral host and its symbiotic partner may break down leading to the expulsion of the zooxanthellae and/or the loss of photosynthetic pigments from within algal cells giving rise to the phenomenon known as coral bleaching (Brown, 1997; Hoegh-Guldberg *et al.*, 2007; Weis, 2008) Fig. 1.10).

Elevations in sea surface temperature, as small as 1–2 °C above the summer average, in combination with high irradiance, are well known to cause mass coral bleaching events on coral reefs around the world (Hoegh-Guldberg *et al.*, 2011; Hoegh-Guldberg *et al.*, 2007). It is forecasted that ocean temperatures will continue to increase with global warming due to anthropogenic emissions of greenhouse gases, placing corals more at risk of having sea temperatures above their upper thermal maximum. Coral bleaching events are therefore predicted to become more frequent, wide spread and severe with climate change (Hoegh-Guldberg *et al.*, 2007). Breakdown in the coral symbiosis has been linked to an initial photosynthetic impairment in the endosymbiotic algae. A sustained loss of photosynthetic performance in coral symbionts is a well-defined characteristic of a thermal stress response (Jones *et al.*, 1998).

A great deal of evidence has been collected, primarily through non-invasive Chl fluorescence techniques, demonstrating the decrease of photosystem II (PSII) efficiency during a bleaching event (Jones *et al.*, 1998). Therefore, such results are indication of impacts on early phase of the light reactions of photosynthesis, it is not clear whether inhibition of PSII activity is the initial site of damage induced by bleaching conditions, or whether it is a secondary or tertiary impact only detected following inhibition or damage at another site (Bhagooli, 2013). Indeed, the initial site of impact remains elusive, with many studies aiming to identify the origin of photosynthetic impairment in *Symbiodinium* under simulated bleaching conditions.

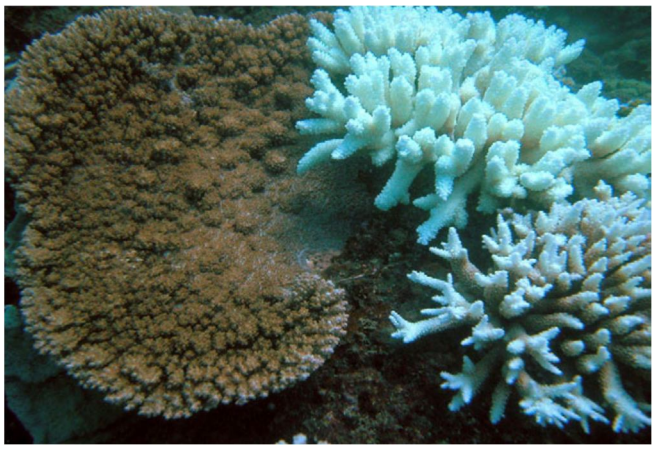

*Fig.1.10. Ccoral bleaching, the normal coral on the left side whereas partially bleached on the lower right side and fully bleached on upper right side Figure is taken from (Weis, 2008).*

Photoinhibition, which is largely an impairment of PSII caused by high irradiance, has been demonstrated in coral symbionts during thermal stress with the rate of photoinactivation of the core D1 protein exceeding the rate of repair. D1 content has been shown to drop under bleaching conditions, in correlation with declines in PSII photochemical efficiency (Ragni *et al.*, 2010).

The application of chemical inhibitors can provide a useful means to manipulate the function of metabolic processes. At low concentrations, glycolaldehyde (GA) and cyanide have been used to inhibit the Calvin-Benson cycle (Wishnick & Lane, 1969; Sicher, 1984). GA is known to inhibit ribulose-1,5-diphosphate synthase in this cycle. Potassium cyanide (KCN) is also an inhibitor of the Calvin-Benson cycle, although its specific mode of action is different to that of GA. KCN inhibits $CO_2$ fixation by binding to Rubisco activase and preventing its release from Rubisco (Wishnick & Lane, 1969; Sicher, 1984) and it also causes inhibition of ascorbate peroxidase and the scavenging of hydroxyl radicals (Jakob & Heber, 1996b). The exact mechanism of cyanide action is not well described in the literature, and because *Symbiodinium* have Type 2 rather than Type 1 Rubisco (i.e. Rubisco is composed of mainly two large subunits rather than eight large and eight small subunits), they may not react in the same way to cyanide, i.e. the binding of substrates and the heat sensitivity may be quite different. Furthermore, in symbiosis such as that exists between the coral host and *Symbiodinium*, the effect of these inhibitors on host (animal) tissue has to be taken into account.

The mechanism(s) that trigger the expulsion of the symbiotic partner are not yet fully understood. However, the involvement of ROS such as superoxide, singlet oxygen, and hydroxyl radicals, which are formed under combined heat and light stress, has been implicated based on a number of different lines of evidence. One key process, first suggested by Lesser and coworkers (Lesser *et al.*, 1990; Lesser, 1997) is the leakage of ROS from the symbiont to the host, which then overwhelms the antioxidant capacity of the host tissue under stressful conditions (Brown *et al.*, 2002; Lesser, 1996; Downs *et al.*, 2002; Richier *et al.*, 2005). Recent studies also revealed the details of pathomorphogenesis and photo-oxidative damage of the symbiont algae under stressful conditions (Downs *et al.*, 2013). A previous study revealed that elevated temperature cause decrease in the photosynthetic activity of the symbiotic partner, by affecting the ability of harvesting light by PSI and PSII (Hoegh-Guldberg, 1999). Heat induced impairment of

photosystems leading to an accumulation of superoxide, as the energy that normally derives from photosynthesis is provided to molecular oxygen via reduced components of PSI and PSII (Hoegh-Guldberg *et al.*, 2007; Hoegh-Guldberg *et al.*, 2011). Under high temperature conditions cellular components, such as superoxide dismutase and ascorbate peroxidase, which quench superoxide molecules, are denatured and depleted; therefore, the accumulation of superoxide cause coral to lose its symbiotic partner (Hoegh-Guldberg, 1999). Other abiotic stress factor, which could affect pigmentation in *Symbiodinium* is salinity. However, evidence show that it may cause only color loss but do not cause corals (*Stylophora pistillata*) to lose their symbiotic partners as in mass bleaching events (Hoegh-Guldberg & Smith, 1989).

There is accumulating evidence for differing antioxidant capacities and stress tolerances linked with different *Symbiodinium* genetic types (Suggett *et al.*, 2008; Krueger *et al.*, 2015). There is also evidence for elevated antioxidant capacity of the host under thermal stress, alongside unaltered photosynthetic capacity in the symbiont. This suggests a decoupling of host redox mechanisms from the symbiotic photo-physiology that may prevent bleaching (Hawkins *et al.*, 2015; Krueger *et al.*, 2015; Wietheger *et al.*, 2015). Although singlet oxygen is one of the most reactive oxygen forms, which can be generated in the zooxanthellae and interact with the animal host, no direct evidence has been provided before our studies for the formation of $^1O_2$ in *Symbiodinium* cells. This was mainly due to the lack of suitable $^1O_2$ detection methods that could be applied in intact microalgal cells.

# 2. AIMS

The general aim of the PhD work was to gain knowledge on the mechanisms of photodamage and photoprotection of the photosystem II complex in cyanobacteria and in dinoflagellate with special emphasis on the role of singlet oxygen production and scavenging processes. The specific aims of this study were:

1. To develop a method for the detection of singlet oxygen ($^1O_2$) in intact cyanobacterial cells and to investigate the correlation of photodamage and the extent of $^1O_2$ production in WT *Synechocystis* sp. PCC 6803 as well as in mutants in which Gln130 residue of the D1 reaction center subunit of PSII is replaced with Glu or Leu.
2. To investigate the role of $^1O_2$ in photoinhibition of the *Synechocystis* sp. PCC 6803 *ΔsigCDE* mutant, which contains the stress responsive SigB as the only functional group 2 σ factor.
3. To understand the role of Orange Carotenoid Protein (OCP) as $^1O_2$ quencher by using mutants lacking, or overexpressing the OCP gene in *Synechocystis* sp. PCC 6803.
4. To characterize $^1O_2$ production by a secondary metabolite of the cyanobacterium *Nostoc* XPORK14A.
5. To investigate the role of flavodiiron proteins (FDPs) in protecting against $^1O_2$-dependent photodamage of PSII in *Synechocystis* sp. PCC 6803.
6. To investigate the effect of moderate salt stress on $^1O_2$-dependent photodamage in *Synechocystis* sp. PCC 6803 mutants, which lack the Scp ABCDE small Chl binding proteins.
7. To develop a method for the detection of $^1O_2$ in the dinoflagellate *Symbiodinium* and to study the correlation of inhibition of the Calvin-Benson cycle by glycolaldehyde (GA) and KCN on the extent of photodamage under heat and light stress.
8. To investigate the effect of chloramphenicol (CAP) on the rate of photodamage in intact cyanobacterial cells.

# 3. MATERIALS AND METHODS

## 3.1. Biological materials

### 3.1.1. *Synechocystis* cell cultures

*Synechocystis* sp. PCC 6803 (which will be abbreviated as *Synechocystis* hereafter) wild type and mutant cells were propagated in BG-11 growth medium in a rotary shaker at 30 °C under a 3 % $CO_2$-enriched atmosphere. The D1-Gln130Leu (which is referred to as Q130L) and D1-Gln130Glu (which is referred to as Q130E) mutants were constructed in the *psbA3* gene of *Synechocystis* (Nixon *et al.*, 1992). *Synechocystis* wild type Orange Carotenoid Protein (OCP), mutant OCP and OCP overexpressing strains were obtained from the Institute de Biologie et Technologies de Saclay, France, whose construction has been described previously (Wilson *et al.*, 2008). The *Synechocystis Δflv4* mutant, the *ΔpsbA2* and *flv4-2*/overexpressing strains were obtained from the Department of Biochemistry, University of Turku, Finland and construction of these mutants has been described (Bersanini *et al.*, 2014). The glucose tolerant control strain of *Synechocystis* (CS) and the σ factor mutant strain *ΔsigCDE* were obtained from the Department of Biochemistry, University of Turku, Finland, and construction has been described previously (Pollari *et al.*, 2010). *Synechocystis* PSI-less and PSI-less/*ScpABCDE*$^-$ strains were obtained from the Department of Chemistry, Umeå University, 90187 Umeå, Sweden and construction of these mutants has been described previously (Shen *et al.*, 1993; Hernandez-Prieto *et al.*, 2011).

### *3.1.2. Symbiodinium* cell cultures

The CS-156 S*ymbiodinium* culture was obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Australian National Algae Culture Collection. Cells were grown in artificial seawater (known as f/2 media) at 26°C and growth light was 20 µmole photons $m^{-2}s^{-1}$, under a 12 h: 12 h cycle of light to dark. The cells were collected during their logarithmic growth phase by centrifugation at 6500 g for 5 min and re-suspended in the same volume of fresh f/2 medium at same concentration of Chl for experiments. Chl concentration was determined by a UV-1601 (SHIMADZU) spectrophotometer after extracting the pigments with 100% methanol (Takahashi *et al.*, 2008). Measurements were performed in cultures with approximately 10 µg Chl/mL (Hill *et al.*, 2014; Takahashi *et al.*, 2008).

### 3.2. Flash-induced fluorescence and variable fluorescence measurements

Flash-induced increase and the subsequent decay of Chl fluorescence yield were measured by a double-modulation fluorometer, PSI Instruments, Brno (Trtilek *et al.*, 1997) in the 150 µs to 100 s time range. The sample concentration was 5 µg Chl/mL. For variable fluorescence measurements the same double-modulation fluorometer was used (Vass *et al.*, 1999a). For *Synechocystis* ΔOCP mutant and OCP overexpressing strain, the cell suspension were irradiated through a gelatin filter (Kodak Wratten 29), which cuts all light below 600 nm and transmits only orange-red light. The light treatment for variable Chl fluorescence measurements was performed by illuminating 2 mL cell suspension through the red filter directly in the chamber of a Hansatech oxygen electrode, resulting in a light intensity of 550 µmole photons $m^{-2}s^{-1}$. PSII activity was checked by measuring variable fluorescence in cells, which were moved from the chamber each 15 min into the fluorometer and then back to the illumination chamber. The initial amplitude of variable Chl fluorescence induced by a 20 µs long saturating light pulse was measured 150 µs after the saturating light pulse. Measurements were taken after 3 min dark adaptation in the fluorometer chamber. DCMU at concentration of 10 µM was added in some measurements to inhibit electron transfer at the $Q_B$ binding site of PSII. Data were collected and the fluorescence curves were plotted after normalization and the data were analyzed using Origin version 8.6.

For *Symbiodinium* cells the culture was kept under constant 26°C temperature. The irradiance was applied for 8 h, followed by 12 h darkness and a further 12 h of light (40 µmole photons $m^{-2}s^{-1}$). Photosynthetic activity was assessed by measuring the so-called OJIP transient of variable Chl fluorescence during application of a 2 s saturating pulse (Strasser *et al.*, 1995). Fv/Fm was obtained by calculating (Fm-Fo)/Fm, where Fo and Fm represent the minimum fluorescence in dark adapted cells, and the maximal fluorescence yield under continuous saturating light, respectively. These measurements were performed by using the FL-3000 Fluorometer (PSI Instruments, Brno). Data were collected and the fluorescence curves were plotted after normalization and the data were analyzed using Origin version 8.6.

### 3.3. Oxygen evolution and oxygen uptake measurements

Steady state oxygen evolution and oxygen uptake rates in *Synechocystis* cells were measured with Hansatech DW2 $O_2$ electrode at 30°C under the illumination of 2300 µmole photons $m^{-2}s^{-1}$ in the presence of 0.5 mM DMBQ, or 1mM pBQ+1mM FeCN as artificial electron acceptors. CAP at concentration of 200 µg/mL was used. The wild-type *Synechocystis* cells were incubated in the presence of 200 µg/mL CAP or 300 µg/mL lincomycin for 30 min under growth conditions and obtained data is presented in figure 4.33b. Freshly re-suspended *Synechocystis* cell suspension of 2 mL was used at 5 µg Chl/mL in each measurement, and three replicates were measured. DCMU at concentration of 10µM was also added in combination with CAP for the comparison with control measurements. In case of *Symbiodinium* cells, the cell suspension of 1 mL at 10 µg Chl/mL was used in each measurement, and three replicates were measured.

### 3.4. His-mediated oxygen uptake measurements

Singlet oxygen ($^1O_2$) production in intact cells was detected by measuring the rate of light-induced oxygen uptake in the presence of 5 mM histidine (abbreviated as His hereafter). *Synechocystis* cells were centrifuged twice and re-suspended in fresh BG-11. The Chl concentration was 5 µg Chl/mL. The cells were kept under normal conditions for one hour before $^1O_2$ measurement. $O_2$ uptake measurements were performed by a Hansatech DW2 $O_2$ electrode in the absence of artificial electron acceptors at 2300 µmole photons $m^{-2}s^{-1}$ light intensity. Oxygen evolution was measured with and without 5 mM His and the differences in oxygen rates were calculated. The rate of His-mediated O2 uptake was used as a measure of $^1O_2$ production (Rehman *et al.*, 2013). Water content of cell culture was replaced with $D_2O$ for the enhancement of $^1O_2$ production, while quenching of $^1O_2$ was achieved by the addition of 10 mM $NaN_3$ which is a $^1O_2$ quencher. The effect of other ROS on His-mediated O2 uptake was probed by oxygen measurements in BG-11 medium containing 5 mM His in the presence of 500 µM $H_2O_2$, or artificially generated hydroxyl radials (500 µM $H_2O_2$ + 200 µMFe$(NH4)_2(SO4)_2$), or artificially generated superoxide (100 µM xanthine + 0.025 unit/mL xanthine oxidase (McCord & Fridovich, 1969; Beauchamp & Fridovich, 1971).

### 3.5. Photoinhibitory treatments of *Synechocystis* cells

For photoinhibitory treatment, re-suspended cells of *Synechocystis* wild-type and mutants strains were illuminated at the irradiation of 500 µmole photons $m^{-2}s^{-1}$ in the presence of the protein synthesis inhibitor lincomycin (300 µg/ml), which blocks the protein synthesis dependent repair of PSII and allows determination of the rate of photodamage. Oxygen evolution of wild type *Synechocystis* and mutant strains was measured in 2mL cell culture using a DW2 oxygen electrode (Hansatech) at 2300 µmole photons $m^{-2}s^{-1}$ visible light intensity in the presence of 0.5 mM DMBQ at different time points and normalized to initial point in percent for comparison. Different photoinhibitory treatment conditions were employed for *Synechocystis* wild type OCP, ΔOCP mutant and OCP overexpressing strains. Oxygen evolution was performed by illuminating a 100 mL cell suspension (5 µg Chl/mL) through the red filter, resulting in a light intensity of 550 µmole photons $m^{-2}s^{-1}$at the surface of the cell culture. The oxygen evolution rate was measured in 2mL cell culture using a DW2 oxygen electrode (Hansatech) at 2300 µmole photons $m^{-2}s^{-1}$ visible light intensity in the presence of 0.5 mM DMBQ.

The ΔOCP strain and wild type *Synechocystis* were also illuminated with 500 µmole photons $m^{-2}$ $s^{-1}$ visible light in the presence of the protein synthesis inhibitors CAP at concentration of 200 µg/mL and lincomycin at concentration of 300 µg/mL. Oxygen evolution of wild type *Synechocystis* and mutant cells were measured in the presence of 0.5 mM DMBQ and combination of 1 mM pBQ+1 mM FeCN at different time points of illumination. Data were normalized to initial point in percent for comparison.

### 3.6. $^1O_2$ production in intact *Symbiodinium* cells

Singlet oxygen production in intact *Symbiodinium* cells was detected by His mediated chemical trapping technique, which is based on oxygen uptake from the suspension due to oxidation of His by $^1O_2$ (Rehman *et al.*, 2013). The *Symbiodinium* cells were collected by centrifugation at 6500 g for 5 min and re-suspended. The final concentration of the cells was approximately 10 µg Chl/mL. The cells were kept under growth conditions for at least one hour before $^1O_2$ measurement. $^1O_2$ production in intact *Symbiodinium* cells was detected by measuring the rate of light induced oxygen uptake. The difference between the rates of light induced oxygen production with and without His was used to calculate the rate of His-induced oxygen uptake as

a measure of $^1O_2$ production. Measurements were performed by using a Hansatech DW2 $O_2$ electrode at 2300 µmole photons $m^{-2}s^{-1}$ light intensity.

### 3.7. Glycolaldehyde and potassium cyanide treatment of *Symbiodinium* cells

The *Symbiodinium* cells were collected by centrifugation at 6500 g for 5 min and then freshly re-suspended cells were shifted into conical flasks with and without 10 mM GA. The flasks were placed in dark for 15 min for control measurements and later shifted to high light at 45 µmole photons $m^{-2}s^{-1}$ to study the effect of GA on the extent of $^1O_2$ production. PSII activity was monitored by measuring maximum quantum yield (Fv/Fm) from control and GA-treated samples after 0, 2, 4, 6 and 8 hours of treatment by using the FL-3000 Fluorometer. Parallel oxygen evolution measurements were made in the absence and presence of 5 mM His and the rates of His-induced oxygen uptake were calculated.

The His-mediated $O_2$ uptake method was also used to monitor the effect of KCN on the extent of $^1O_2$ production in *Symbiodinium* cells. Freshly re-suspended cells were used and mixed with 10 µM KCN and incubated in the dark for 30 min. Dark adapted cells were used to measure the oxygen evolution with and without 5 mM His and the rate of His-induced oxygen uptake were calculated. The light intensity during the oxygen measurements was 2300 µmole photons $m^{-2}s^{-1}$.

### 3.8. Singlet oxygen imaging in *Symbiodinium* cells

Singlet Oxygen Sensor Green (SOSG) reagent (Molecular Probes Inc., Eugene, OR, USA) was used for $^1O_2$ imaging in cultured *Symbiodinium* cells. SOSG was added to a culture of re-suspended *Symbiodinium* cells at concentration of 250 µM. The cells containing SOSG were illuminated at 2300 µmole photons $m^{-2}$ $s^{-1}$ intensity of visible light for 5 min either directly, or through a filter which transmits only in the 380-450 nm range. Imaging of green fluorescence, which is emitted by SOSG after capturing $^1O_2$ was performed by a confocal laser scanning microscope (Leica SP5, Leica Microsystems, Germany) using excitation at 504 nm and emission detection at 525 nm. The images were obtained by transmitted light detection module with 488 nm excitation using laser diode (LD). Simultaneously, fluorescence excited by a 488 nm line of argon laser was also detected. SOSG green fluorescence was detected by using excitation at 504 and emission at 525 nm, while Chl fluorescence was detected with 650–750 nm emission filter.

The images were also obtained from control samples without SOSG and without light illumination. The images were analyzed by using computer software Leica.

Fluorescence Stereo Microscope (Leica Microsystem) was also used for imaging SOSG in *Symbiodinium* cells with its old supernatant and fresh supernatant to observe the fluorescence signal from outside the cell. The cells containing SOSG were illuminated for 3 and 5 min, the visible light irradiation during the cells treatment was approximately 2300 µmole photons $m^{-2}s^{-1}$. The fluorescence signal from the obtained images was analyzed by a MATLAB based software.

### 3.9. Heat and light treatment of *Symbiodinium* cells

The *Symbiodinium* cells were collected by centrifugation at 6500 g for 5 min and re-suspended in fresh medium. 5 mL volume of cells was placed in glass tube and approximately 10 µg Chl/mL. The tubes with and without His were kept at different conditions in automated incubator. These treatment conditions were; growth light 20 µmole photons $m^{-2}s^{-1}$ and growth temperature, 26 °C (control); high light, 100 µmole photons $m^{-2}s^{-1}$ and growth temperature, 26 °C (HL); growth light, 20 µmole photons $m^{-2}s^{-1}$and high temperatures, 34°C (HT); high light, 100 µmole photons $m^{-2}s^{-1}$and high temperatures, 34°C (HLHT). Maximum quantum yield of PSII (Fv/Fm) was measured from each sample at time zero and after 24 hours treatment by using the FL-3000 fluorometer. After 24 hours treatment 1 mL cells from each sample were also harvested for photometric analysis of pigmentation at 665nm. Chl concentration was determined by a UV-1601 (SHIMADZU) spectrophotometer after extracting the pigments with 100% methanol (Takahashi *et al.*, 2008).

# 4. RESULTS AND DISCUSSION

## 4.1. Characterization of $^1O_2$ production and its relation to PSII photodamage in *Synechocystis* sp. PCC 6803

### 4.1.1. Detection of $^1O_2$ production in *Synechocystis* by His-mediated chemical trapping

Light induced production of singlet oxygen ($^1O_2$) via interaction of ground state molecular oxygen with Chl triplets has been demonstrated in isolated PSII reaction center complexes by His- or imidazole mediated chemical trapping (Telfer *et al.*, 1994a) and also by direct 1270 nm luminescence measurements (Macpherson *et al.*, 1993). In thylakoid membrane particles EPR spin trapping was applied successfully for $^1O_2$ detection by using either TEMP (Hideg *et al.*, 1994c) or TEMPD-HCl (Fischer *et al.*, 2006b). The direct 1270 nm luminescence measurements could also be used in isolated PSII complexes (Tomo *et al.*, 2012). In intact plant systems the fluorescent $^1O_2$ traps DanePy (Hideg *et al.*, 1998; Hideg *et al.*, 2007) and Fluorescence Sensor Green (SOSG) have been applied (Flors *et al.*, 2006). However, detection of $^1O_2$ production in intact cyanobacterial cells presents a significantly larger challenge since the EPR and fluorescent spin traps do not penetrate the cells and application of the direct 1270 nm luminescence method is hampered by the influence of background Chl fluorescence (Li *et al.*, 2012).

In order to establish a method that can be applied for detection of $^1O_2$ production in intact *Synechocystis* cells we used a chemical trapping method in which a good $^1O_2$ acceptor, such as His (or imidazole) reacts with $^1O_2$ to form $HisO_2$ (or $ImO_2$) (Verlhac *et al.*, 1984). In the absence of added His the $^1O_2$, which is formed inside the cells, is mostly converted back to triplet ground state molecular oxygen via physical quenching mechanisms, e.g. via energy transfer to carotenoids. This process does not consume $O_2$, and therefore does not lead to $O_2$ uptake. His can penetrate the cell wall of cyanobacteria and efficiently competes with the physical quenching processes of $^1O_2$ inside the cells. The reactions of $^1O_2$ with His are very selective and result in the production of short lived endoperoxides, followed by the production of stable oxidation products, which chemically trap singlet oxygen (Méndez-Hurtado *et al.*, 2012). Since $O_2$ inside the cells equilibrates with dissolved $O_2$ in the aqueous phase of the suspension outside the cells, the $O_2$ uptake which occurs due to $^1O_2$ induced oxidation of His inside the cells results in the decrease of dissolved $O_2$ outside the cells as well, which can be measured by the oxygen electrode. It is of note that a small fraction of $^1O_2$ is trapped by the pigment, lipid and protein constituents of the

thylakoid membrane even in the absence of added His, resulting in a background level of $O_2$ uptake, which cannot be separated from $O_2$ uptake arising from respiration.

His-mediated $O_2$ uptake has been applied for $^1O_2$ detection earlier in isolated PSII reaction center complexes (Telfer *et al.*, 1994a; Telfer *et al.*, 1994c). In order to show that there is no $O_2$ consuming interaction between the ingredients of the BG-11 culture medium and the added His we tested the effect of His addition on the level of dissolved $O_2$ in BG-11. As shown in Fig. 4.1, the $O_2$ level was not affected by illumination in the BG-11 medium in the presence of 5 mM His alone. When BG-11 was supplemented with Rose Bengal (RB), which is a strong sensitizer of light-induced $^1O_2$ production, switching on the light (2300 µmole photons $m^{-2}s^{-1}$), induced a rapid $O_2$ uptake in the presence of His, which is due to the removal of dissolved $O_2$ by His-mediated $^1O_2$ trapping (Fig. 4.1a). It is of note that a small light induced decrease of the $O_2$ level was also observed when RB was added without His. This effect is most likely the consequence of $^1O_2$ trapping by organic ingredients of BG-11.

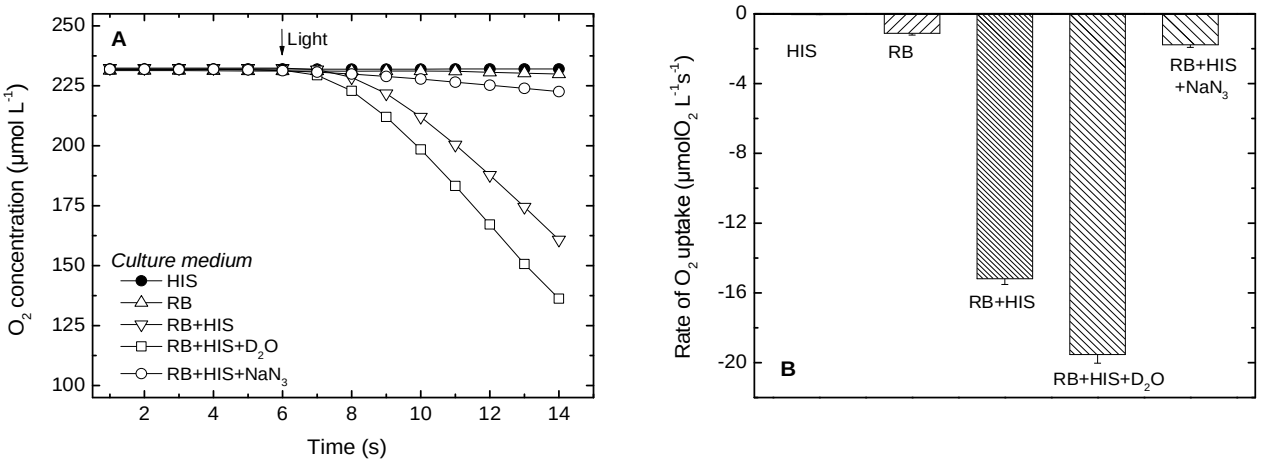


*Fig.4.1a. His-mediated oxygen uptake in culture medium containing Rose Bengal (RB): A, Concentration changes of dissolved oxygen were measured by a Clark type oxygen electrode in cell free BG-11 culture medium in the presence of 5 mM His (closed circles), 1 µM Rose Bengal (RB) (up triangles), 5 mM His+1 µM RB (down triangles), 5 mM His+1 µM RB in $D_2O$ containing BG-11 (open squares), 5 mM His+1 µM RB+10 mM $NaN_3$ (open circles). B, Rate of oxygen uptake calculated from the traces of fig A. Data are means ± s.d. (n=3). The light intensity during the measurements was 2300 µmole photons $m^{-2}s^{-1}$.*

The correlation of His-mediated $O_2$ uptake with $^1O_2$ trapping was further confirmed by adding the $^1O_2$ quencher $NaN_3$, which suppressed the $O_2$ uptake effect. Furthermore, water

content of BG-11 was replaced with $D_2O$, which extends the lifetime of $^1O_2$ (Ogilby & Foote, 1982; Egorov *et al.*, 1989) and therefore increases the probability for its trapping by His, enhanced the rate of $O_2$ uptake. The calculated $O_2$ uptake rates confirm that the presence of $D_2O$ significantly enhances the rate of His-mediated oxygen removal, whereas $NaN_3$ almost completely eliminates it (Fig. 4.1a). We have also applied another $^1O_2$ sensitizer, methylene blue for the confirmation test, which led essentially to the same results as RB (Fig. 4.1b). The above data demonstrate that His-mediated chemical trapping can be applied for $^1O_2$ detection in BG-11 containing media.

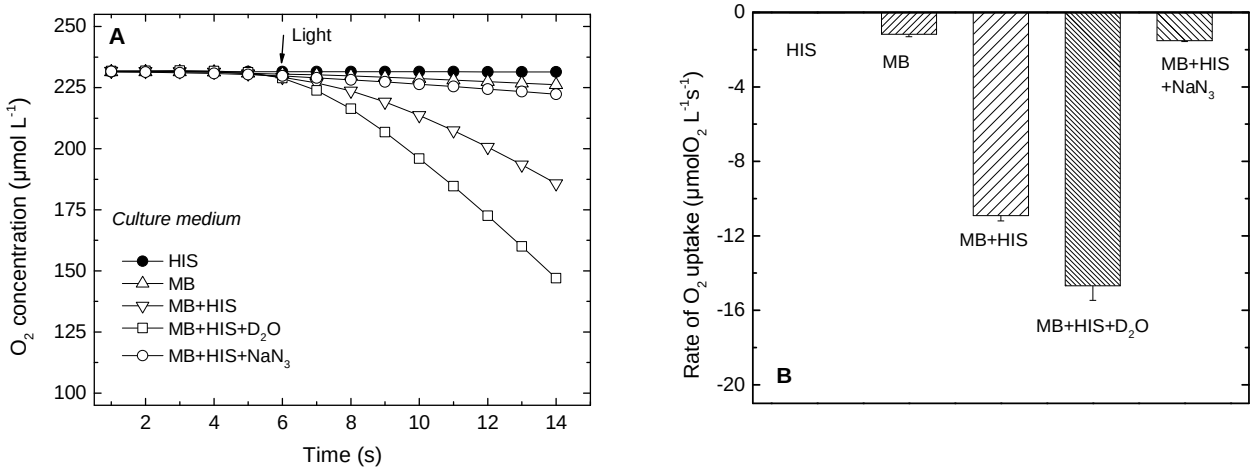


*Fig.4.1b. His-mediated oxygen uptake in culture medium containing methylene blue (MB): A, Concentration changes of dissolved oxygen in BG-11 culture medium in the presence of 5 mM His (closed circles), 1µM MB (up triangles), 5 mM His+1µM MB (down triangles), 5 mM His+1 µM MB in $D_2O$ containing BG-11 (open squares), 5 mM His+1 µM MB+10 mM $NaN_3$ (open circles).. B, Rate of oxygen uptake calculated from the traces of fig A. Data are means ± s.d of three independent experiments. The light intensity during the measurements was 2300 µmole photons $m^{-2}s^{-1}$.*

Since His may also react with reactive oxygen species (ROS) other than $^1O_2$ we checked the effect of $H_2O_2$, hydroxyl radicals and superoxide on the level of dissolved oxygen in the presence of His. As shown in Fig. 4.2, the addition of 0.5 mM $H_2O_2$ in the presence of 5 mM His did not induce any oxygen uptake. Hydroxyl radicals, which were produced via the Fenton reaction by mixing 0.5 mM $H_2O_2$ + 200 µM $Fe^{2+}$ added in the form of $Fe(NH_4)_2(SO_4)_2$, did not lead to His mediated $O_2$ uptake either. Production of superoxide by mixing 100 µM xanthine + 0.025 unit/mL xanthine oxidase induced an $O_2$ uptake effect by converting $O_2$ to superoxide (McCord & Fridovich, 1969; Beauchamp & Fridovich, 1971). However, the $O_2$ uptake was not

affected by the addition of His together with xanthine and xanthine oxidase (Fig. 4.2). These data show that the presence of $H_2O_2$, hydroxyl radicals, or superoxide is not expected to induce His-mediated $O_2$ uptake that would compromise the effect arising from the trapping of $^1O_2$.

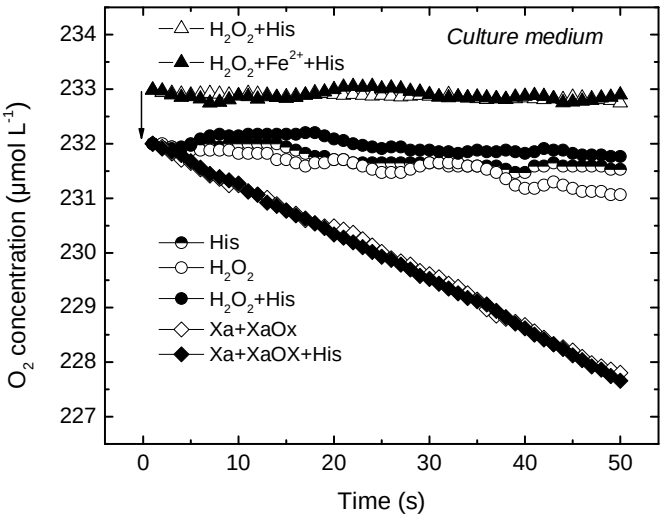


*Fig.4.2. Effect of His on the production of other ROS: $O_2$ uptake measurements were performed in the dark in the presence of 500 µM $H_2O_2$ (open circles), 5 mM His (half-closed circles), 500 µM $H_2O_2$+5 mM His (closed circles), 100 µM xanthine+0.25 unit/mL xanthine oxidase (open diamonds), 100 µM xanthine +0.25 unit/mL xanthine oxidase (open diamonds) +5 mM His (closed diamonds).*

*In a separate experiment the effect of 500 µM $H_2O_2$ +200µM $Fe^{2+}$ (added in the form of Fe $(NH_4)_2(SO_4)_2$) + 5 mM His was also checked (closed triangles). This curve is shown together with its 500 µM $H_2O_2$+5 mM control (open triangles). For the sake of clarity the $H_2O_2$+His, and $H_2O_2$+His+ $Fe^{2+}$ curves are shown after shifting along $O_2$ concentration axis by 1 µmole $O_2L^{-1}$. The actual position of these curves is shown by the arrow. The light intensity during the measurements was 2300 µmole photons $m^{-2}s^{-1}$.*

An important prerequisite of $^1O_2$ trapping in intact cells is that the trap molecules should reach cell compartments where $^1O_2$ is produced. Due to their short lifetime the mobility of $^1O_2$ molecules is very limited (ca. 200 nm in water and 400 nm in membranes (Baier *et al.*, 2005)), which prevents their migration from the cells to the external medium. Therefore, the observation that His induces light-dependent $O_2$ uptake in the suspension of *Synechocystis* cells (Fig. 4.4), while it has no effect in cell free BG-11 medium (Fig. 4.1a) shows that exogenous His penetrates inside the cells reaching close vicinity of the site of $^1O_2$ production. A further requirement for the applied trap is that it should not inhibit PSII electron transport activity. Such an effect would represent an artifact in the $O_2$ uptake measurements in *Synechocystis* cells, which produce $O_2$ themselves during illumination. Such an effect can be partly circumvented by complete inhibition of $O_2$ evolution, e.g. by DCMU, however, electron transport inhibitors, which act at

the $Q_B$ site modify the redox potential of $Q_A$ and influence charge recombination pathways in PSII and modify $^1O_2$ production efficiency.

The effect of exogenously added His on PSII was assessed by flash induced variable Chl fluorescence transients, which reflect the functioning of electron transport both at the donor and acceptor side of PSII. Illumination of *Synechocystis* cells with a single turnover saturating flash extracts an electron from the catalytic Mn cluster of water oxidation and transfers it to the $Q_A$ acceptor, which leads to increased fluorescence yield (Fig. 4.3). Subsequent reoxidation of $Q_A^-$ in the dark results in the relaxation of fluorescence yield exhibiting three main decay phases. The two faster decay components reflect the reoxidation of $Q_A^-$ by PQ molecules which are already bound to the $Q_B$ site at the time of the flash ($\tau$~500-600 μs) or bind from to PQ pool after the flash ($\tau$~5-10 ms), respectively. Whereas, the slow phase of the decay ($\tau$~ 10-15 s) arises from back reaction of the $S_2$ state of the water-oxidizing complex with $Q_A^-$, which is populated via the equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ (Vass *et al.*, 1999a; Rutherford *et al.*, 1982; Robinson & Crofts, 1983).

It is clear from Fig. 4.3a that neither the amplitude nor the kinetics of the fluorescence signal is affected at 5 mM His concentration in the absence or presence of DCMU. As a consequence His does not induce modification of PSII electron transport at the level of the $Q_A$ and $Q_B$ quinone electron acceptors, or the water oxidizing complex. However, at higher concentrations (above 10 mM) His addition induces a small decrease in the amplitude of flash induced Chl fluorescence relaxation traces (data not shown), which shows that exogenously added His can reach the immediate vicinity of the PSII complex. We have also measured the so-called OJIP variable Chl fluorescence transient, which reflects the efficiency of electron transfer from the water oxidizing complex. Again, this variable fluorescence transient was not affected by 5 mM His either in the absence or presence of DCMU (Fig. 4.3b). Based on these data we can conclude that up to 5 mM concentration His does not interfere with PSII activity and potentially suitable for $^1O_2$ trapping in intact *Synechocystis* cells.

The $^1O_2$ quencher $NaN_3$ is known to affect photosynthetic activity and expected to decrease the rate of oxygen evolution (Forti & Gerola, 1977), which would interfere with the $O_2$ uptake assay if it is applied in cells with functional oxygen evolving activity. Therefore, $NaN_3$ was applied in the presence of DCMU, which blocks the $Q_B$ site and eliminates $O_2$ evolution. As

shown in Fig. 4.3b addition of 10 mM $NaN_3$ induced a decrease of variable fluorescence in the presence of DCMU showing a partial inhibition of PSII activity. However, this effect is not expected to interfere significantly with the His-mediated $O_2$ uptake when performed in the presence of DCMU (Fig. 4.3b).

We have also tested the effect of imidazole on PSII function, which was used earlier as a chemical trapping agent for $^1O_2$ detection in isolated PSII reaction center complexes (Telfer *et al.*, 1999). However, in contrast to His, 5 mM imidazole induced a significant inhibition of PSII activity as revealed by the decreased amplitude and retarded relaxation kinetics of the flash induced Chl fluorescence traces, as well as by the decreased induction of the OJIP fluorescence transient (data not shown). The mechanism of this inhibitory effect of PSII activity by imidazole is not clear at present, and we restricted our studies to using His as chemical $^1O_2$ trap.

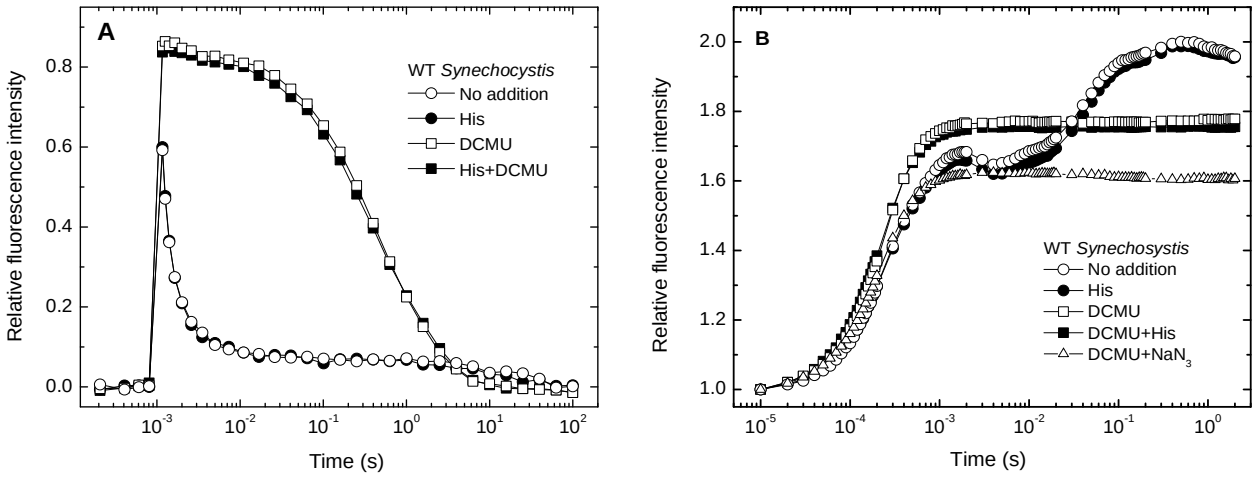


*Fig.4.3. Effect of His addition on variable fluorescence characteristics of Synechocystis cells, A, Flash induced Chl fluorescence traces in the absence (open symbols) and presence of 5 mM His (closed symbols) and DCMU. 20 µs long saturating light pulse was used; B, Variable Chl fluorescence traces in the absence (open symbols) and presence of 5 mM His (closed symbols). The traces were measured either without electron transport inhibitor (circles), or in the presence of 10 µM DCMU (squares). The effect of 10 mM $NaN_3$ is shown in the presence of DCMU (triangles). 2 s long saturating pulse was used in case of OJIP transient. The curves in fig A and B are presented after normalized to same initial Fo fluorescence level.*

When the effect of 5 mM His was checked on the $O_2$ evolving activity of *Synechocystis* cell cultures no modification on the rate of dark respiration was observed. In contrast, the

apparent rate of light induced oxygen evolution decreased (Fig. 4.4a). Since inhibition of PSII electron transport does not occur at this His concentration, the apparent decrease of the $O_2$ evolution rate indicates that His induces an $O_2$ removal process from the cell suspension. This process is in competition with light induced oxygen evolution and can be related to His mediated $^1O_2$ trapping. This hypothesis was verified by using $D_2O$ in the suspension medium instead of $H_2O$, which extends the lifetime of $^1O_2$ (Ogilby & Foote, 1982; Egorov *et al.*, 1989) and therefore enhances the probability of $^1O_2$ trapping by His (Fig. 4.4a and 4.4b). Although $D_2O$ in itself decreased the rate of $O_2$ evolution the extent of $O_2$ uptake by His addition was clearly increased relative to that obtained in the $H_2O$ containing medium, which supports the idea that His-mediated $O_2$ uptake reflects $^1O_2$ trapping.

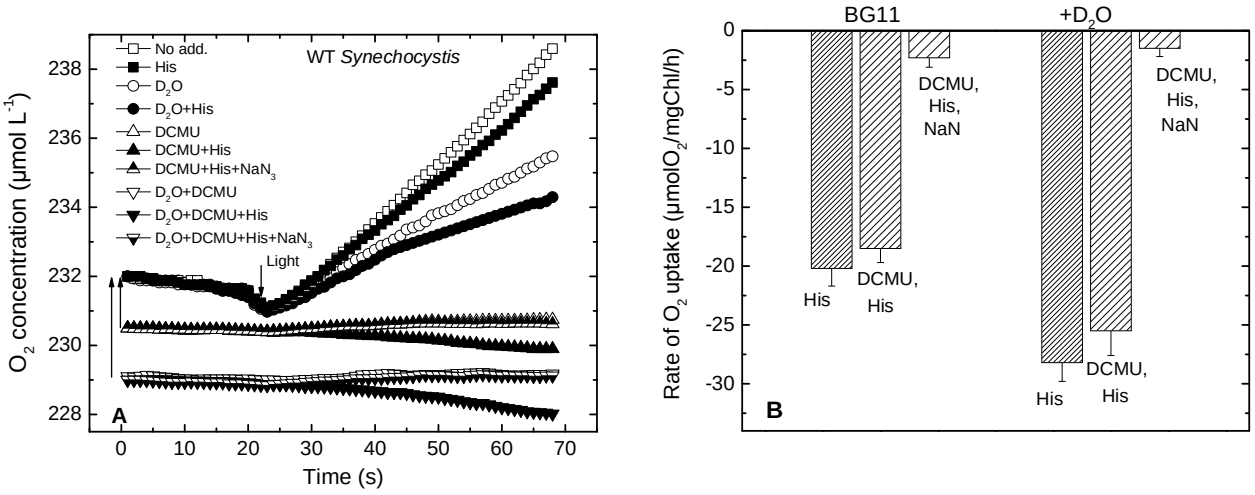


*Fig.4.4. The effect of $D_2O$ and sodium azide ($NaN_3$) on His-mediated $O_2$ uptake in Synechocystis. A, Light induced changes in the concentration of dissolved $O_2$ in Synechocystis cells containing $H_2O$ (squares and up triangles) or $D_2O$ (circles and down triangles) in the absence (open symbols), and in the presence of 5 mM His (closed, and half closed symbols). The measurements were also performed in the presence of 10 µM DCMU (triangles) where the effect of 10 mM $NaN_3$ in the presence of 5 mM His was also tested (half closed symbols).. For the sake of clarity some curves are shown after shifting along $O_2$ concentration axis. The actual position of these curves is shown by the arrow. B, Rate of His-mediated $O_2$ uptake in $H_2O$ and $D_2O$ containing cell suspensions, the light intensity during the measurements was 2300 µmole photons $m^{-2}s^{-1}$. Data are ±s.d of three independent experiments.*

Further support for the idea that His-mediated $O_2$ uptake arises from $^1O_2$ trapping is provided by the observation of real $O_2$ uptake in the presence of DCMU, which blocks $O_2$ evolution by occupying the $Q_B$ binding site. When only DCMU was added to the *Synechocystis* cells light induced increase in the level of dissolved oxygen was almost completely eliminated (Fig. 4.4a, open up triangles). The apparent small residual $O_2$ evolution could arise from the non-complete inhibition of PSII activity by the applied 10 μM DCMU. However, this effect must be very small since the flash induced fluorescence relaxation curves (Fig. 4.3a), and steady state variable fluorescence transients (Fig. 4.3b), show a large extent inhibition of electron transport at the acceptor side of PSII. Another reason for the apparent residual $O_2$ evolution in the presence of DCMU could be a light induced decrease in the rate of respiration, as was shown earlier for another cyanobacterium *Trichodesmium* spp. by membrane inlet mass spectrometry, which effect was assigned to partial diversion of electrons that would end up at the terminal oxidase(s) in the dark towards $NADP^+$ in the light via PSI (Kana, 1992).

The rate of respiration in the presence of DCMU is apparently smaller than in the absence of this inhibitor. This effect most likely arises from the usage of different cell culture batches for the measurements, which were performed in the absence and presence DCMU. We did not observe DCMU effect on the respiration rate when the measurements were performed in the same cell culture with and without DCMU. The previous membrane inlet mass spectrometry data did not show DCMU effect on the respiration rate either (Kana, 1992). Illumination in the presence of DCMU + His resulted in an absolute $O_2$ uptake (Fig. 4.4a, closed up triangles), which effect was enhanced in the presence of $D_2O$ (Fig. 4.4a, closed down triangles) in agreement with the increased lifetime of $^1O_2$ in $D_2O$. These observations confirm that His-mediated $O_2$ uptake in intact *Synechocystis* cells in the absence of DCMU cannot be caused by a small inhibition of $O_2$ evolution by His addition, which may cause such a small extent of change in the variable fluorescence characteristics in Fig. 4.3 that, is masked by experimental error.

Based on these findings DCMU inhibition of $O_2$ evolution could be a standard part of the His-mediated $O_2$ uptake measurement protocol. However, electron transport inhibitors, which act at the $Q_B$ site modify the redox potential of $Q_A$ and influence charge recombination pathways in PSII and modify $^1O_2$ production efficiency (Krieger-Liszkay & Rutherford, 1998a; Rutherford & Krieger-Liszkay, 2001). Such an effect may interfere with the modifications of charge

recombination characteristics induced by the studied mutations (see below). Therefore, we prefer to perform the $O_2$ uptake measurements without DCMU unless other treatments, such as $NaN_3$, induce partial inhibition of $O_2$ evolution which should be masked by complete inhibition of $O_2$ evolving activity.

Further support for $^1O_2$ trapping by His comes from the suppression of $O_2$ uptake in the presence of the $^1O_2$ quencher $NaN_3$. Since $NaN_3$ inhibits $O_2$ evolution (Forti & Gerola, 1977) its $^1O_2$ quenching effect was tested in the presence of DCMU where the artifact that would arise from the decreased of $O_2$ evolution rate could be avoided. Addition of $NaN_3$ almost completely reversed $O_2$ uptake either in $H_2O$, or in $D_2O$ containing cell suspensions (Fig. 4.4a, half closed up and down triangles, respectively). It has to be noted that $NaN_3$ causes a partial inhibition of PSII activity even in the presence of DCMU (Fig. 4.3b). However, this inhibitory effect is not expected to eliminate $^1O_2$ production, which can be observed even in isolated PSII reaction center complexes that lack completely $O_2$ evolving activity (Telfer *et al.*, 1994a). It is also of note that $NaN_3$ is not only a $^1O_2$ quencher, but also an inhibitor of superoxide dismutatse (SOD) (del Rio *et al.*, 1989) and catalase (Forti & Gerola, 1977).

Therefore, $NaN_3$ addition could lead to accumulation of $O_2^-$ and/ or $H_2O_2$ in the cells besides quenching $^1O_2$. However, it is highly unlikely that this effect could cause an artifact that would be responsible for the observed elimination of His-mediated $O_2$ uptake in the presence of $NaN_3$ as observed in Fig. 4.4. Since our data show that neither $O_2^-$, nor $H_2O_2$ interacts with His (Fig. 4.2) that would modify the level of dissolved $O_2$, the $NaN_3$ induced elimination of His-mediated $O_2$ uptake in the presence of DCMU provides strong support for the idea that the $O_2$ uptake effect is indeed due to $^1O_2$ trapping by His. Based on the above presented key observations, which show that illumination in the presence of His induces $O_2$ uptake both in the absence and presence of DCMU, which is enhanced by $D_2O$ and suppressed by $NaN_3$, we can conclude that His at 5 mM concentration is a suitable chemical trap for $^1O_2$ detection in intact *Synechocystis* cells.

The rate of $O_2$ uptake at 2300 μmole photons $m^{-2}s^{-1}$ is ca. 20 μmole $O_2$/mg Chl/h when measured in a *Synechocystis* cell suspension at 5 μg Chl/mL, corresponding to 5.6 μM Chl concentration (Fig 4.6b). In absolute terms this corresponds to 0.055 μmol $O_2$/L/s rate of changing the level of dissolved $O_2$ in the suspension. Considering that in cyanobacteria PSII and

PSI contains ca. 35 and 96 Chl molecules based on their crystal structures (Fromme *et al.*, 2001; Jordan *et al.*, 2001; Barber, 2001; Barber *et al.*, 2004) respectively, and also that the ratio of PSII:PSI is ≈3 (Aizawa *et al.*, 1992), the 5.6 μM total Chl corresponds to ≈17 nM PSII concentration. In comparison, 1 μM RB in cell free BG-11 induces ca. 16 μmole $O_2$/L/s uptake rate at 2300 μmole photons $m^{-2}s^{-1}$ light intensity due to $^1O_2$ trapping by His (Fig. 4.1a). Although the $^1O_2$ producing efficiency of RB and Chl cannot be exactly compared due to their different absorption characteristics, from the above data we can conclude that under illumination with white light the overall $^1O_2$ producing efficiency of Chls in intact *Synechocystis* cells is ca. 0.06% that of RB when equal number of sensitizing molecules are considered. However, if we consider whole PSII units as $^1O_2$ sensitizers and assume also that the contribution of antenna Chls in PSI to the overall $^1O_2$ production is negligible in comparison to PSII, which is supported by EPR spin trapping measurements (Hideg & Vass, 1995; Hideg *et al.*, 2001), we obtain that one PSII complex can produce $^1O_2$ with ca. 20% efficiency of one RB molecule when identical white light illumination is used. Since the main source of $^1O_2$ production in PSII is expected to be $^3$P680 this represents very efficient $^1O_2$ formation in the heart of PSII.

### 4.1.2. Involvement of $^1O_2$ in photodamage of PSII

The production of $^1O_2$ in photosynthetic systems involves the formation of triplet Chl states in the PSII reaction center, or in the light harvesting antenna (Krieger-Liszkay, 2005; Krieger-Liszkay *et al.*, 2008; Vass, 2011; Vass, 2012). This is followed by the interaction of Chl triplets with $O_2$, which also has triplet configuration in its ground state, and leads to the formation of highly reactive $^1O_2$. Since the amount of $O_2$ is usually high in comparison to the amount of Chl triplets, the yield of $^1O_2$ production is limited by the availability of $^3$Chl. On the other hand, the amount of Chl triplets is expected to be linearly dependent on light intensity not only in the light harvesting antenna, but also in the case of the PSII reaction center Chl P680.Therefore, the yield of $^1O_2$ production is expected to be linearly increasing with increasing light intensity. This expectation has been supported earlier in isolated PSII membranes by using EPR spin trapping (Fufezan *et al.*, 2002).

The hypothesis that light intensity dependence of $^1O_2$ production can be followed by His-mediated $O_2$ uptake was tested first in an *in-vitro* system, which is consisted of the BG-11 culture medium and Rose Bengal as $^1O_2$ sensitizer. As shown in Fig. 4.5 $^1O_2$ production, as

measured by the His-mediated $O_2$ uptake assay indeed showed linear light intensity dependence in the 34-2300 µmole photons $m^{-2}s^{-1}$ intensity range when sensitized by Rose Bengal in cell free culture medium.

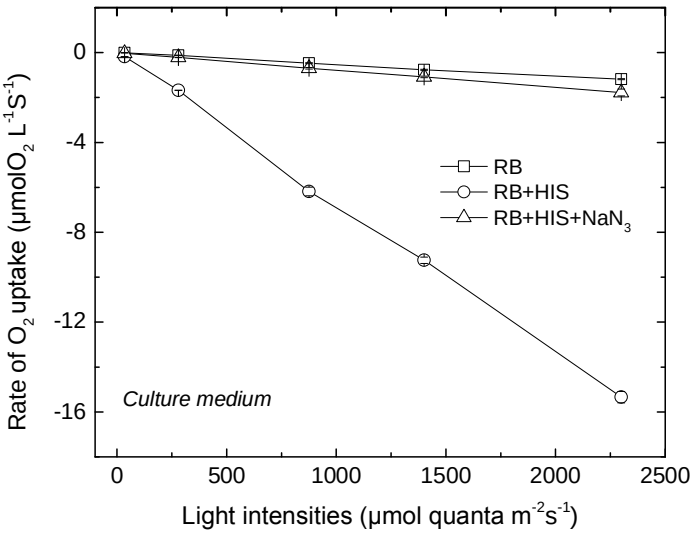


*Fig.4.5. Light intensity dependence of $^1O_2$ production in cell free culture medium, $O_2$ uptake was measured in BG-11 medium in the presence of 1µM RB alone (open square), with RB+5 mM His (open circle), or RB + His+ 10 mM $NaN_3$ (open up triangles). Data are ± s.d of three independent experiments.*

A computer simulation using the rate constants (Deák *et al.*, 2014; Rehman *et al.*, 2013) demonstrate that the concentration of $^3$P680 increases linearly in a wide intensity range even after $Q_A$ is fully reduced (Fig. 4.6a). This prediction could also be verified experimentally when $^1O_2$ production was measured in a suspension of intact *Synechocystis* cells and yielded linear light intensity dependence in the 30– 2300 µmole photons $m^{-2}s^{-1}$ range (Fig. 4.6b).

This result is highly important from the point of view of the light intensity dependence of photodamage. Experimental data demonstrate that the initial rate of photodamage linearly increases with light intensity even above the saturation of photosynthetic electron transport (Tyystjarvi & Aro, 1996; Tyystjarvi *et al.*, 1994). It has been argued that this finding can be explained only by assuming that photodamage of PSII is initiated by direct light induced inactivation of the Mn cluster of the water oxidizing complex (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005). The data, which were obtained earlier in isolated

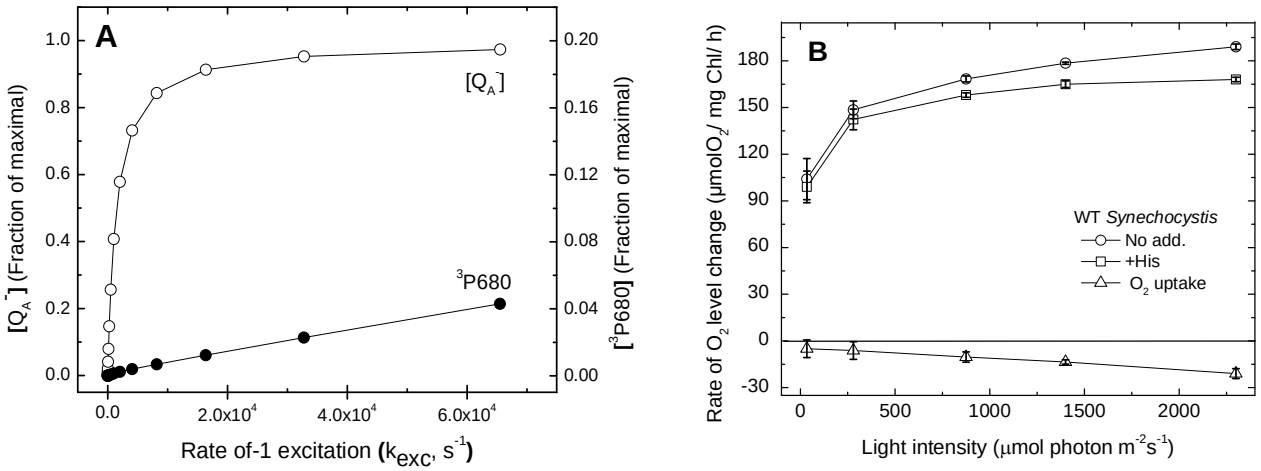


*Fig.4.6. Light intensity dependence of $^3$P680 formation and singlet oxygen production, A, Light induced changes in the amount of $Q_A^-$ and $^3$P680 were calculated by using a differential equation system. The fraction of PSII centers with $Q_A^-$ (open circles) and with $^3$P680 (closed circles) are plotted as a function of the rate of excitation, which is proportional to the light intensity. B, Measured rates of $O_2$ evolution in Synechocystis cells without addition (open circles) and in the presence of 5 mM His (open squares), as well as the rate of $O_2$ uptake, which reflects the rate of $^1O_2$ production (open triangles). The data in fig. B are means ± s.d of three independent experiments.*

PSII membranes (Fufezan *et al.*, 2002) and those presented here in intact *Synechocystis* cells demonstrate that the charge recombination mechanism of photodamage, in which $^3$P680 mediated $^1O_2$ production is a key event, can also explain the linear light intensity dependence of photoinhibition in a wide light intensity range, which extends well above the saturation level of photosynthesis. It has been reported recently that in higher plants ΔpH dependent dissipation of absorbed light energy via the non-photochemical quenching (NPQ) mechanism decreases $^1O_2$ formation and partially protects against photodamage (Roach & Krieger-Liszkay, 2012). In cyanobacteria NPQ does not depend on ΔpH, but occurs via light induced conformational change of the so called OCP (Wilson *et al.*, 2006). Since OCP dependent NPQ is induced when light intensity increases one could expect a retardation of $^1O_2$ production at the onset of light energy dissipation via NPQ. Our data did not indicate such a process in the investigated light intensity range, which might be due to the saturation of the OCP-dependent quenching effect at high light intensities, or to some other effect whose background is not clear at present. This interesting question could be investigated in future studies by using *Synechocystis* mutants in which the level and activity of OCP dependent NPQ can be controlled.

It has been shown earlier that the amino acid residue at the 130th position of the D1 PSII reaction center subunit influences the redox potential of the primary electron acceptor Phe by modifying H-bonding interactions (Loll *et al.*, 2008; Merry *et al.*, 1998). Higher plants have a Glu residue at this position, whereas numerous cyanobacteria have multiple D1 protein forms, which have either D1-130Glu or D1-130Gln. The Glu residue occurs in the so called high light D1 forms, which are expressed under high light exposure, whereas Gln occurs in the so called low light D1 forms, which are expressed under low light exposure of the cells (Kós *et al.*, 2008). Studies with site directed mutants of *Synechocystis*, which have the same D1 form with D1-Gln130 under both low and high light conditions have demonstrated that the replacement of Gln with Glu increases the midpoint redox potential of Phe by 33 mV, whereas the D1-Gln130Leu mutation decreases the Em(Phe/Phe$^-$) by 74 mV (Merry *et al.*, 1998).

Biophysical characterization of these mutants has revealed that the D1-Gln130Glu accelerates charge recombination of the $S_2Q_A^-$ state, whereas the D1-Gln130Leu slows it down (Rappaport *et al.*, 2002a; Cser & Vass, 2007; Cser & Vass, 2008). These effects have been assigned to the modulation of the rate of nonradiative recombination pathway from the primary charge separated state $P680^{+\bullet}Phe^{-\bullet}$ (Rappaport & Lavergne, 1997; Rappaport *et al.*, 2002b; Rappaport *et al.*, 2005; Cser & Vass, 2007; Cser & Vass, 2008). It has also been suggested earlier that nonradiative charge recombination within PSII (Cser & Vass, 2008; Cser & Vass, 2007; Vass & Aro, 2008; Vass & Cser, 2009; Vass, 2011; Vass, 2012) and in particular from the singlet state of the primary radical pair $^1[P680^{+\bullet}Phe^{-\bullet}]$ acts as an important photoprotective pathway, which competes with $^3P680$ formation and the consequent $^1O_2$ production (Cser & Vass, 2007; Vass & Cser, 2009; Vass, 2011). Although this hypothesis is well supported by various lines of experimental evidence, so far only preliminary data were reported about the comparison of $^1O_2$ production and photodamage in intact cyanobacterial cells (Cser & Vass, 2008).

Here we applied the His-mediated $O_2$ uptake assay of $^1O_2$ detection in the D1- Gln130Glu and D1-Gln130Leu mutants in which the non-radiative recombination pathway is enhanced and retarded, respectively, in parallel with the measurement of PSII photodamage. As shown in Fig. 4.7a, the rate of His-mediated oxygen uptake is smaller in the D1-Gln130Glu mutant, and higher in the D1-Gln130Leu mutant as compared to the WT. In order to confirm that the different rates

of oxygen uptake in the mutants reflect different rates of $^1O_2$ production the measurements were performed in the presence of $D_2O$, which increases the lifetime of $^1O_2$. As expected $D_2O$ increased the rate of $O_2$ uptake in both mutants and the WT (Fig. 4.7a) supporting its origin from $^1O_2$ trapping.

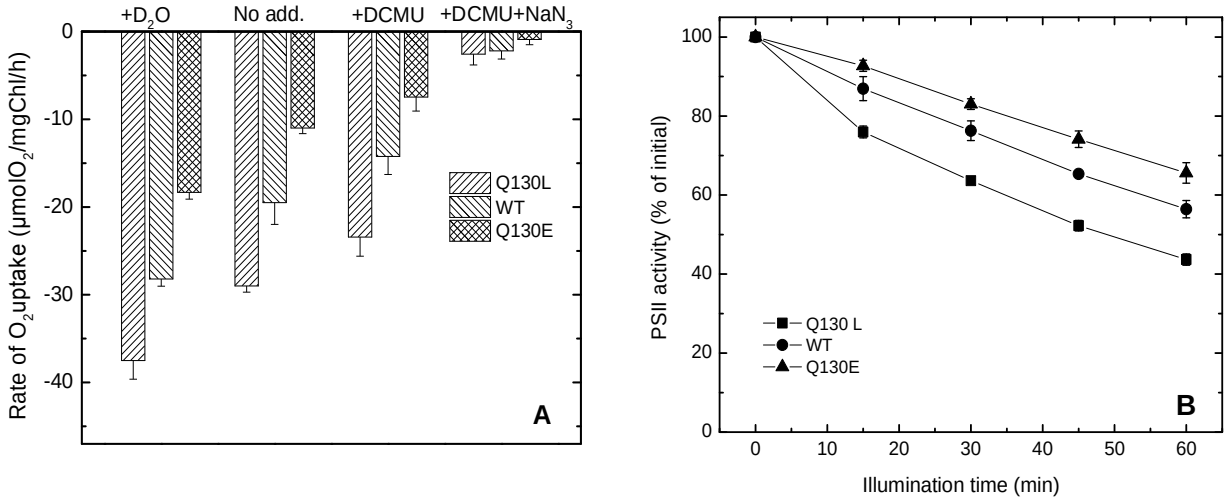


*Fig.4.7. The effect of D1-Gln130Glu and D1-Gln130Leu mutations on singlet oxygen production and photodamage in Synechocystis cells, A, The $^1O_2$ production was assessed by measuring the rate by His-mediated oxygen uptake in WT, D1-Gln130Glu and D1-Gln130Leu Synechocystis strains. The measurements were performed either without addition, or in the presence of $D_2O$. B, Light induced damage of PSII activity was assessed by measuring the rate of $O_2$ evolution in the presence of 0.5 mM DMBQ as artificial electron acceptor during exposure of WT (circles), D1-Gln130Glu (triangles) and D1-Gln130Leu (squares) to illumination with 500 µmole photons $m^{-2}s^{-1}$ light intensity. Data are means ± s.d of three independent experiments.*

This idea was further confirmed by the effect of $NaN_3$, which is a specific $^1O_2$ quencher. Since $NaN_3$ partially inhibits oxygen evolution the $O_2$ uptake assay was performed after blocking oxygen evolution by DCMU. In the presence of DCMU the extent of $O_2$ uptake was somewhat decreased relative to that obtained in the absence of DCMU (Fig. 4.7A), which effect can be explained by the effect of DCMU on the $^3P680$ producing charge recombination efficiency. However, addition of $NaN_3$ to DCMU treated cells drastically decreased the His-mediated $O_2$ uptake, which supports further the correlation of $^1O_2$ production with $O_2$ uptake in the presence of His. The residual rate of $O_2$ uptake in the presence of $NaN_3$ may arise either from the inability

of $NaN_3$ to react with all produced $^1O_2$, or could reflect His oxidation by other ROS forms than $^1O_2$. Therefore, the difference of the $O_2$ uptake rates in the presence and absence of $NaN_3$ reflect the lower limit for the rate of $^1O_2$ production. These values, which can be obtained from the measurements in the presence of DCMU, are significantly higher in the D1-Gln130Leu than in the D1-Gln130Glu mutant, whereas the WT rate shows an intermediate level (Fig. 4.7a).

These data confirm that the D1-Gln130Leu mutant produces $^1O_2$ at a significantly higher rate than the D1-Gln130Glu mutant, while the WT shows an intermediate rate. Fig. 4.7a also shows that the rate of $O_2$ uptake due to His-mediated $^1O_2$ trapping is smaller in the presence of DCMU than without addition. It has been shown earlier that binding of electron transport inhibitors to the $Q_B$ site shifts the redox potential of $Q_A$ (Vass & Demeter, 1982; Krieger-Liszkay & Rutherford, 1998b). In case of DCMU $Em(Q_A/Q_A^-)$ is shifted by +50 mV (Krieger-Liszkay & Rutherford, 1998b), which increases the free energy gap between Phe and $Q_A$. As a consequence the rate of reverse electron transfer from the $P680^{+\bullet}Phe^{-\bullet}$ charge separated state to $^3[P680^{+\bullet}Phe^{-\bullet}]$ will decrease at the expense of direct recombination, which decreases the probability of $^3P680$ and $^1O_2$ formation (Krieger-Liszkay & Rutherford, 1998b; Rutherford & Krieger-Liszkay, 2001). This prediction has been validated earlier by EPR detection of $^1O_2$ in isolated PSII (Fufezan *et al.*, 2002; Hideg *et al.*, 2011) and supported further by our data in intact cells.

When susceptibility to photoinhibitory damage of PSII electron transport was measured in the presence of the protein synthesis inhibitor lincomycin, which prevents protein synthesis dependent repair of PSII, the D1-Gln130Glu cells were less inhibited than the WT (Fig. 4.7b). In contrast, the D1-Gln130Leu cells were damaged to a larger extent than either the D1-Gln130Glu or the WT cells. This shows that the D1-Gln130Glu amino acid change provides protection against photodamage, while the D1-Gln130Leu change enhances it. It is very important to note that the extent of photodamage is correlated with the rate of $^1O_2$ production. Since the replacement of D1-Gln130 with Glu accelerates the efficiency of nonradiative charge recombination from the $^1[P680^{+\bullet}Phe^{-\bullet}]$ charge separated state, while the Leu replacement slows it down (Cser & Vass, 2008; Cser & Vass, 2007), these data provide further support for the earlier proposed (Ohad *et al.*, 2010; Vass & Cser, 2009) protective role of nonradiative charge recombination against $^1O_2$ mediated photodamage.

## 4.2. Characterization of $^1O_2$ production in *Synechocystis* mutants

### 4.2.1. The role of $^1O_2$ in photoinhibition of *Synechocystis* sp. PCC 6803 *ΔsigCDE* mutant

The sigma (σ) factor mutant *ΔsigCDE* contains the multifunctional, stress responsive SigB as its only functional group 2 σ factor. Previous studies have shown that inactivation of SigB results in a salt (Nikkinen *et al.*, 2012) and heat (Tuominen *et al.*, 2006) sensitive phenotype. On the contrary, *ΔsigCDE* acclimates slightly better to high salt than the control strain (CS) due to upregulation of the compatible solute producing enzyme glucosylglycerol phosphate synthase, heat shock protein HspA and carotenoids, all of which are known to increase high salt tolerance (Tyystjärvi *et al.*, 2013). Hakkila and coworkers showed that ΔsigCDE has high carotenoid and *flv*4-2 contents and it suffers from oxidative stress but protected PSII against light induced damage. The His-mediated $O_2$ uptake method of $^1O_2$ detection was used to investigate the role of oxidative stress and photoinhibition in high light acclimation using a regulatory *ΔsigCDE* mutant of the cyanobacterium *Synechocystis*

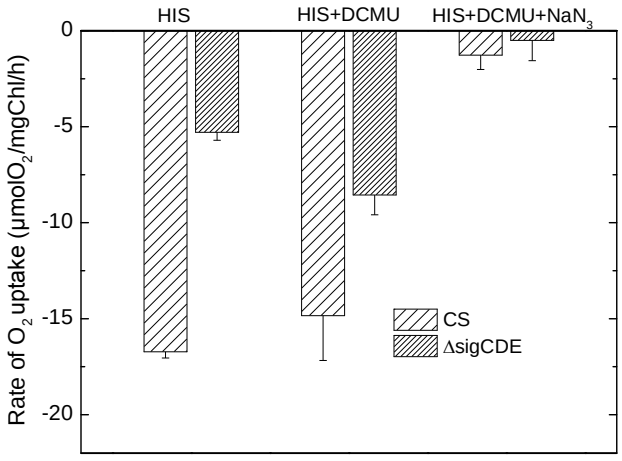


*Fig.4.8. $^1O_2$ production in ΔsigCDE mutant of Synechocystis, $^1O_2$ production was quantified by measuring the rate of His-mediated oxygen uptake in control strain, CS (right hatch) and ΔsigCDE (dense right hatch). The measurements were also performed either in the presence of 10 µM DCMU or in the presence of DCMU plus 10mM $NaN_3$ .The light intensity during the measurements was 2300 µmole photons $m^{-2}s^{-1}$. Data are means ± s.d. of three independent experiments.*

We measured $^1O_2$ production in the *ΔsigCDE* mutant and in its control strain by our His-mediated chemical trapping method. Figure 4.17 shows the rate of $^1O_2$ production in the *ΔsigCDE* mutant compared to the control strain. The rate of $O_2$ uptake, which is induced by the presence of His, is proportional to the amount of produced $^1O_2$. As shown in Fig. 4.17, we confirmed $^1O_2$ production by applying the $^1O_2$ quencher $NaN_3$ in the presence of DCMU, which blocks the $Q_B$ site and eliminates $O_2$ evolution. Our result reveals that $^1O_2$ production in

*ΔsigCDE* cells was significantly decreased as compared to control strain (Fig. 4.8). Therefore, the decreased rate of $^1O_2$ production in the *ΔsigCDE* mutant is correlated with the enhanced extent of photoprotection (Hakkila *et al.*, 2014).

### 4.2.2. The role of orange carotenoid protein as $^1O_2$ quencher in *Synechocystis* sp. PCC 6803

The orange carotenoid protein (OCP) is a soluble cyanobacterial protein that binds the keto-carotenoid 39-hydroxyechinenone (hECN) (Kerfeld *et al.*, 2003). OCP is present in the vast majority of cyanobacteria containing phycobilisomes, a large extramembrane antenna formed by blue and red phycobiliproteins. OCP is essential for a photoprotective mechanism decreasing the energy arriving at the reaction centers (Wilson *et al.*, 2006). OCP is a photoactive protein (Wilson *et al.*, 2008) and the only photosensory protein described to date that has a carotenoid as the active chromophore. Since carotenoids in plants have dual activities as energy and $^1O_2$ quenchers, we investigated if the carotenoid in OCP has $^1O_2$ quenching activity in addition to its function as an energy quencher.

We measured the production of $^1O_2$ by the His-mediated $O_2$ uptake method in *Synechocystis* cells containing or lacking OCP, as well as in an OCP overexpressing mutant during illumination with visible light. Since the OCP overexpressing mutant was constructed in a strain which lacks the *psbA2* gene, $^1O_2$ was also measured in the Δ*psbA2* mutant. The lack of *psbA2* significantly increased the production of $^1O_2$ as compared to the OCP overexpressed and WT (Fig. 4.9a). The comparison of $^1O_2$ production between the OCP overexpressed and the Δ*psbA2* control strain, shows that OCP overexpression decreased $^1O_2$ production by ca. 50% (Fig. 4.9a).

We also measured and compared the production of $^1O_2$ in *Synechocystis* cells containing or lacking OCP during illumination with orange-red light and white light. The wild-type and mutant cells were illuminated either strong white light , 2300 µmole photons $m^{-2}$ $s^{-1}$ or red-orange light, 1400 µmole photons $m^{-2}$ $s^{-1}$. Our results show the same trends in $^1O_2$ production as we observed in Fig. 4.18a under both light conditions. However, the OCP lacking mutant clearly shows increased production of $^1O_2$ as compared to WT (Fig. 4.9b). Our data suggest that the highest accumulation of $^1O_2$ was in *Synechocystis* cells lacking OCP under both illumination conditions (Fig. 4.9). The lowest accumulation was observed in the strain overexpressing OCP.

The measurements shown in figure 4.9a were repeated in the presence of DCMU, which blocks oxygen evolution by binding to the $Q_B$ site, or in the presence of DCMU and $NaN_3$, a strong $^1O_2$ quencher, to confirm that the $O_2$ uptake is related to $^1O_2$ trapping. These results strongly suggested that OCP could have a high $^1O_2$ quenching activity.

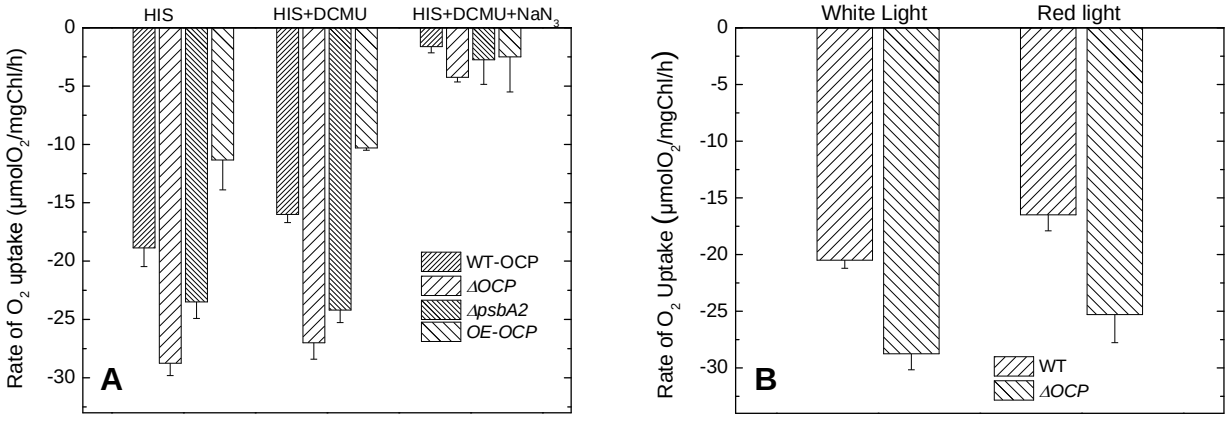


*Fig.4.9. Effect of OCP contents on the production of $^1O_2$ as measured by His-induced $O_2$ uptake using white or red light. A, The production of $^1O_2$ in WT (dense right hatch), ΔOCP (right hatch), OCP overexpressing (left hatch) and its background ΔpsbA2 strains (dense left hatch), the measurements were also performed either in the presence of 10 µM DCMU or in the presence of DCMU+10 mM $NaN_3$. The measurements were performed using white light (2300 µmole photons $m^{-2} s^{-1}$). B, The production $^1O_2$ in WT (right hatch), and ΔOCP (left hatch) strains, the measurements were performed either using strong white light (2300 µmole photons $m^{-2} s^{-1}$) or red-orange light (1400 µmole photons $m^{-2} s^{-1}$). Each bar represents the means ± s.d. of three independent experiments*

We studied the role of OCP as $^1O_2$ quencher in *Synechocystis*. Our results indicated that the exposure of wild-type and ΔOCP *Synechocystis* cells to high intensities of red-orange light induced photoinhibition, which was manifested as a decrease of PSII variable fluorescence (Fig. 4.10a) and initial amplitude of Chl fluorescence, Fv′/Fm′ (Fig. 4.10b). The variable fluorescence decreased faster in the ΔOCP mutant than in the wild type, suggesting a photoprotective function of OCP even under orange-red illumination, when the conformation change dependent excitation energy dissipation does not occur.

We also tested the effect of OCP overexpression on the rate of photodamage. We compared the decrease of variable fluorescence and Fv′/Fm′ in a *Synechocystis* strain overexpressing OCP and in the Δ*psbA2* background strain. Both strains lack the *psbA2* gene, and this inactivation increases the sensitivity to photoinhibition compared with the wild type (Fig. 4.10a and Fig. 4.10b). The OCP overexpressing strain contains the *ocp* gene expressed under the control of the strong *psbA2* promoter and has higher OCP concentrations (Sedoud *et al.*, 2014). The variable Chl fluorescence and Fv′/Fm′ decreased more slowly in the mutant strain overexpressing OCP than in the Δ*psbA2* background strain and in the wild type (Fig. 4.10a and Fig. 4.10b), showing a photoprotective effect of OCP, which is proportional with OCP concentration.

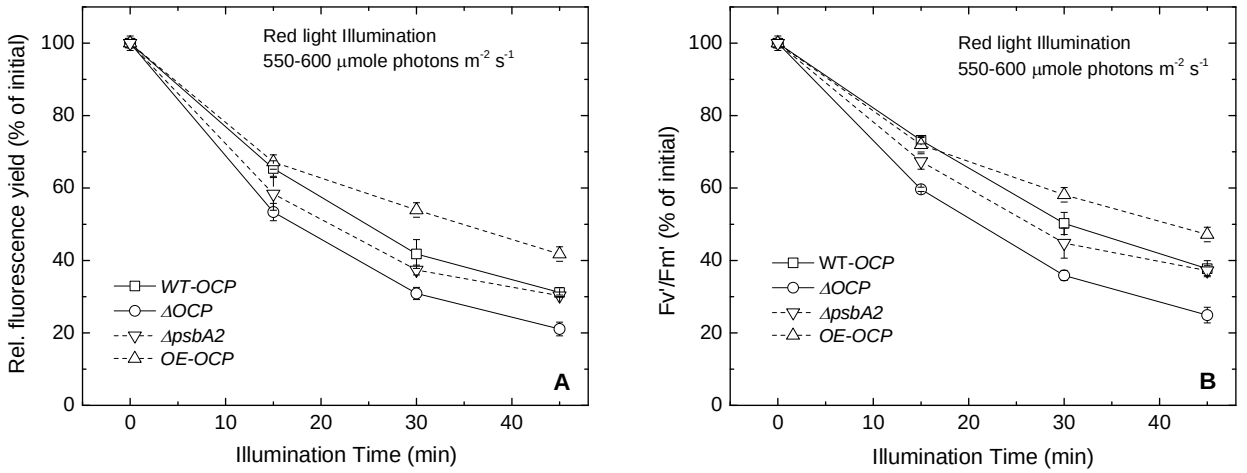


*Fig.4.10. Effect of OCP amount on the extent of red light induced photodamage. A, Variable fluorescence calculated from the initial amplitude of flash induced Chl fluorescence transients. Synechocystis cells were illuminated with strong red light in the presence of lincomycin, and changes in the activity of PSII were followed by measuring variable Chl fluorescence. The measurements were performed by using WT (open squares), and ΔOCP cells (open circles), as well as an OCP overexpressing mutant (dotted line and open up triangles) and its OCP containing background strain (dotted line and open down triangles). B, Fv'/Fm' calculated from the initial amplitude of flash induced Chl fluorescence transients. The initial amplitude of variable Chl fluorescence induced by a 20 μs long saturating light pulse. Data are means ± s.d. of three independent experiments.*

In addition, we studied the changes in PSII activity by measuring oxygen evolution and observed decline of PSII activity during the exposure to strong orange-red light. The illumination

conditions for this experiment differed from those used for the fluorescence measurements. These samples experienced on average a lower light intensity and oxygen evolution decreased more slowly than fluorescence (Fig. 4.11). Nevertheless, the much higher resistance to high intensities of orange-red light of the overexpressing OCP strain compared with the ΔOCP mutant was also observed when photoinhibition was followed by oxygen evolution measurements (Fig. 4.11). OCP is photoactivated only by strong blue-green light, which induces carotenoid and protein conformational changes converting OCP orange to OCP red (Wilson *et al.*, 2008). These data show that OCP can photoprotect *Synechocystis* cells also under conditions in which OCP is not photoactivated and does not induce light energy quenching. These experiments clearly demonstrate that OCP has a photoprotective function as a $^1O_2$ quencher in addition to its function as an excitation energy quencher in cyanobacterial cells.

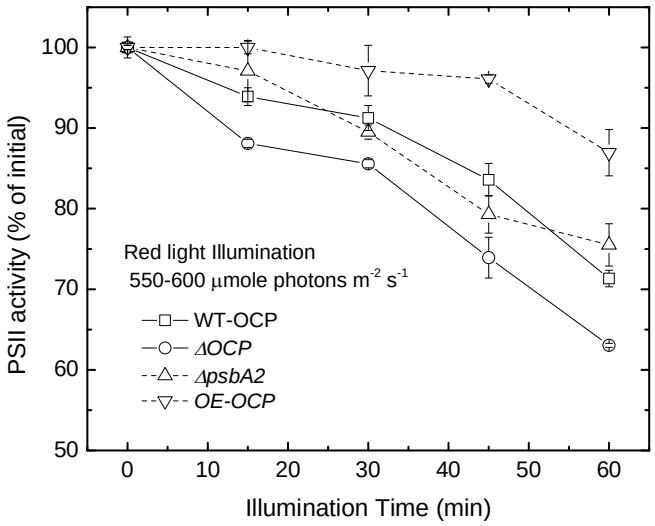


*Fig.4.11. Effect of red light illumination on PSII activity of mutant OCP, $O_2$ evolution rate was measured in WT (open squares), and ΔOCP cells (open circles), as well as an OCP overexpressing mutant (dotted line and open up triangles) and its OCP containing background strain (dotted line and open down triangles). The $O_2$ evolution was recorded in the presence of 0.5 mM DMBQ and the light intensity during measurement was 1000 μmole photons $m^{-2}$ $s^{-1}$. The data are shown after normalization to their initial values and are means ± s.d of three independent experiments.*

We demonstrated that OCP, in addition to its function as an energy quencher, has an important role as a $^1O_2$ quencher in *Synechocystis* cells. Under high-light conditions, the production of $^1O_2$ is higher in a *Synechocystis* mutant lacking the OCP than in the wild type or in a mutant overexpressing the *ocp* gene, even under strong orange-red light. Under these conditions, OCP is not photoactivated and cannot act as an energy quencher. Thus, under strong orange-red light, OCP must protect the cells from photodamage by another mechanism. Our results strongly suggest that in the cells, OCP diminishes the concentration of $^1O_2$ by direct

quenching. OCP activity as an energy quencher needs the photoactivation of the protein, since only OCP red is able to bind to phycobilisomes (Wilson *et al.*, 2008). By contrast, the $^1O_2$ quenching activity is present in both forms of the protein, OCP orange and OCP red.

### 4.2.3. $^1O_2$ production by a secondary metabolite of the cyanobacterium *Nostoc* XPORK14A

Cyanobacteria produce a wide spectrum of secondary metabolites with diverse range of biological activities (O'Brien & Wright, 2011). Some studies have suggested that these compounds could be important for successful adaptation to different extreme environments (Gademann & Portmann, 2008). Chemical structures of bioactive compounds are diverse and include novel cyclic and linear peptides, fatty acids, alkaloids and other organic chemicals (Moore, 1996). The majority of these bioactive compounds are cyanotoxins that have received worldwide attention. The genetic basis and toxicity of the most common cyanotoxins like microcystin and nodularin on vertebrates, aquatic organisms and higher plants has been extensively reviewed (Wiegand & Pflugmacher, 2005; van Apeldoorn *et al.*, 2007; Wiegand & Pflugmacher, 2005). Shunmugam and coworkers described a novel secondary metabolite denoted as M22. It was purified from N2-fixing filamentous cyanobacterium *Nostoc* XPORK14A isolated from the Baltic Sea. M22 has a non-peptide structure and containing several 106 Da subunits, two 168 + 18 Da subunits and two 278 + 16 + 16 Da subunits forming together the native molecule of 1626 Da (Shunmugam *et al.*, 2014).

They proposed that M22 compound induced retardation of cell growth and inhibition of photosynthesis in *Synechocystis* cells, both in light and in darkness. They showed that the purified M22 compound absorbs light within the UV-range and, interestingly, also in the blue region of visible light. The absorption of the compound in the blue range makes highly possible the light-induced formation of an excited state of M22 that could interact with $O_2$ to produce $^1O_2$. In order to reveal this characteristic of the M22 compound, we employed His-mediated chemical trapping technique by using Clarke-type oxygen electrode.

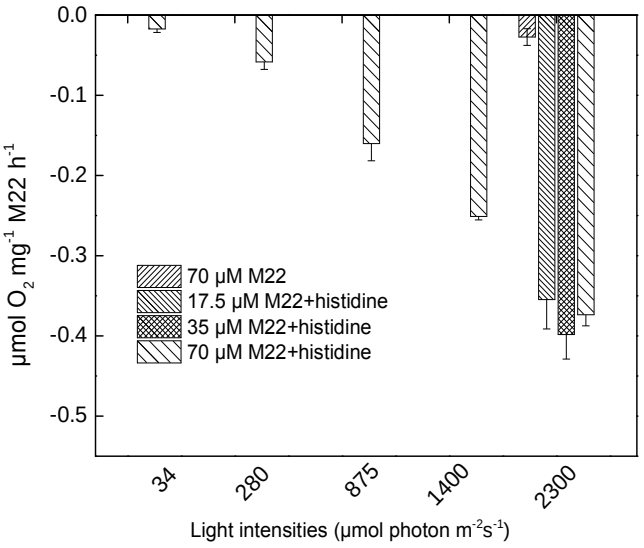


*Fig.4.12. Detection of $^1O_2$ production by M22. $^1O_2$ production was determined as light-induced $O_2$ uptake in the presence of 5 mM His in BG-11 medium. The measurements were performed by using 70 µM M22 (left hatch) at all light intensities. At the 2300 µmole photons $m^{-2}s^{-1}$ 35 and 17.5 µM M22 was also tested. The data are shown after normalization to the amount of M22. Data are means ± s.d of three independent experiments.*

The extent of $^1O_2$ production was expected to be proportional to the amount of $O_2$ uptake from the BG-11 medium. Our results show that the addition of 70 µM M22 to BG-medium in the presence of 5 mM His induced the $O_2$ uptake already at 34 µmole photons $m^{-2}s^{-1}$ (Fig. 4.12), However, 70 µM M22 to BG-medium without His shows no $O_2$ uptake or very small $O_2$ uptake even at 2300 µmole photons $m^{-2}s^{-1}$, thus confirming the light induced production of $^1O_2$ by M22. Interestingly, the $O_2$ uptake rate was linearly dependent on the light intensity from 34 to 2300 µmole photons $m^{-2}s^{-1}$, and slightly deviated from linearity above that (Fig. 4.12). Different concentrations of M22 (17.5, 35 and 70 µM) were used at 2300 µmole photons $m^{-2}s^{-1}$. Interestingly, our result shows that there are no significant changes in $^1O_2$ production at different M22 concentrations when 2300 µmole photons $m^{-2}s^{-1}$ was employed. This indicates that the light induced linear production of $^1O_2$ is disturbed at light intensity of 2300 µmole photons $m^{-2}s^{-1}$, which might result from high-light-induced destruction of oxidized M22 or saturation of the His oxidation capacity at high light intensity. Altogether the results showed that M22 generates $^1O_2$ in the presence of light, which in turn affects the photosynthetic machinery of *Synechocystis* (Shunmugam *et al.*, 2014).

### 4.2.4. The role of flavodiiron proteins (FDPs) in protecting against $^1O_2$-dependent photodamage of PSII in *Synechocystis* sp. PCC 6803

The His-mediated $O_2$ uptake method was also used to study the role of flavodiiron proteins (FDPs) in PSII photoprotection. FDPs are specific electron transfer enzymes present mainly in anaerobic bacteria, and they are found also in cyanobacteria. Cyanobacterial FDPs constitute a specific group that evolved differently to cooperate with oxygenic photosynthesis. In *Synechocystis* there are four FDPs, Flv1-Flv4. Two of them, Flv2 and Flv4, are encoded by an operon together with an Sll0218 protein (Zhang *et al.*, 2009). Several recent studies have suggested that the FDPs have an important role in photoprotection of the photosynthetic machinery in cyanobacteria (Zhang *et al.*, 2012; Allahverdiyeva *et al.*, 2013). To test whether the production of $^1O_2$ is altered in the presence of different Flv2 and Flv4 amounts we assessed $^1O_2$ production by measuring a His-mediated oxygen uptake in all four Flv mutant strains.

Our results (Fig. 4.13).show that the lack of the Flv4, Sll0218 and Flv2 proteins (Δ*flv4* strain) results in the increase of $^1O_2$ production in comparison to WT cells, even though the carotenoid content of the Δ*flv4* strain was increased as compared to WT (Bersanini *et al.*, 2014). These data show that in the absence of the *flv4-2* operon generation of $^1O_2$ is enhanced to such an extent, which effect induces increased carotenoid content, which however is not sufficient to keep the $^1O_2$ amount at the WT level (Fig. 4.13). This idea was further confirmed by a large decrease in $^1O_2$ production in the *flv4-2*/OE mutant as compared to all other strains, even though it contains smaller carotenoids content than the WT (Bersanini *et al.*, 2014). Since the *flv4-2*/OE mutant was constructed in a strain which lacks the *psbA2* gene, $^1O_2$ was also measured in the Δ*psbA2* mutant. The lack of *psbA2* significantly increased the production of $^1O_2$ as compared to the WT (Fig. 4.13).

When we compared $^1O_2$ production between the *flv4-2*/OE strain and the Δ*psbA2* control strain, we found that overexpression of *flv4-2* operon decreased $^1O_2$ production by ca. 60%. These data are consistent with high light sensitivities of the strains, and provide a strong support for the idea that the *flv4-2* operon has an important role in photoprotection via decreasing the production of $^1O_2$, which is a key factor of PSII photodamage (Vass *et al.*, 1992; Vass & Cser, 2009; Vass & Aro, 2008).

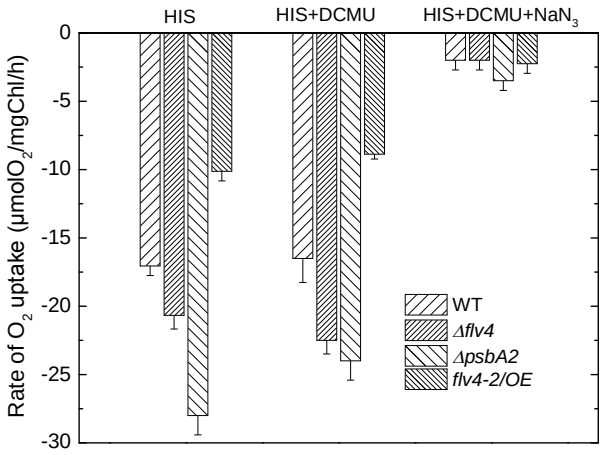


*Fig.4.13. Production of $^1O_2$ in WT, ΔpsbA2, flv4-2/OE and Δflv4 strains upon illumination of the cells at 2300 µmole photons $m^{-2}$ $s^{-1}$. Amounts of $^1O_2$ oxygen in the assay system are proportional to $O_2$ uptake. The cultures were adjusted to a Chl concentration of 5 µg $mL^{-1}$. The results are a mean (± SD) of three independent experiments.*

### 4.2.5. The effect of moderate salt stress on $^1O_2$-dependent photodamage in *Synechocystis* sp. PCC 6803 mutants

Small CAB-like proteins (SCPs) are single-helix light-harvesting-like proteins found in all organisms performing oxygenic photosynthesis. In this study we used a *Synechocystis* mutant, which lack the ScpABCDE small Chl binding proteins. In the *Synechocystis* most Chl is bound to PSI; mutants deficient of PSI therefore contain only 20% of the wild type Chl amount (Shen *et al.*, 1993). In this PSI-less background PSII is very vulnerable to photooxidation (Sandström *et al.*, 2002). Additional deletion of the five SCPs leads to further reduction in Chl on OD basis (Xu *et al.*, 2004), giving the PSI-less/ScpABCDE- mutant a very pale phenotype. Deletion of the SCPs has been shown to increase $^1O_2$ production (Sinha *et al.*, 2012) and to cause high oxidative damage; long-term exposure to high light even leads to the death of the culture (Havaux *et al.*, 2003). The bleached phenotype of the PSI-less/ScpABCDE- mutant strain could be restored in the presence of NaCl; addition of 0.2 M NaCl to the medium led to an increased amount of Chl per cell without affecting the strain´s division time (Tibiletti *et al.*, 2016).

We employed the His-mediated $O_2$ uptake method was to understand the effect of moderate salt stress conditions on $^1O_2$-dependent photodamage in PSI-less and PSI-less/ScpABCDE$^-$ strains of *Synechocystis.* In moderate salt stress conditions, our result shows decreased production of $^1O_2$ in the PSI-less/ScpABCDE$^-$ mutant compared to the PSI-less strain (Fig.4.14). $^1O_2$ was measured in intact mutant cells by using His-mediated chemical trapping (Rehman *et al.*, 2013). Figure 4.14 shows the rate of $^1O_2$ production in the PSI-less/ScpABCDE$^-$ mutant compared to the PSI-less strain without and with 0.2 M NaCl. It was also demonstrated

that that the addition of 0.2 M NaCl to the growth medium in the PSI-less/ScpABCDE$^-$ mutant strain decreased $^1O_2$ formation and induced resistance to photoinhibition compared to control strain.

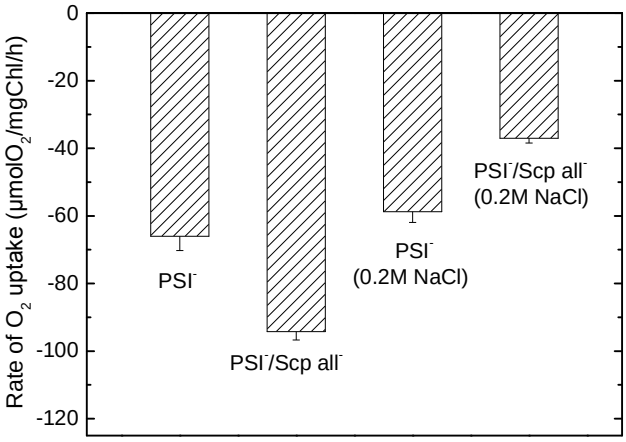


*Fig.4.14. $^1O_2$ production measured as rate of His-mediated oxygen uptake in the PSI-less and the PSI-less/ScpABCDE$^-$ strains of Synechocystis grown in the presence or absence of NaCl. The measurements were performed in the presence of 5 mM His at 2300 µmole photons $m^{-2}s^{-1}$ light intensity. Data are means ± s.d of three independent experiments.*

Recently Sinha *et al.* (2012) studied the role of small CAB-like proteins (SCPs) on the production of $^1O_2$ in *Synechocystis* by electron paramagnetic resonance (EPR) spin-trapping technique and by Singlet Oxygen Sensor Green (SOSG) fluorescence imaging and suggested that SCPs prevent $^1O_2$ formation. They showed increased production of $^1O_2$ in the PSI-less/ScpABCDE$^-$ strain compared to the PSI-less control (Sinha *et al.*, 2012).

Our result obtained by His-mediated chemical trapping confirm the previous study by (Sinha *et al.*, 2012) and reveals 30% increase in the rate of $^1O_2$ production in the PSI-less/ScpABCDE$^-$ strain compared to the PSI-less strain (Fig. 4.14). However, the PSI-less/ScpABCDE$^-$ strain shows 60% decrease in the rate of $^1O_2$ production, when it is grown in the presence of 0.2 M NaCl compared to when cultured without 0.2 M NaCl. Furthermore, our result shows the production of $^1O_2$ was 38% decreased in the PSI-less/ScpABCDE$^-$ mutant compared PSI-less strain in the presence of NaCl indicates protective effect against $^1O_2$ production in the PSI-less/ScpABCDE$^-$ mutant.

Adaptation to moderate salinity requires energy to produce osmo-protectant molecules and to extrude $Na^+$ from the cell (Allakhverdiev *et al.*, 2005). This energy is provided mainly by the respiratory electron transport, which in cyanobacteria shares common components with the

photosynthetic electron transport (Schultze *et al.*, 2009). Increased electron transport within the respiration chain induced by NaCl addition (Jacoby *et al.*, 2015; Hamilton & Heckathorn, 2001), therefore might generate an effective sink for PSII-generated electrons. It is well known that oxidases i.e. dehydrogenases (Howitt *et al.*, 1999), cytochrome oxidases and/or quinol oxidases (Howitt *et al.*, 1999; Vermaas, 2001; Vermaas *et al.*, 1994) in *Synechocystis* can function as effective electron sinks. In the presence of NaCl the electrons generated by PSII in the PSI-less/ScpABCDE- mutant are efficiently consumed by oxidases, generating a shield against excess light and ROS production, particularly $^1O_2$, and cellular damages therefore are avoided. Both the decreased amount of $^1O_2$, but very likely also the increased amount of ATP available in the PSI-less/ScpABCDE mutant in the presence of NaCl contributes to the higher rate of Chl biosynthesis and PSII assembly.

## 4.3. $^1O_2$-mediated photooxidative stress in symbiotic dinoflagellate cells

### 4.3.1. Production of singlet oxygen in *Symbiodinium* cells

*Symbiodinium* resides within the endodermal tissue of the anthozoan host and form endosymbiosis (Wooldridge, 2010). Since *Symbiodinium* is an oxygenic photosynthetic dinoflagellate which contains very efficient $^1O_2$ sensitizers in the form Chl pigments, it is highly likely that *Symbiodinium* also produces significant amount of intracellular $^1O_2$ which can interact with the animal host. No direct evidence has been provided before our studies for the formation of $^1O_2$ in *Symbiodinium* cells. This was mainly due to the lack of suitable $^1O_2$ detection methods that could be applied in intact *Symbiodinium* cells. We employed His-mediated chemical trapping method to study the $^1O_2$ production in *Symbiodinium* cells.

The result in (Fig. 4.15) shows that His itself does not inhibit PSII activity in *Symbiodinium* cells, which was confirmed by variable Chl fluorescence measurements (Fig. 4.15). Therefore, the $O_2$ uptake observed in the presence of His in *Symbiodinium* cells (Fig. 4.16) indicates $^1O_2$ production. In order to fully verify this hypothesis we probed the *Symbiodinium* culture for His-mediated $O_2$ uptake in the absence and presence of $^1O_2$ enhancers ($D_2O$) and scavengers ($NaN_3$). $O_2$ uptake was indeed enhanced when the culture medium, which was used for the $O_2$ measurements, contained $D_2O$ instead of $H_2O$. On the contrary, when the reaction mixture contained 10 mM $NaN_3$ the $O_2$ uptake was decreased (Fig. 4.16a). Since the lifetime of $^1O_2$ is enhanced in the presence of $D_2O$ and decreased in the presence of $NaN_3$ these findings provide a firm support for the idea that His-mediated $O_2$ uptake arises from the oxidation of His by $^1O_2$. The travel distance of $^1O_2$ is in the range of 70-250 nm in cellular environment due to its short lifetime (Moan, 1990; Skovsen *et al.*, 2005), which limits the possibility of its presence close to the site of production in the thylakoid membranes. Therefore, the observation of His-mediated $O_2$ uptake demonstrates intracellular production of $^1O_2$ in *Symbiodinium* cells.

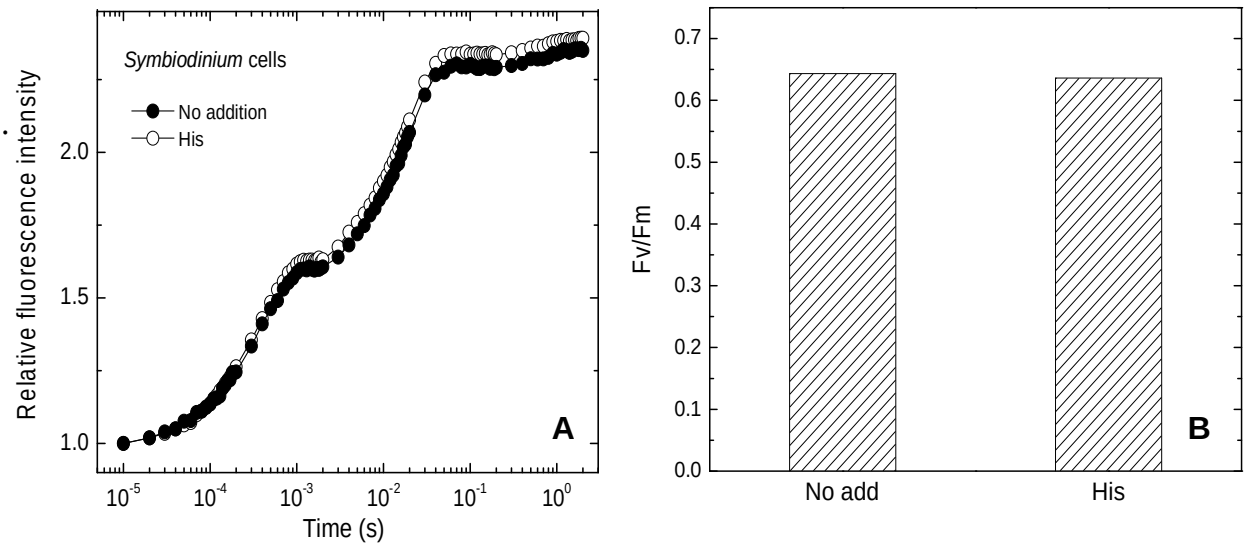


*Fig.4.15. Effect of His addition on PSII activity in intact Symbiodinium cells as measured by fast Chl fluorescence rise, A, Variable Chl fluorescence traces in the absence (closed circles) and presence of 5 mM His (open circles). The curves are presented after normalization to same initial Fo fluorescence level and 2 s long saturating light pulse was used B, The Fv/Fm values of cultured Symbiodinium in the absence and presence of 5 mM His.*

The His-mediated oxygen uptake method was also employed to study the $^1O_2$ production in intact coral *Pocillopora damicornis*, which mean *Symbiodinium* is living within its animal host. We observed $O_2$ uptake in the presence of His in coral (Fig. 4.16b). The $^1O_2$ detection was confirmed and indeed generated by its symbiotic partner *Symbiodinium.*

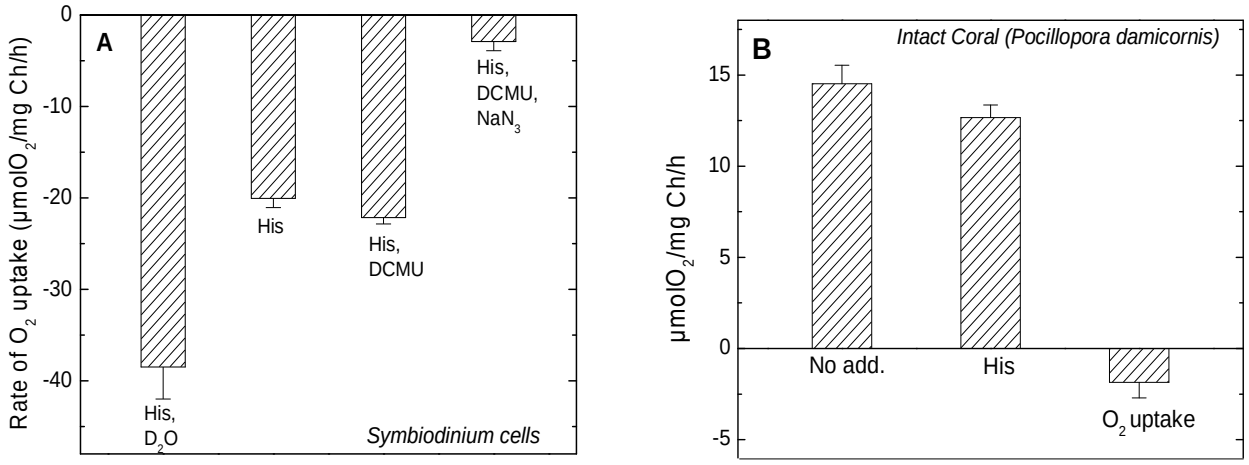


*Fig.4.16. The production of $^1O_2$ in Symbiodinium cells and intact coral (Pocillopora damicornis), A, His-mediated oxygen uptake in the presence of $^1O_2$ lifetime enhancer, deuterium oxide ($D_2O$), DCMU, and in the presence for $^1O_2$ quencher, sodium azide ($NaN_3$). B, His-mediated oxygen uptake in intact coral, The light intensity during the oxygen measurements was 2300 µmole photons$m^{-2}s^{-1}$. Data are means ± s.d. of three independent experiments.*

Stress conditions, which induce coral bleaching, include parallel increase of light intensity and temperature above the levels to which *Symbiodinium* cells are adapted. In order to check if intracellular $^1O_2$ production is affected by combined light and heat stress *Symbiodinium* cells, which were grown at 26 °C (GT) and 20 µmole photons $m^{-2}s^{-1}$ (GL), they were exposed to 34 °C (HT) and 45 µmole photons $m^{-2}s^{-1}$ (HL) conditions for several hours. As shown in Fig. 4.17a incubation of cells under HT + HL conditions lead to a gradual increase of $^1O_2$ production. During 7 hour incubation at HT + HL the detectable amount of $^1O_2$ doubled to the level that can be observed under GT + GL conditions. Under natural conditions coral embedded *Symbiodinium* cells experience a significantly higher light intensities than applied here as HL, therefore we wanted to clarify if our HT+HL conditions indeed represent stress situation to the cells and variable Chl fluorescence transients were also recorded. These data show a significant loss in the Fm level (Fig. 4.17b) and, as a consequence, in the maximal quantum yield of PSII (Fv/Fm), and thus confirm that the cells experienced stress during the HT + HL incubation.

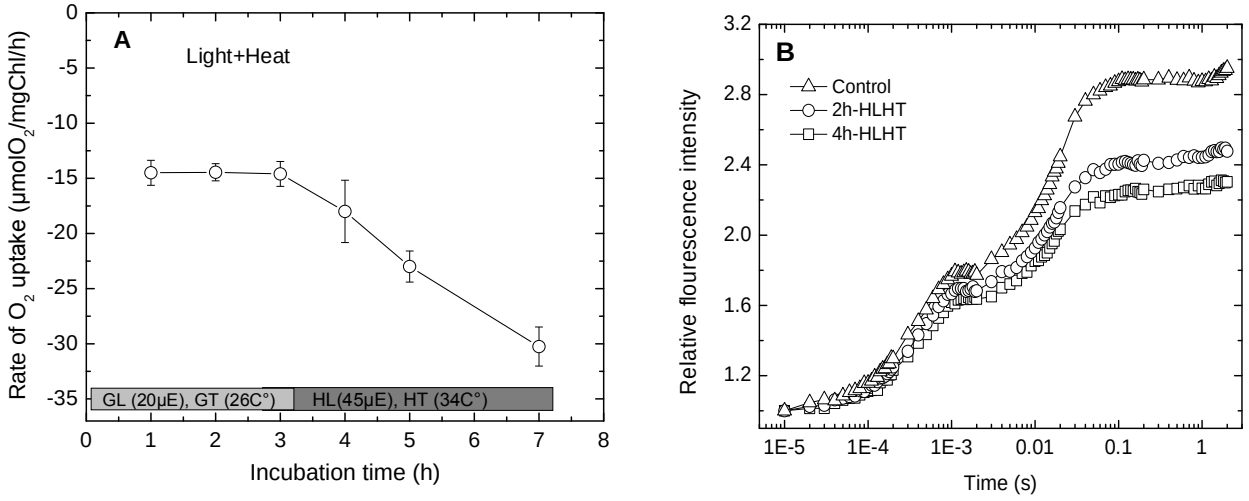


*Fig.4.17. Effect of heat and light stress on the production of $^1O_2$ in Symbiodinium cells A, His-mediated $O_2$ uptake under high temperature, 34°C and high light, 45 µmole photons $m^{-2}s^{-1}$. Light intensity during the oxygen measurements was 2300 µmole photons $m^{-2}s^{-1}$. Data are means ± s.d. of three independent experiments, B, Variable Chl fluorescence traces of Symbiodinium cells under controlled light and temperature (open up triangles), 2h (open circles) and 4h (open square) high temperature (34°C)and high light (45 µmole photons $m^{-2}s^{-1}$) treatment. The curves are presented after normalization to same initial Fo fluorescence level.*

Recent results have demonstrated that inhibition of the Calvin-Benson cycle either by thermal stress or chemical treatments can trigger coral bleaching (Lilley *et al.*, 2010; Bhagooli, 2013; Hill *et al.*, 2014). The Calvin-Benson cycle acts as terminal sink for electrons arising from photosynthetic light reactions. Therefore, inhibition of its function leads to the reduction of photosynthetic electron transport chain, which is expected to enhance $^1O_2$ production that could play a role in bleaching induction. However, it was also shown that GA and KCN, which both act as Calvin-Benson cycle inhibitors have differential effects and only KCN treatment leads to bleaching symptoms, GA not (Hill *et al.*, 2014). In order to obtain better insight into to consequences of Calvin-Benson cycle inhibition the effects of KCN or GA treatments on $^1O_2$ production were investigated in *Symbiodinium* cells.

Incubation of cells with 10 mM GA at 45 µmole photons $m^{-2}s^{-1}$ high light illumination lead to gradual loss of photosynthetic activity (Fig.4.18a), and this effect was accompanied by an enhanced level of $^1O_2$ production (Fig. 4.18b). Similar effect was also obtained when cells were treated with KCN (Fig. 4.19). It is important to note that the enhancement of $^1O_2$ production proceeded gradually during several hours (Fig. 4.18b) although GA inhibits the Calvin-Benson cycle in few tens of minutes. This effect indicates that in case of GA treatment other factors than reduction of the electron transport chain due to the inhibition of $CO_2$ fixation also contribute or actually cause $^1O_2$ production, which is in agreement with our previous finding that GA causes significant inhibition of photosynthetic activity of *Symbiodinium* cells during incubation even in darkness (Hill *et al.*, 2014). It is also of note that intracellular $^1O_2$ reaches the same level even after 30 min incubation with KCN as observed after 8 hours of GA treatment (Fig. 4.18b and 4.19). These data indicate a differential response of $^1O_2$ production to KCN and GA treatment of *Symbiodinium* cells, which is agreement with the differential bleaching response induced by these treatments in intact corals (Hill *et al.*, 2014).

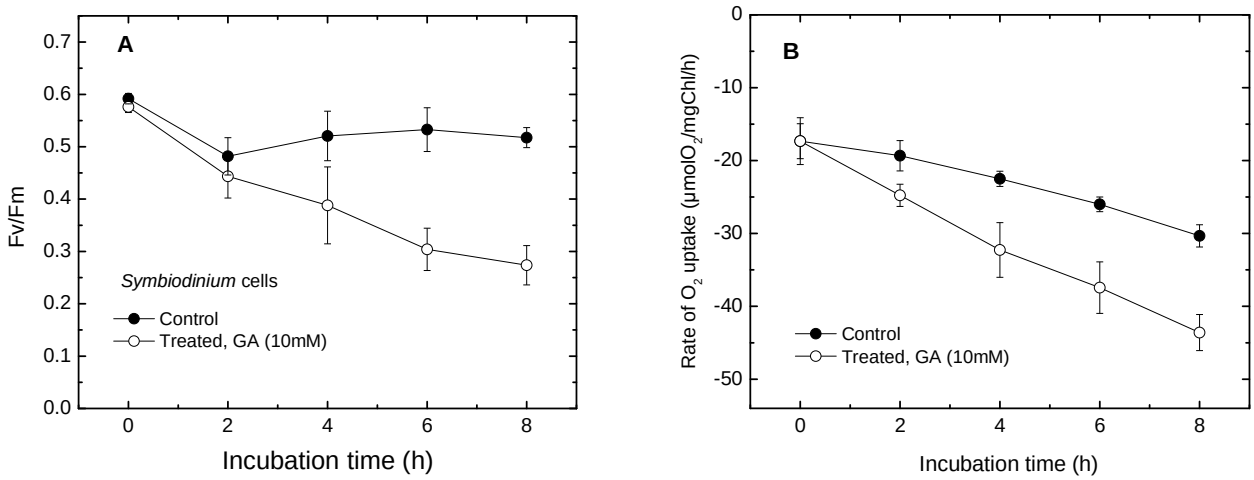


*Fig.4.18. Effect of glycolaldehyde (GA) under high light stress on the extent of $^1O_2$ production in Symbiodinium cells, A, The Fv/Fm values in control (closed circles) and treated cells with 10mM GA under high light, 45 µmole photons $m^{-2}s^{-1}$(open circles). B, His-mediated $O_2$ uptake under high light in control (closed circles), and treated cells with 10 mM GA (open circles). Light intensity during the oxygen measurements was 2300 µmole photons $m^{-2}s^{-1}$ and the data are shown in fig. 4.18b after shifting them to same initial uptake value of control. Data are means ± s.d. of three independent experiments.*

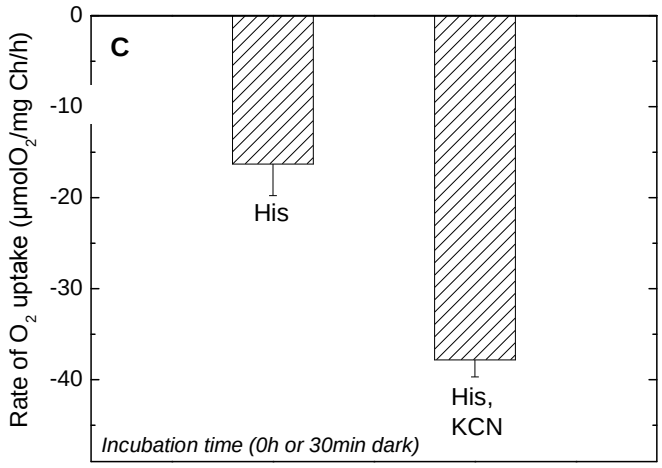


*Fig.4.19. Effect of potassium cyanide (KCN) on the extent of $^1O_2$ production in Symbiodinium cells, His-mediate $O_2$ uptake in control and with 10 µM KCN. Light intensity during the oxygen measurements was 2300 µmole photons $m^{-2}s^{-1}$. The data represent mean values from 3 independent experiments with the indicated standard errors.*

Our data presented above demonstrate the production of $^1O_2$ inside *Symbiodinium* cells, which is enhanced under stress conditions, as well as chemical treatments which lead to release of zooxanthellae from coral tissue. Therefore, $^1O_2$ might participate in the chain of events that lead to coral bleaching.

### 4.3.2. The role of Calvin-Benson cycle inhibition in coral bleaching

Calvin–Benson cycle which is essential in the fixation of organic carbon from carbon dioxide has been shown to be impaired in *Symbiodinium* cells, when corals are exposed to high temperature stress. This impairment of Calvin–Benson cycle in *Symbiodinium* cells has been reported to be due to the inhibition of Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Jones *et al.*, 1998). It has also been shown that the inhibition of the Calvin–Benson cycle accelerate photoinhibition and it causes bleaching of *Symbiodinium* cells within corals (Takahashi & Murata, 2005; Takahashi & Murata, 2008). At low concentrations, GA and cyanide have been used to inhibit the operation of the Calvin–Benson cycle (Wishnick & Lane, 1969; Sicher, 1984). Therefore, the application of chemical inhibitors can provide a useful means to manipulate the function of metabolic processes. Here we used cultured *Symbiodinium* while manipulating the function of the Calvin–Benson cycle using chemical inhibitors to investigate whether inhibition of the Calvin–Benson cycle by these chemical inhibitors promotes PSII photodamage under thermal stress.

Our results show a concentration dependent drop in Fv/Fm for GA and KCN in the *Symbiodinium* culture (Fig. 4.20). We used concentration series from 0 to 10 mM for GA, and 0 to 100 µM for KCN to find suitable concentration to induce Calvin-Benson cycle inhibition in *Symbiodinium* cells while not affecting the animal host under light exposure conditions. Moreover, our results show recovery of Fv/Fm in all KCN concentrations under dark conditions (Fig. 4.20b), while no recovery has been observed for GA (Fig. 4.20a). This means that the long time GA treatment damages the photochemical activity at higher concentration than 1 mM in *Symbiodinium* cells. However, the decline of photosynthetic activity by KCN was recovered in darkness up to 50 µM.

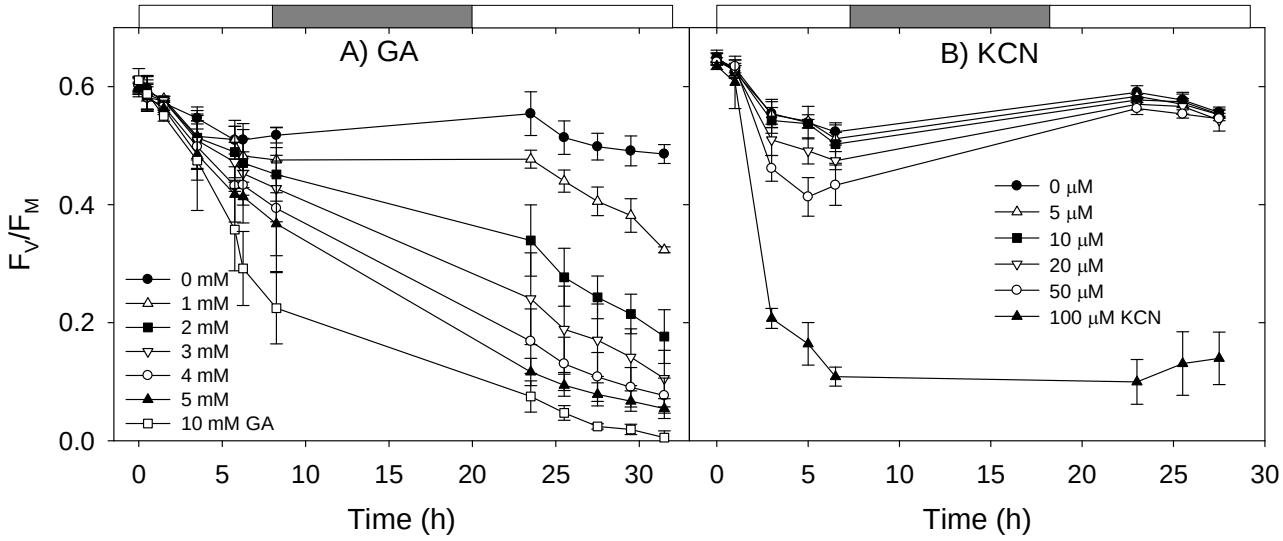


*Fig.4.20. Fv/Fm of cultured Symbiodinium in the presence of A) GA (0-10 mM) and B) KCN (0-100 µM) during 8 h of exposure to 40 µmole photons $m^{-2}s^{-1}$, followed by 12 h darkness and a further 12 h visible light. The white bars on the top of figure indicate periods of light and the grey bars indicate darkness. Data are means ± s.d. of three independent experiments.*

It has been observed that *Symbiodinium* cells suffer Chl pigment bleaching during heat and light stress, which lead to coral bleaching under natural conditions. In order to understand the role of the inhibition of the Calvin-Benson cycle and Chl pigment bleaching, GA (3 and 5 mM) as well as KCN (20µM) was used with and without His in cultured *Symbiodinium*. Photosynthetic efficiency (Fv/Fm) was measured at different time intervals. Our results show a loss in the maximal quantum yield of PSII in the presence of both inhibitors of the Calvin-Benson cycle (Fig. 4.21). In addition, Chl pigment bleaching was also observed (Fig. 4.22) demonstrating that the stress caused either by GA- or KCN-induced inhibition of Calvin-Benson cycle enhances Chl pigment bleaching, as well loss of PSII activity in *Symbiodinium* cells. Interestingly, His provides protection against GA- and KCN- induced loss of Fv/Fm and also against Chl bleaching. The recovery of Chl bleaching and PSII activity by externally added His provides strong support for the idea that $^1O_2$ is involved as damaging agent in the inhibition of PSII activity, as well as Chl pigment bleaching.

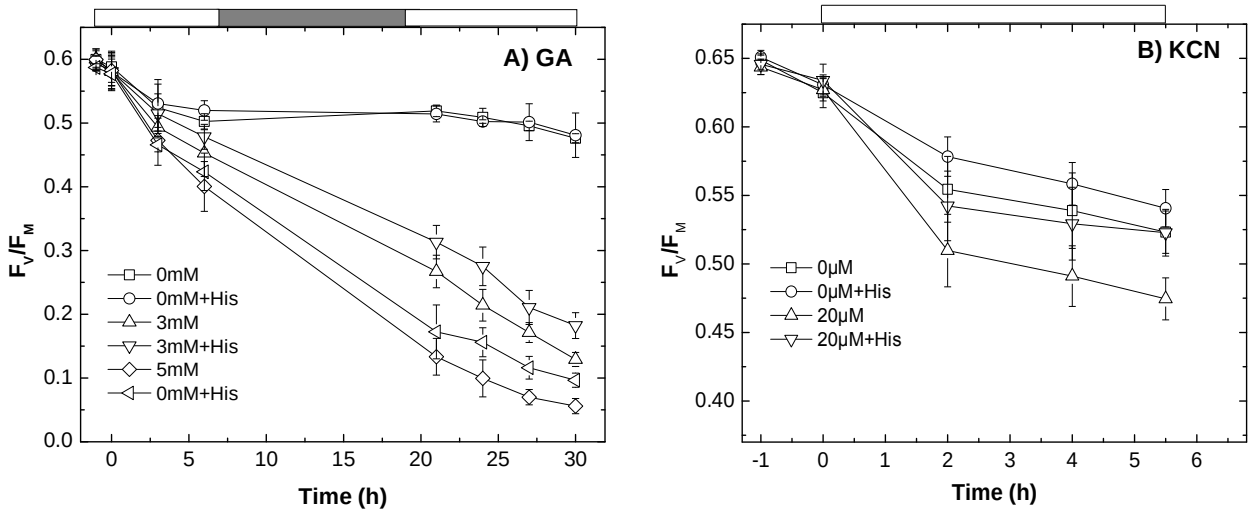


*Fig.4.21. Recovery of Fv/Fm in the presence of His A) Fv/Fm of cultured Symbiodinium in the presence of GA (0 mM, 3 mM and 5 mM ± His) during 5 h of exposure to 40 µmole photons $m^{-2}s^{-1}$, followed by 12 h darkness and a further 11 h light B) KCN (0 µM and 20 µM with or without His) during 5.5 h of exposure to 40 µmole photons $m^{-2}s^{-1}$ visible light. The white bars on the top of figure indicate periods of light and the grey bars indicate darkness. Data are means ± s.d. of three independent experiments.*

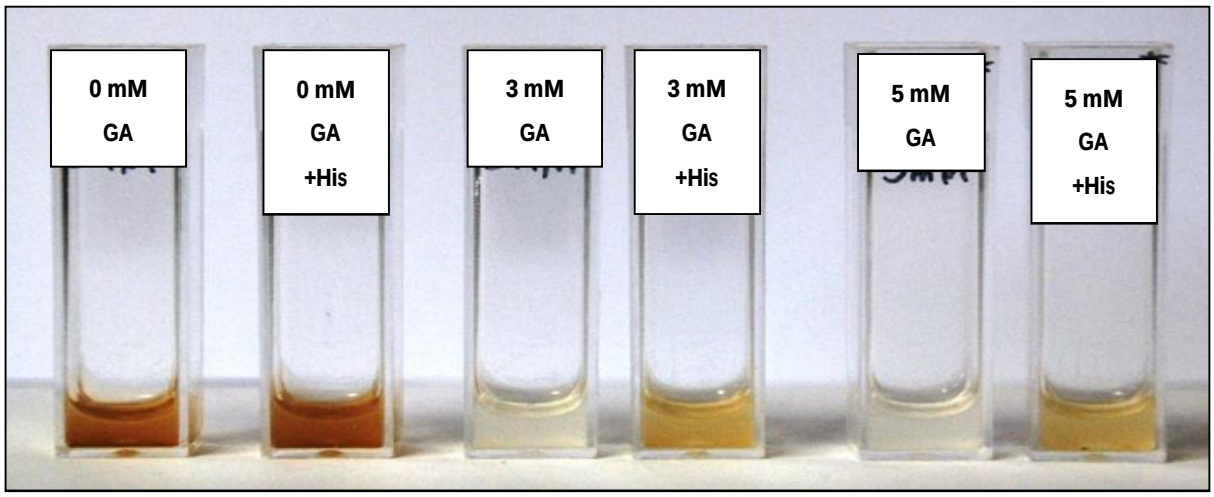


*Fig.4.22. Representative cuvettes showing glycolaldehyde (GA) mediated bleaching of Symbiodinium cells and Chl pigment bleaching protection by His (0mM, 3 mM and 5 mM with and without His).*

To clarify the effect of GA and KCN treatment on the function of PSII in cultured *Symbiodinium*, the variable Chl fluorescence was measured at 0h and 5h of treatment of GA and KCN under exposure to 40 µmole photons $m^{-2}s^{-1}$ and the curves were double normalized [(Ft–

Fo)/(Fm–Fo)]. Dramatic changes in the fluorescence kinetics were observed in the treated cells after 5 h as compared to control and 0 h (Fig. 4.23). In the presence of both GA and KCN the J step increases after incubation of the cells in growth light. These findings demonstrate that the reduction level of the PQ pool increases (Hill *et al.*, 2014) due to partial inhibition of the Calvin-Benson cycle, which limits electron flow towards $CO_2$ that acts as the final electron acceptor. These results show that 3mM GA and 20 µM KCN caused minimal inhibition of photosynthetic loss and suitable to use for the study of pigment bleaching in cultured *Symbiodinium* (Hill *et al.*, 2014).

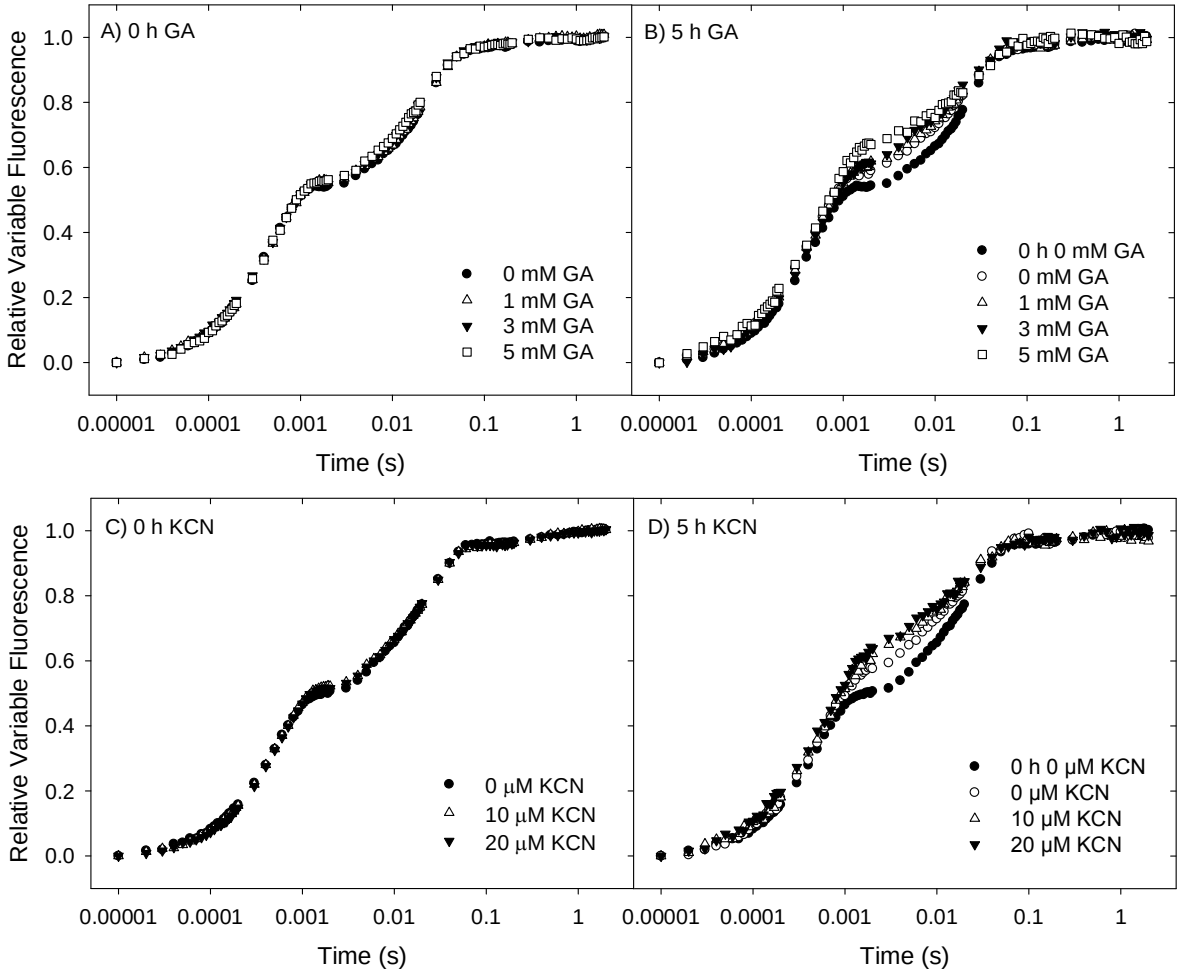


*Fig.4.23. Double normalized curves showing relative variable fluorescence [(Ft – Fo)/ (Fm–Fo)] of cultured Symbiodinium in the presence of GA (0, 1, 3 and 5 mM; A, B) and KCN (0, 10 and 20 µM) C, D), after 3 min exposure to inhibitors (A, C) and after a further 5 h exposure to 40 µmole photons $m^{-2}s^{-1}$ (B, D). The control 0 h measurements are also shown for comparison*

### 4.3.3. Extra-cellular production of $^1O_2$ in *Symbiodinium* culture

For a direct interaction with coral tissue either $^1O_2$ or its sensitizer has to exit from the *Symbiodinium* cells. Due to the short lifetime and travel distance exit of $^1O_2$ from the intracellular production site is unlikely, although not impossible. On the other hand, there is a precedence for the production of $^1O_2$ sensitizing metabolites in cyanobacteria (Shunmugam *et al.*, 2014), whose release to the cell exterior can provide a mechanism for extracellular $^1O_2$ production. In order to check the capacity of *Symbiodinium* cells to produce extracellular $^1O_2$ sensitizers we monitored the level of $^1O_2$ in cell free media of *Symbiodinium* cultures. Interestingly, we observed light-induced $^1O_2$ production in the cell free culture medium, which was separated from *Symbiodinium* cells by centrifugation (Fig. 4.24). This was typically observed in well grown cultures of 2-3 weeks of age. When the separated cells were re-suspended in fresh culture medium it took a couple of days until $^1O_2$ production appeared again in the extracellular medium. These data clearly demonstrate that *Symbiodinium* cells excrete one or more metabolite, which are capable for $^1O_2$ production under illumination.

In order to provide the first step in the characterization of the $^1O_2$-sensitizing metabolite(s) the absorption spectrum of the cell free medium was measured using a fresh culture medium as reference. As shown in Fig. 4.24b the absorption spectrum of the excreted metabolite(s) shows the main peak in the UV region which has a broad and gradually declining band extending in the visible and far-red region. Since we used a visible light source for the detection of $^1O_2$ in the His assay, which can extend to the blue and perhaps UV-A spectral range the UV-B and UV-C, part of the absorption spectrum can be excluded as playing role in $^1O_2$ sensitization.

Another useful method of $^1O_2$ detection is microscopy imaging by using sensor molecules whose fluorescence yield either decreases (DaNePy) or increases (SOSG) after interaction with $^1O_2$. Both of these sensors were successfully applied in cells of intact leaves (Hideg *et al.*, 1998), however, neither of them could be used in microalgae cells due to their limited penetration inside the cells. In spite of this difficulty microscopy imaging by fluorescent $^1O_2$ sensors could be a useful approach to study the distribution of extracellular $^1O_2$ production in the vicinity of *Symbiodinium* cells. In order to clarify the applicability of SOSG for detection of $^1O_2$ in

*Symbiodinum* cultures we used confocal microscopy imaging of SOSG stained cells under different illumination conditions.

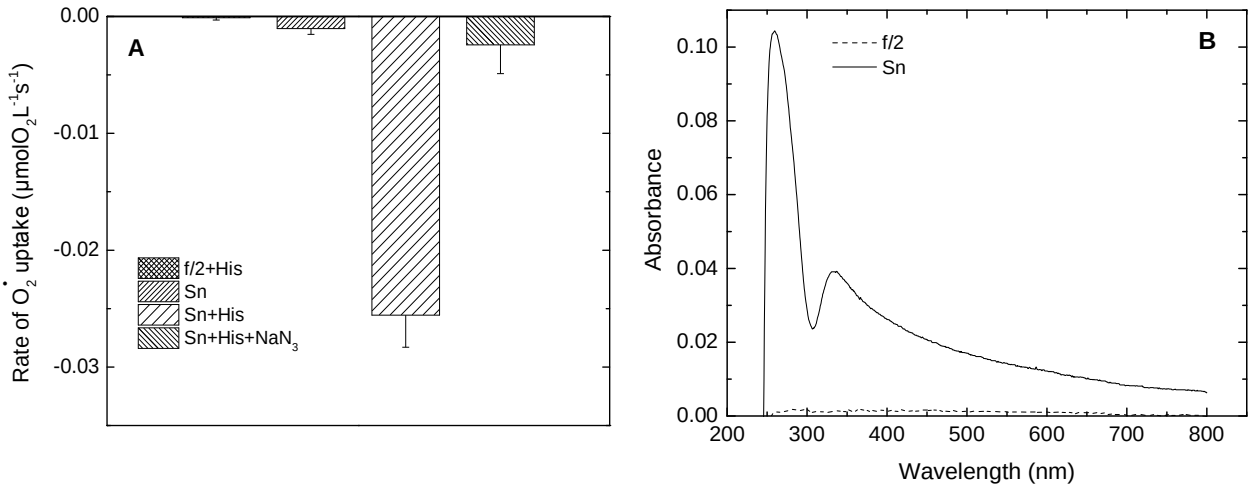


*Fig.4.24. Absorption spectrum and $^1O_2$ production in Symbiodinium cell free medium, A, His-mediated light induced $O_2$ uptake measured in the presence of 5 mM His and $^1O_2$ quencher, sodium azide ($NaN_3$). Light intensity during the oxygen measurements was 2300 μmole photons $m^{-2}s^{-1}$. Data are means ± s.d. B, the absorption spectrum of Symbiodinium cell free medium.*

As shown in Panel A of Fig. 4.25 only a minor trace of SOSG fluorescence could be detected if cells were kept in darkness after SOSG addition. For visualization of cell size and thylakoid membranes the images obtained on the same cell in the transmission and red fluorescence (Chl) channels, respectively, are also presented. The light green halo around the cell in green fluorescence channel arises from SOSG, which shows some fluorescence even without interacting with $^1O_2$. In addition illumination by the exciting green laser light also induces some increase of SOSG fluorescence due to trapping of $^1O_2$, which is produced by the excited SOSG molecules themselves. Similarly, the bright green spot outside the cell in Fig. 4.25A (channel a) is related to SOSG fluorescence induced by the scanning laser beam in a location where SOSG is accumulated due to an unknown reason. When cells were illuminated with red light, which excites Chls within the cells and produces intracellular $^1O_2$, SOSG fluorescence increase was observed only outside the cells, in the close vicinity of the cell surface.

Since cells were re-suspended in fresh culture medium before the SOSG addition, $^1O_2$ production, which is shown by the SOSG fluorescence increase can arise either from leakage of intracellular produced $^1O_2$ to the exterior of the cells, or due to the presence of extracellular $^1O_2$

sensitizers. In order to verify this important aspect we also used illumination through a bandpass filter with transmission maximum at 425 nm (i.e. violet-blue light), and half-bandwidth of 120 nm, to induce $^1O_2$ production. This light, although still exciting Chls in the Soret band, shows a larger overlap with the absorbance of the cell-free medium and therefore is expected to produce more $^1O_2$ from non-Chl sensitizers than red light does. As shown in Fig. 4.25C $^1O_2$ production under violet-blue pre-illumination occurs with the same pattern, i.e. outside or in the outer cell layer, as under red pre-illumination. Although this finding is not conclusive regarding the origin of $^1O_2$ detected here, it shows that $^1O_2$ is present in a relatively high amount in the outer layer of *Symbiodinium* cells, under these conditions, which has a potential importance for the interactions with the coral host.

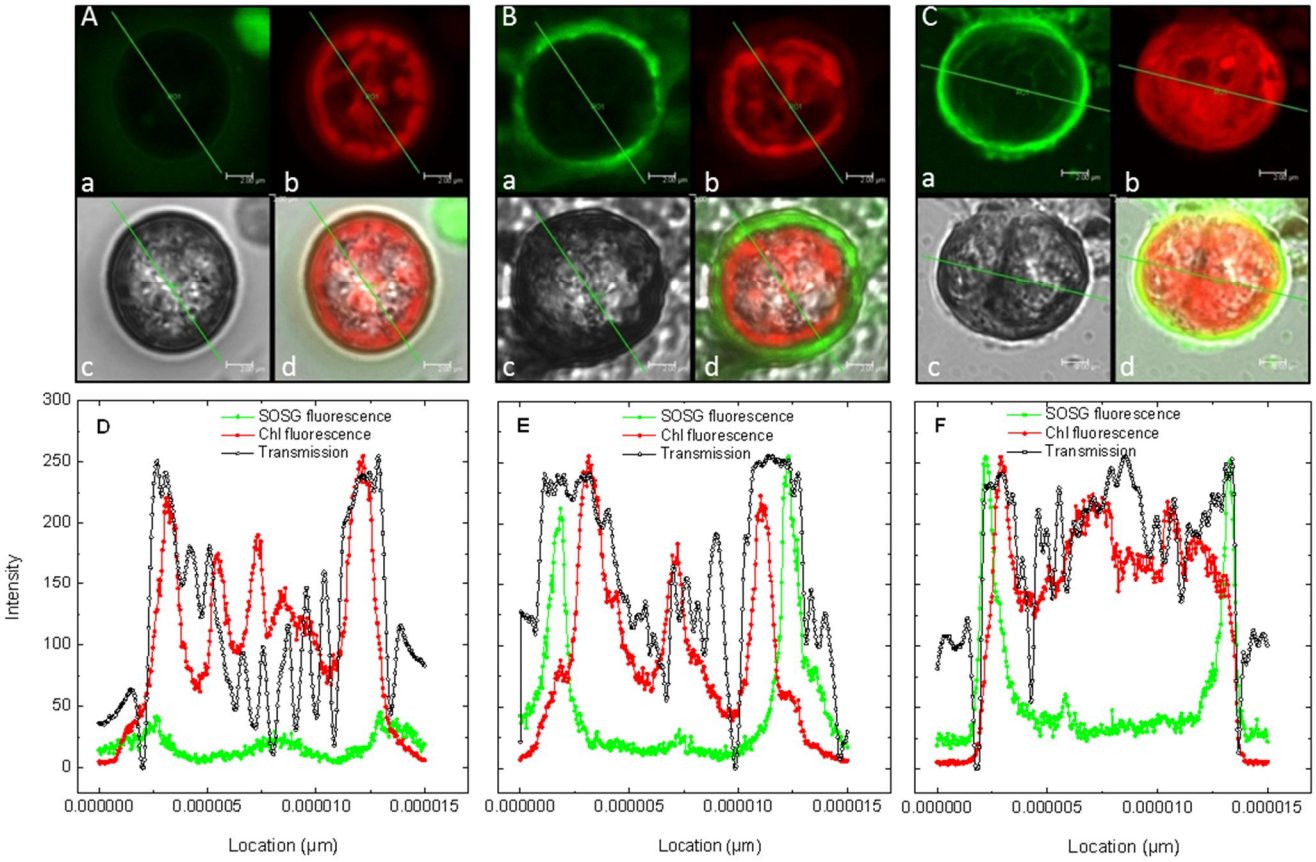


*Fig.4.25. Confocal fluorescence LSM imaging with SOSG in Symbiodininium cells, four channels are represented: a) SOSG fluorescence, b) Chl fluorescence, c) transmission channel, d) merged images of both signals with scale bars 5 µm. A) LSM imaging in cells with no pre-light illumination and D) Quantitative analysis of fluorescence signal from fig A. B) LSM imaging in cells with pre red light illumination for 5min and E) quantitative analysis of fluorescence signal from fig B. C) LSM imaging in cells with pre violet light illumination for 5 min and F) quantitative analysis of fluorescence signal from fig C. The visible light intensity during the cells treatment was approximately 2300 µmole photons $m^{-2}s^{-1}$.*

When *Symbiodinium* cells were left without resuspension in their original medium before SOSG imaging $^1O_2$ production could be detected in a more or less uniform way in the medium far from the cell surface and also in cell free medium ( Fig. 4.26). This confirms the finding obtained by His-trapping of $^1O_2$ described above.

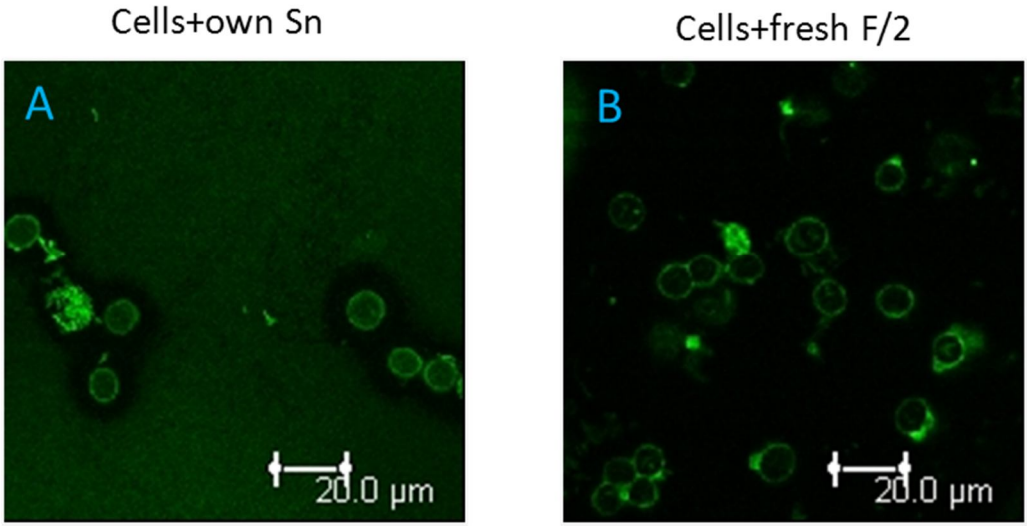


*Fig.4.26. Confocal fluorescence imaging of SOSG stained Symbiodininium cultures, A, The cells were kept either in their original medium in which they were cultured for 2 weeks, B, or were re-suspended in fresh culture medium before SOSG imaging, In order to trap $^1O_2$ the cells were illuminated by 2300 µmole photons $m^{-2}s^{-1}$ intensity visible light in the presence of SOSG for 5 min.*

In order to confirm light-induced production of $^1O_2$ by cell free media of *Symbiodinum* cultures which was observed by the His uptake method, we used fluorescence stereo microscopy imaging of SOSG stained cell free medium of *Symbiodinum* cultures under 3 min and 5 min light illumination conditions. As shown in Fig. 4.27 SOSG fluorescence signal in cell free medium of *Symbiodinum* cultures is significantly higher as compared to the control and this increase in SOSG fluorescence signal was light intensity dependent (Fig. 4.27b). Similarly, SOSG fluorescence increase has been observed when SOSG signal was compared with cells with original supernatant and re-suspend with fresh culture medium (Fig. 4.28). This data confirm that *Symbiodinium* cells produce extracellular $^1O_2$ sensitizers.

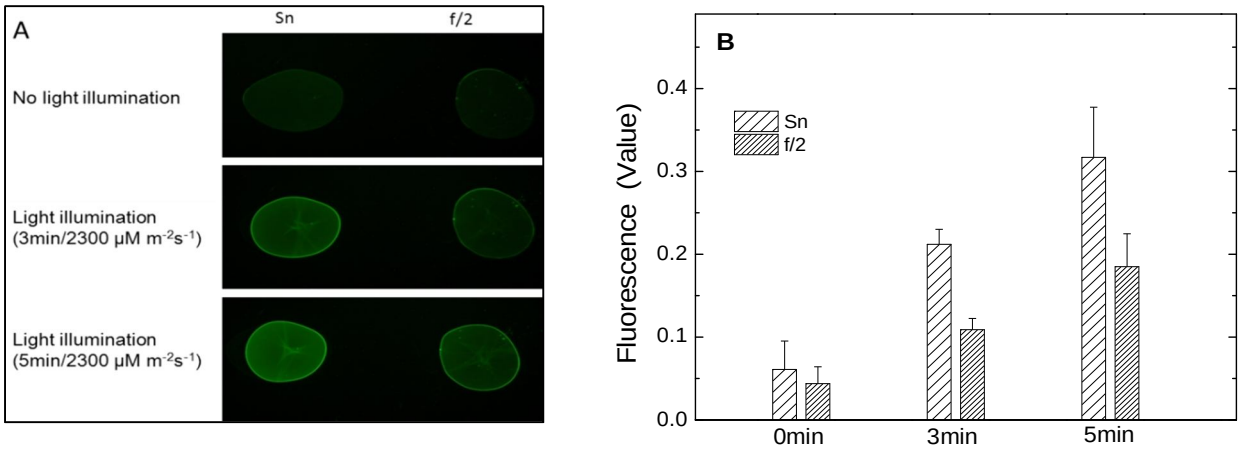


*Fig.4.27. Stereoscopic imaging with SOSG in Symbiodinium cell free medium A, Images of cell free medium and fresh medium with SOSG for 0, 3, and 5 min light irradiation of 2300 µmole photons $m^{-2}$ $s^{-1}$ B, Quantitative analysis of fluorescence signal from panel A which were quantified by a custom-made image analysis software.*

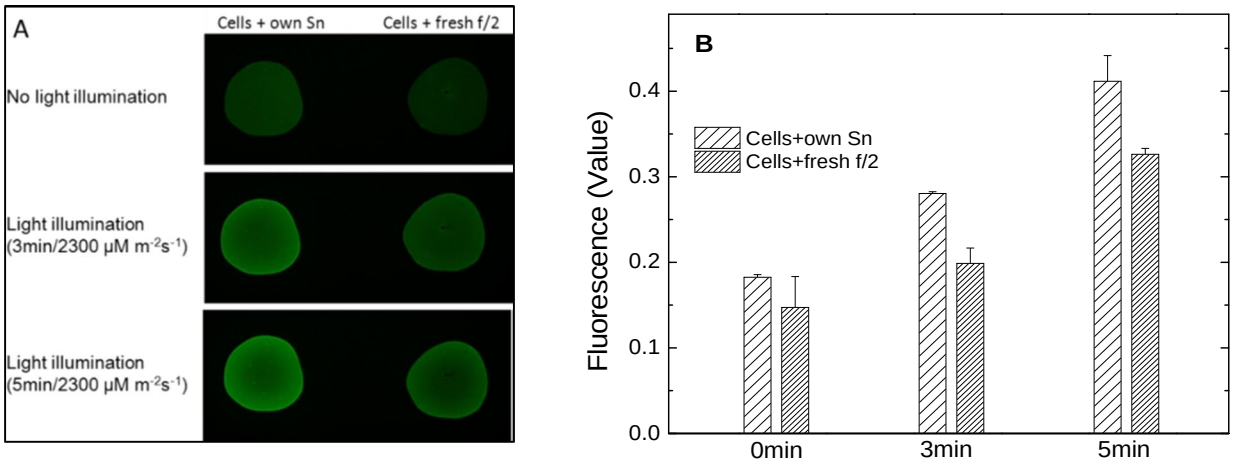


*Fig.4.28. Stereoscopic imaging with SOSG in Symbiodinium cells, A, Images of cells with own old and fresh medium and SOSG for 0min, 0, 3, and 5 min light illumination of 2300 µmole photons $m^{-2}$ $s^{-1}$ B, Quantitative analysis of fluorescence signal from panel A which were quantified by a custom-made image analysis software.*

Under conditions of HL and HT, which lead to the release of zooxanthellae from corals, *Symbiodinium* cells suffer Chl pigment bleaching as well loss of PSII activity. If $^1O_2$ is involved as damaging agent its trapping by externally added His could ameliorate Chl bleaching and PSII activity loss. In order to test this idea cells were incubated under HL, HT and HL+HT conditions

in the absence and presence of 5 mM His. Exposure of the cells in the absence of His to HL or HT, or to the combination of the two stress conditions induced a significant decrease in the level of Fm fluorescence (Fig. 4.29a) and the maximal quantum yield of PSII (Fv/Fm, Fig. 4.29b). In addition, Chl bleaching was observed as shown by the loss of absorbance in the red band of Chl (Fig. 4.29c, d and 4.30). Importantly, the addition of 5 mM His partially prevented all of these effects, as shown by the respective data in Figs. 4.29A, B, C, D, and 4.30. Therefore, these data provide a firm support for the idea that $^1O_2$ is involved as damaging agent in the inhibition of PSII activity, as well as Chl bleaching under HL, HT, and HL+HT stress conditions.

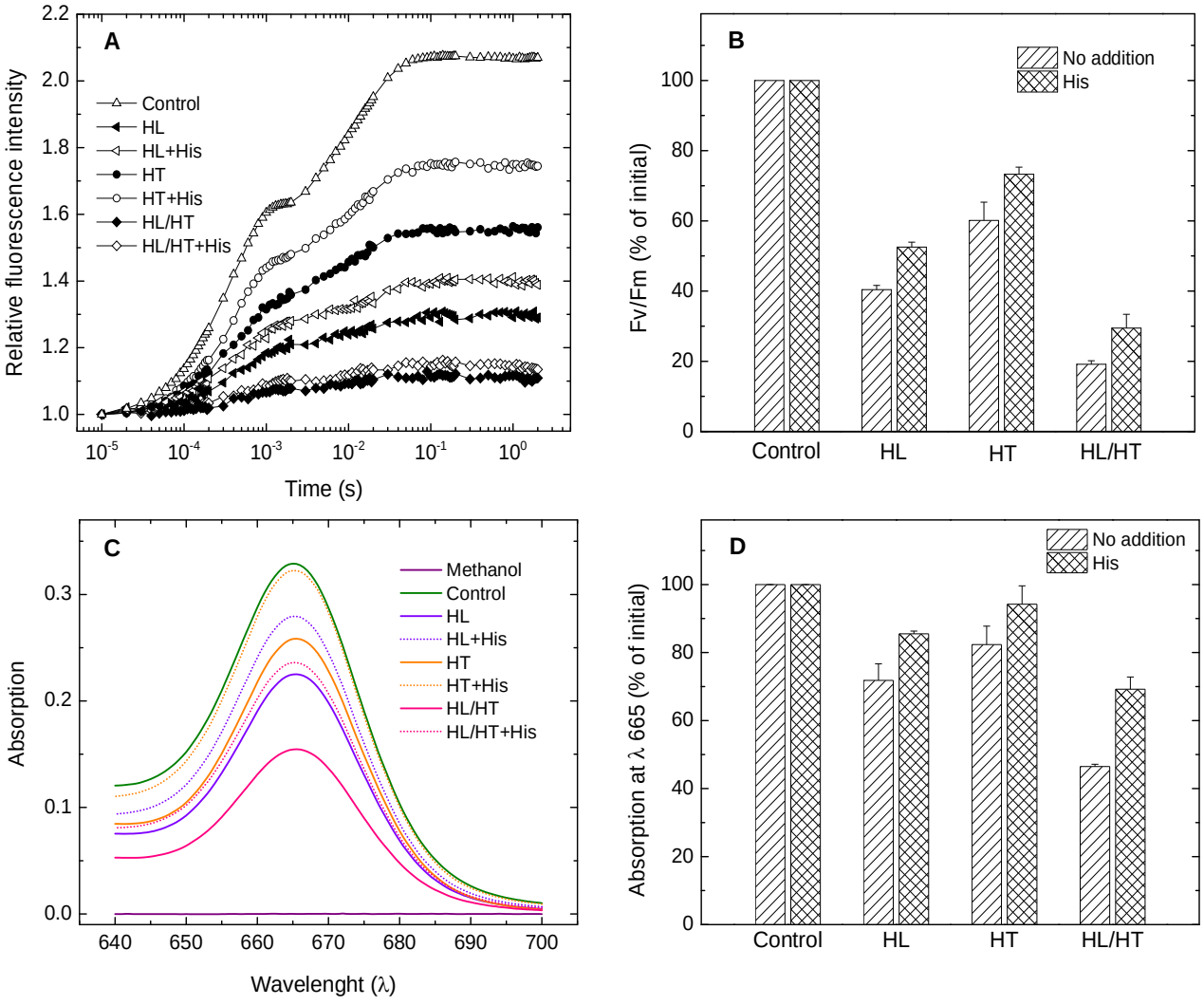

*Fig.4.29. Effect of His on heat and light stress induced PSII photoinactivation and pigment bleaching in Symbiodinium cells. A, Variable Chl fluorescence traces were measured in cells exposed to growth conditions (open, up triangle), or to high light (HL: 100 µmole photons* $m^{-2} s^{-1}$*, closed arrows), high temperature (HT: 34 °C, closed circles) and high light/high temperature (HL/HT, closed diamond). The traces were also measured in the presence of 5 mM His under high light (HL + His, open arrows), high temperature (HT + His, open circles) and high light/high temperature (HL/HT + His, open diamond). The curves are presented after normalized them to same initial Fo fluorescence level and 2 s long saturating pulse was applied. B, Fv/Fm values calculated from data in panel A, and shown after normalization to their initial control values C, Absorbance spectrum of methanolic extract of Symbiodinium cells after 24 h HL, HT and HL/HT treatment with and without 5 mM His D, Optical density at 665 nm. The data presented in panels B and D are means ± s.d. of three independent experiments.*

$^1O_2$ is an important reactive oxygen species, which is involved in light induced damage of the photosynthetic apparatus. The environmental conditions which lead to coral bleaching also involve high light, which in combination with high temperatures induce the release of zooxanthellae from coral tissues. Since our recent results paved the way for studying $^1O_2$ formation in intact microalgal cells, in the present work we aimed to investigate the occurrence of $^1O_2$ inside and outside of *Symbiodinium* cells, as well as the physiological consequences of $^1O_2$ formation. By using His-mediated chemical trapping we demonstrated that $^1O_2$ is formed in illuminated *Symbiodinum* cells.

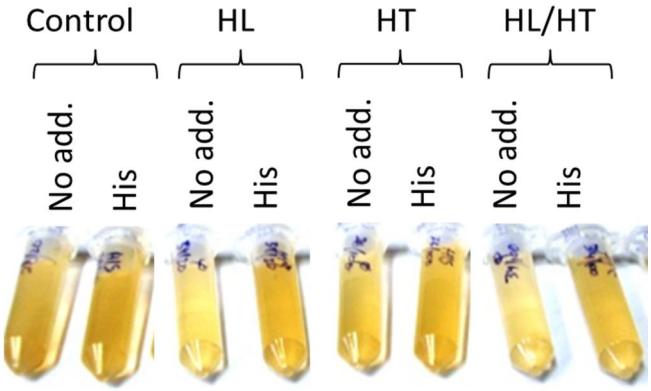


*Fig.4.30. The effect of His on Symbiodinium pigment bleaching, The representative photograph of the data represented in fig.4.29 shows pigment bleaching in Symbiodinium cells during high light (HL), high temperature (HT,) and HL/HT condition after 24 h treatment in the presence and absence of 5 mM His.*

The level of intracellular $^1O_2$ is increased when cells are exposed to high temperature (34 C), or inhibitors of the Calvin-Benson cycle, such GA or KCN (Fig. 4.31). Since HT and inhibition of the Calvin-Benson cycle are elicitors of zooxanthellae release from coral tissue our

data point to the possibility that $^1O_2$ might participate in the chain of events, which lead to coral bleaching. This idea is further supported by the finding that trapping of intercellular $^1O_2$ by externally added His ameliorates the inhibition of PSII electron transport as well as bleaching of Chl, which both occur under conditions of coral bleaching. On the other hand $^1O_2$ in itself may not be sufficient to trigger the release of zooxanthellea since GA treatment, although inhibits the Calvin- Benson cycle and enhances $^1O_2$ production in *Symbiodinium* cells, failed to induce the zooxanthellae from corals (Hill *et al.*, 2014).

This effect might indicate that intracellular $^1O_2$ formation is only coincident with other factors, which actually trigger the release of zooxanthellae, or that the consequences of GA treatment of corals are more complicated than could be expected from inhibition of the Calvin-Benson cycle alone. This latter idea is supported by our finding, which shows that incubation of *Symbiodinium* cells with GA for several hours, which was applied in the coral experiment, induces inhibition of photosynthetic activity even in darkness when light induced ROS production does not occur. Therefore, our data are more in favor of an additional effect of GA, which prevents the actual release of zooxanthellae in spite of Calvin-Benson cycle inhibition and $^1O_2$ production than the lack of $^1O_2$ involvement in the process that triggers the expulsion of *Symbiodinium* cells.

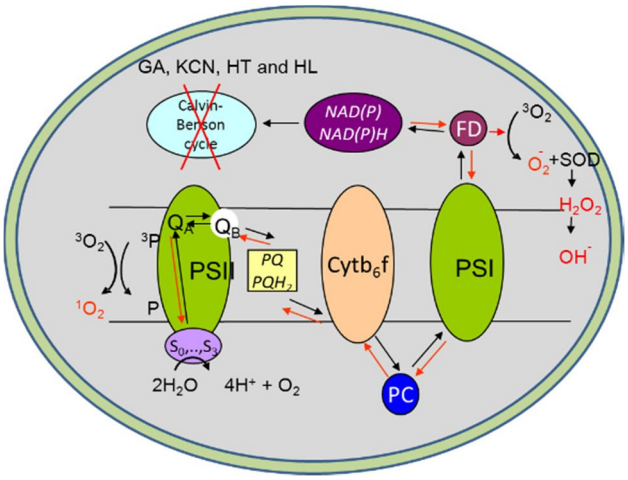


*Fig.4.31. The schematic proposed overview of $^1O_2$ and other ROS formation in the photosynthetic apparatus of Symbiodinium cells via the inhibition of Calvin-Bensin cycle by different stresses i.e. GA, KCN, high light (HL) and high temperature (HT).*

A highly important finding of our work was also the detection of $^1O_2$ production in cell free culture medium by His-mediated chemical trapping, which demonstrates the excretion of $^1O_2$ sensitizing metabolite(s) from *Symbiodinium* cells. Extracellular $^1O_2$ production was

confirmed by confocal microscopy imaging using the fluorescent $^1O_2$ sensor SOSG. These data showed not only the excretion of $^1O_2$ sensitizing metabolite(s) on the timescale of 1-2 days, but also that the compound(s) accumulate(s) at the outer surface of the cells, and cannot be removed by simple centrifugation. Microalgae cells often contain an exo-polysaccharide layer, which seems to collect the $^1O_2$ sensitizing chemical(s) after being excreted from the *Symbiodinium* cell. However, besides accumulation at the outer cell surface the $^1O_2$ sensitizing compound(s) can be found in the whole extracellular medium. This makes the so far unidentified compound(s) possible candidate for acting as a signaling component that can carry information from the intercellular space to the cell exterior.

### 4.4. Chloramphenicol mediated damage of Photosystem II via superoxide production in *Synechocystis* cells

Previous studies of photoinhibition revealed that light stress damages PSII directly (Aro *et al.*, 1992; Aro *et al.*, 1993b; Aro *et al.*, 1993a; Tyystjärvi, 2013; Nishiyama & Murata, 2014; Zivcak *et al.*, 2015; Giovagnetti & Ruban, 2015). The main consequence of photodamage under high light condition is PSII impairment of electron transport and damage to the D1 reaction center subunit (Baroli & Melis, 1996; Tyystjarvi, 2008; Wu *et al.*, 2011; Krinsky, 1968; Aro *et al.*, 1993c; Prasil *et al.*, 1992). In intact systems PSII photodamage can be at least partly repaired via *de novo* synthesis of the D1 protein. It has been reported previously (Okada *et al.*, 1991) that chloramphenicol (CAP) has side effects, namely it mediates superoxide production in thylakoids by transferring electrons from the PSI acceptor side to oxygen. This process on the one hand competes with $CO_2$ reduction, while on the other hand leads to the production of reactive superoxide by oxygen reduction. Very recently we have reported that CAP functions not only as PSI electron acceptor, but takes up electrons also from PSII and it has a potential to produce superoxide in PSII complexes (Rehman *et al.*, 2016b)

Light stress to PSII becomes a problem for photosynthetic capacity when the rate of photodamage exceeds the capacity of repair processes. Therefore, it is important to monitor separately the rates of photodamage and of the protein synthesis dependent repair. The rate of PSII photodamage can be separated from the effect of the ongoing protein synthesis dependent repair by applying inhibitors of D1 protein synthesis, such as lincomycin or CAP, which inhibit translation elongation in chloroplasts (Mulo *et al.*, 2003; Chow *et al.*, 2005; Tikkanen *et al.*,

2014) or in cyanobacterial cells (Constant *et al.*, 1997; Nishiyama *et al.*, 2005; Nishiyama *et al.*, 2001; Takahashi & Murata, 2005; Takahashi *et al.*, 2009; Sicora *et al.*, 2003). While there are no reports concerning the participation of lincomycin in photosynthetic electron transport, Superoxide radicals have high reactivity, therefore, it is expected that locally generated superoxide will induce damaging effects in the vicinity of its production. Therefore, several research groups have avoided the application of CAP and used lincomycin instead.

However, some research groups continued to use CAP until recently. An interesting example of controversy, which arose from the use of CAP, concerns the role the OCP protein in photoprotection. Previously the rate of PSII photodamage in wild type *Synechocystis and* OCP (Wilson *et al.*, 2008) lacking mutant was evaluated in the presence of lincomycin and it has been reported that ΔOCP is more sensitive to high intensities of white light than *Synechocystis* wild type. It has been reported that OCP has a very efficient $^1O_2$ scavenging activity, and also that $^1O_2$ induces direct photodamage to the PSII complex (Sedoud *et al.*, 2014). In a subsequent study Kusama and coworkers reinvestigated the $^1O_2$ scavenging role of OCP. They confirmed that OCP has an efficient $^1O_2$ scavenging capacity, but concluded also that the sensitivity of PSII to photodamage was unaffected in wild type *Synechocystis* and the ΔOCP mutant, and the enhanced photodamage was related to the inhibition of *de novo* D1 protein synthesis by $^1O_2$ (Kusama *et al.*, 2015). Detailed comparison of the experimental conditions used in these two studies has revealed interesting differences. In the work of Sedoud *et al*. lincomicyn was used as protein synthesis inhibitor, DMBQ as artificial electron acceptor for $O_2$ evolution measurements. In contrast, Kusama *et al*. used CAP as protein synthesis inhibitor and the combination of PBQ + FeCN as artificial electron system.

In order to clarify the role of OCP in photoprotection via $^1O_2$ scavenging, we performed photoinhibitory experiments in WT and ΔOCP cells using either lincomycin or CAP as protein synthesis inhibitor in combination with DMBQ or PBQ+FeCN as electron acceptor. PSII activity was monitored during the course of illumination by measuring the oxygen evolving activity of the cells using 0.5 mM DMBQ, 1mM pBQ and 1mM FeCN as an artificial electron acceptors. Our results show that the ΔOCP mutant cells were photodamaged faster than the wild type *Synechocystis* cells in the presence of lincomycin using either DMBQ or PBQ+FeCN as electron acceptor system (Fig 4.32a and b). However, this difference could not be observed when CAP

was used as protein synthesis inhibitor independent of the electron acceptor, being DMBQ or PBQ+FeCN (Fig 4.32c and d).

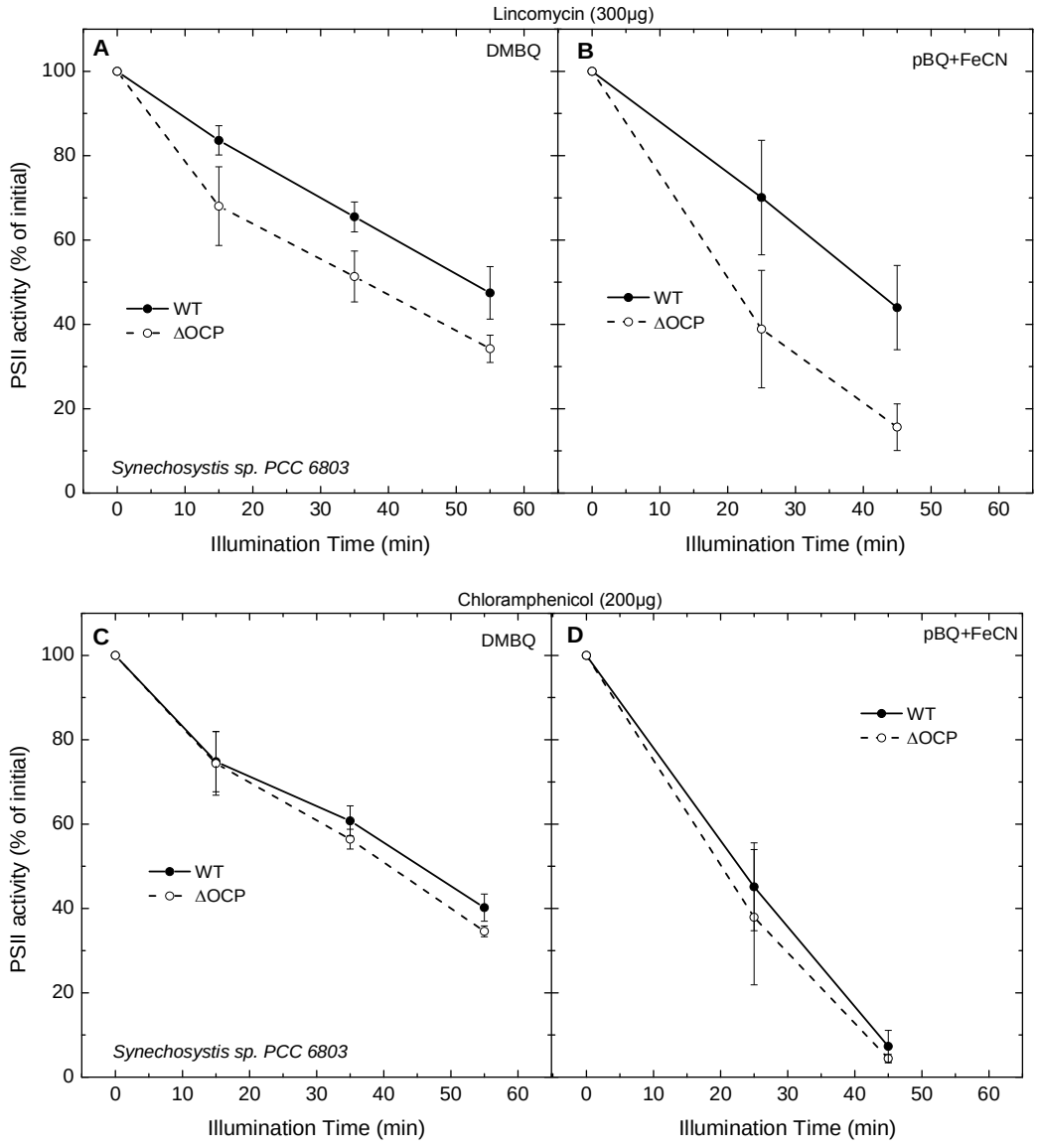


*Fig.4.32. CAP mediated PSII photodamage in Synechocystis cells, A, Light induced damage of PSII was assessed by measuring the rate of oxygen evolution in the presence of 0.5 mM DMBQ during exposure of wild type (closed circles) and ΔOCP strain (dash line, open circle) to illumination with 500 µmole photons $m^{-2}s^{-1}$ with lincomycin as protein synthesis inhibitor. B, the rate of oxygen evolution in the presence of 1 mM pBQ and 1mM FeCN with lincomycin, C, the rate of oxygen evolution in the presence of 0.5 mM DMBQ during exposure of wild type (closed circles) and ΔOCP strain (dash line, open circle) to illumination with 500 µmole photons $m^{-2}s^{-1}$ with CAP. D, the rate of oxygen evolution in the presence of 1 mM pBQ and 1 mM FeCN with CAP, Light intensity during the $O_2$ measurements was 2300 µmole photons $m^{-2}s^{-1}$. Data are means ± s.d. of three independent experiments.*

These data confirm that the applied electron acceptor has no influence on the detected rate of photodamage of the WT and the ΔOCP mutant strains. Therefore, the usage of DMBQ and PBQ + FeCN in the work of (Sedoud *et al.*, 2014; Kusama *et al.*, 2015) respectively, cannot explain the contrasting conclusions about the role of $^1O_2$ in direct photodamage of PSII. On the other hand, it is clear that photodamage is significantly faster in the ΔOCP mutant than in the WT when lincomycin is used as protein synthesis inhibitor, which confirms the earlier data of (Sedoud *et al.*, 2014). On the other hand, it is also clear that when CAP is used to inhibit protein synthesis dependent repair of PSII, the difference between the rates of photodamage of the WT and the *ΔOCP* mutant becomes much less than observed in the presence of lincomycin.

This finding brings credit to the experimental observations presented in the (Kusama *et al.*, 2015) paper since the small difference between the photodamage rates of WT and the ΔOCP could be overlooked and interpreted as the lack of difference. Interestingly, comparison of the data obtained with lincomycin and CAP also shows that the rate of photodamage is accelerated by CAP as compared to that obtained with lincomycin. This effect is especially clear when CAP was used together with PBQ+FeCN acceptor system. It appears for a reason, which is not fully clear at the moment that the photodamage accelerating effect is more pronounced in the WT than in the ΔOCP strain. This effect leads to the apparent loss of the difference between the two strains. However it can be clearly observed in the presence of lincomycin, which does not interfere with the photodamage process. These data demonstrate that CAP induces enhanced PSII photodamage, most likely via production of superoxide in wild type *Synechocystis* 6803 and the ΔOCP mutant (Fig 4.32).

In contrast to molecular oxygen, superoxide does not produce amperometric signal in Clark-type oxygen electrodes. Therefore, conversion of $O_2$ to $O_2^-$ leads to oxygen consumption, which can be easily followed by oxygen uptake measurements. In order to investigate CAP mediated superoxide production we measured $O_2$ uptake under various conditions. From the results, which we have obtained in isolated BBY particles (Rehman *et al.*, 2016b), it follows that the photodamaging effect of CAP arises from superoxide production in PSII. In order to clarify if the same mechanism is also effective *in vivo* we measured the effect of CAP on the rate of oxygen uptake in wild type *Synechocystis*. The data show that the addition of CAP decreases the rate of $O_2$ evolution *Synechocystis* cells in the absence of added artificial acceptors (Fig. 4.33a).

The $O_2$ evolution rate was also measured by using artificial electron acceptor pBQ and FeCN. The results show significant decrease in the $O_2$ evolution rate when CAP was added to the cells. The results were confirmed by measuring $O_2$ evolution with CAP in the presence of DCMU to avoid the artifact that would arise from a direct effect of CAP on the $O_2$ evolving activity. Therefore, the loss of $O_2$ evolution observed in the presence of CAP shows $O_2$ consumption due to $O_2^-$ formation.

Furthermore, the CAP mediated effect on $O_2$ consumption was compared with the effect of lincomycin. Cells were incubated with the same concentration of lincomycin and CAP, which were used in Fig. 4.33, for 30 min under normal growth conditions and $O_2$ evolution was measured by using artificial electron acceptor DMBQ, pBQ and FeCN. The results show that the loss of $O_2$ evolution which was observed in the CAP-treated cells was not observed in lincomycin-treated cells (Fig. 4.33b). These results further confirm that CAP induces superoxide production in intact cells as well, which is most likely responsible for the CAP mediated acceleration of photodamage in *Synechocystis* cells.

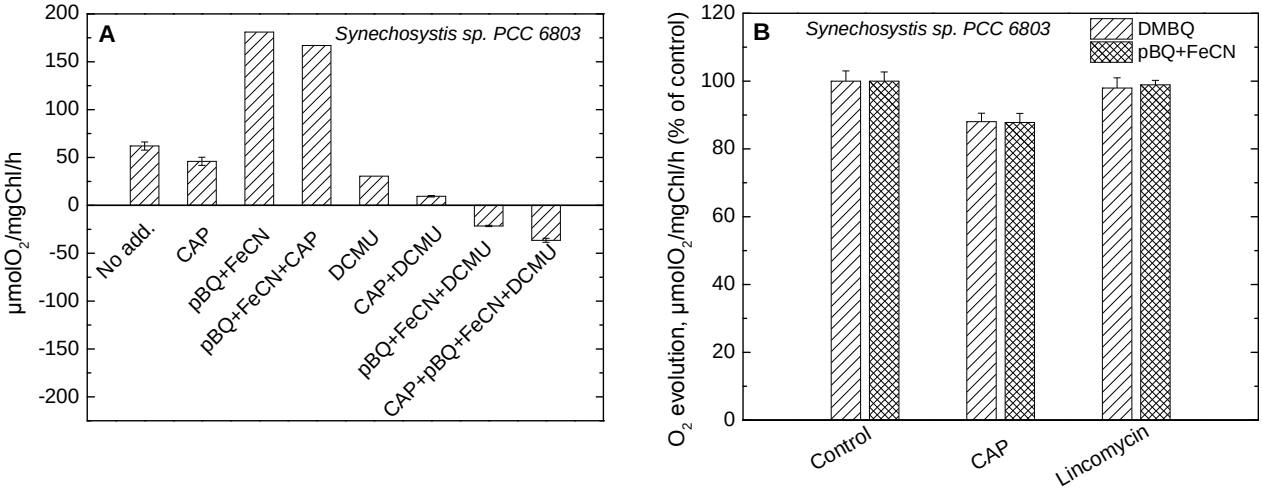


*Fig.4.33. Effect of CAP and lincomycin on the rate of oxygen evolution in Synechocystis cells A, Rate of oxygen evolution in Synechocystis cells having pBQ and ferric cyanide as an electron acceptor with and without CAP, the CAP effect was also monitored with/without DCMU, B, Rate of oxygen evolution in Synechocystis cells by using DMBQ and pBQ + FeCN as an electron acceptor in the presence of CAP and lincomycin, the cells were incubated for 30 min under normal growth conditions and measurements were performed. Light intensity during the $O_2$ measurements was 2300 µmole photons $m^{-2}s^{-1}$. Data are means ± s.d. of three independent experiments.*

On the basis of our results we propose that CAP acts as an electron acceptor, which takes electrons from the acceptor side of both PSI and PSII to molecular oxygen to generate superoxide in intact cells (Fig. 4.34).

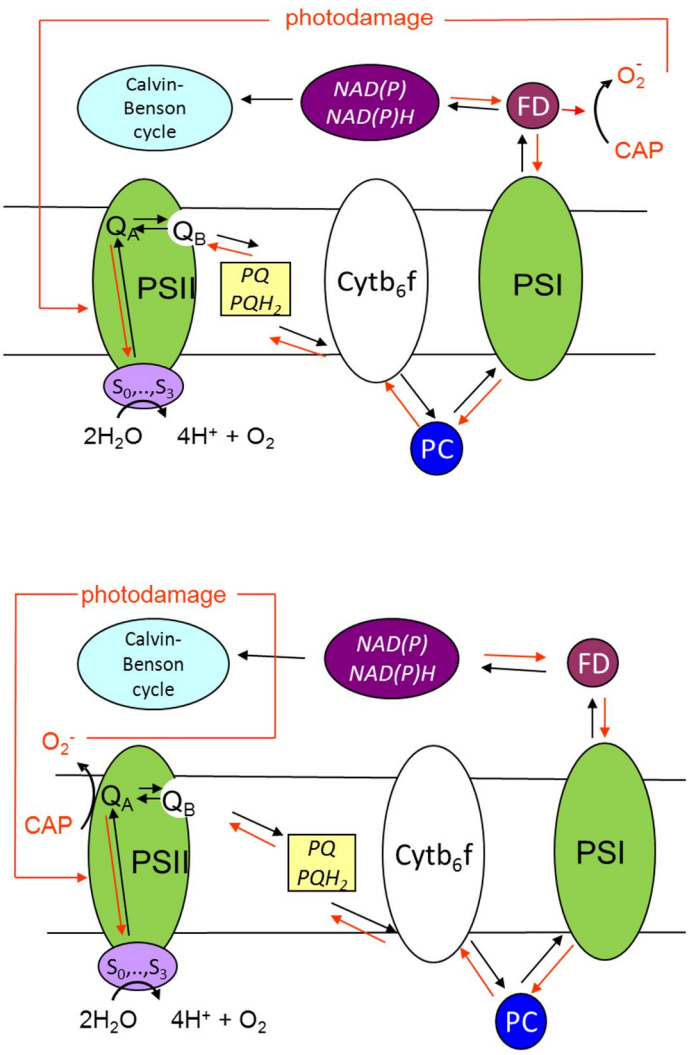


*Fig.4.34. CAP mediated proposed mechanism of $O_2^-$ production and PSII photodamage in the photosynthetic machinery of photosynthetic organisms.*

*A, The proposed mechanism of $O_2^-$ production at accepter side of PSI and PSII photodamage by CAP; the scheme illustrates the production of superoxide via PSI.*

*B, The proposed mechanism of $O_2^-$ production and PSII photodamage in PSII by CAP; the scheme illustrates the production of superoxide via PSII.*

Our research shows that when CAP is used to monitor the rate of photodamage of PSII in photosynthetic organisms, it has additional damaging effect to PSII (Fig. 4.35). This finding has been considered as a source of potential artifact by several research groups, who used lincomycin instead of CAP in photoinhibition studies (Tyystjarvi & Aro, 1996; Tikkanen et al., 2014; Tyystjarvi et al., 2002; Campbell & Tyystjärvi, 2012; Constant et al., 1997; Chow et al., 2005; Miyata et al., 2012). However, other groups kept using CAP in measurements of PSII

photodamage (Nishiyama *et al.*, 2005; Nishiyama *et al.*, 2001; Takahashi & Murata, 2005; Takahashi *et al.*, 2009; Nishiyama & Murata, 2014). Therefore, we propose that CAP must not be used to study the photoinhibiton of PSII in photosynthetic organisms, since besides blocking the repair cycle of PSII, CAP also accelerates the rate of photodamage, which can lead to misleading conclusions regarding the mechanism of photoinhibition.

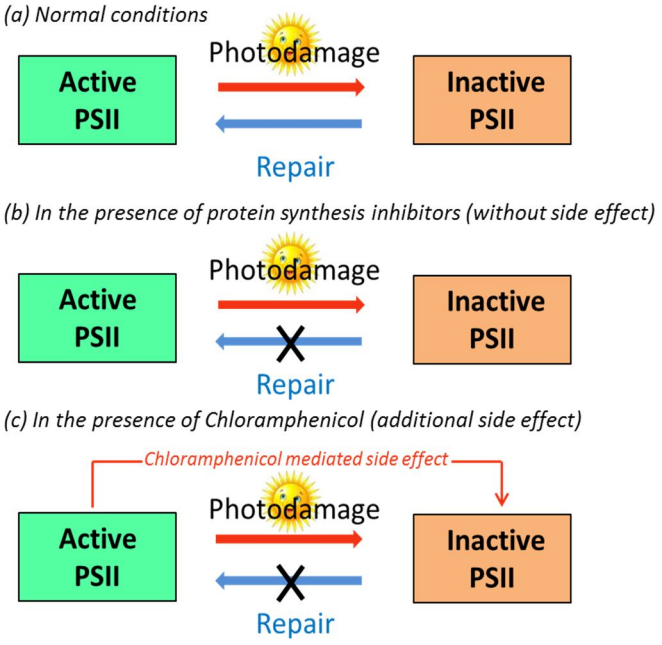


*Fig.4.35. Novel scheme of photosystem II (PSII) photoinhibition in photosynthetic organisms, A, The rate of PSII photodamage and protein synthesis dependent repair without using protein synthesis inhibitors; B, The rate of PSII photodamage and its repair monitored independently by using protein synthesis inhibitors, such as lincomycin; C, The rate of PSII photodamage and its repair monitored individually by using CAP which show additional damage to PSII and it is represented by red arrow. Fig. modified from (Nishiyama & Murata, 2014).*

## 5. CONCLUSIONS

We have developed a method for $^1O_2$ detection in intact cyanobacteria by His-mediated chemical trapping (Rehman *et al.,* 2013). The method is based on chemical trapping of $^1O_2$ by His, which leads to $O_2$ uptake during illumination that can be detected and quantified by commercial oxygen electrodes. We have observed oxygen uptake in intact *Synechocystis* PCC 6803 cells, during illumination in the presence of His. The His-mediated oxygen uptake effect was enhanced when the water content of the BG-11 culture medium was partly replaced with $D_2O$, which enhances the lifetime of $^1O_2$ and therefore facilitates its interaction with His. In contrast, the His-mediated oxygen uptake effect was suppressed by $NaN_3$, which is a $^1O_2$ quencher. Due to the limited mobility of $^1O_2$, these data demonstrate that exogenous His reaches the vicinity of $^1O_2$ production sites inside the cells, becomes oxidized by $^1O_2$ and also that the His-mediated oxygen uptake assay can be used for $^1O_2$ detection in intact cells.

In a previous study the mechanism of charge recombination in PSII was investigated by using flash induced Chl fluorescence and thermoluminescence measurements in the D1-Q130E and D1-Q130L mutants of *Synechocystis* 6803 (Vass & Cser, 2009). We have found a regulatory mechanism of photo-tolerance in the Q130E mutant, which prevents the formation of triplet $^3$P680, and our results suggest that the differential photodamage of Q130E, wild type, and Q130L is correlated with the extent of $^1O_2$ production in these strains (Rehman *et al.*, 2013). We investigated the roles of $^1O_2$ production with relation to the mechanisms of PSII photodamage and photoprotection in cyanobacteria and dinoflagellate by employing His-mediated oxygen uptake method.

**Applications of His-mediated O2 uptake method *in Synechocystis mutants***

- The His-mediated $O_2$ uptake method was used to investigate the roles of oxidative stress and photoinhibition in high light acclimation using a regulatory mutant of the cyanobacterium *Synechocystis* sp. PCC 6803. The mutant strain *ΔsigCDE* contains the stress responsive SigB as the only functional group 2 σ factor. We observed that ΔsigCDE suffers from oxidative stress in standard conditions. ΔsigCDE has high carotenoid and Flv4-2 contents and protected PSII against light induced damage, which is accompanied more efficient $^1O_2$ quenching than in the control strain. Therefore, the

decreased extent of photo-damage in *ΔsigCDE* mutant is correlated with suppressed rate of $^1O_2$ production (Hakkila *et al.*, 2014).

- We have employed the His-mediated $O_2$ uptake method to characterize the properties of a secondary metabolite of *Nostoc* XPORK14A denoted as M22. This compound induced retardation of cell growth and inhibition of photosynthesis in *Synechocystis* PCC 6803 cells, both in light and in darkness. We showed that M22 generates $^1O_2$ in the presence of light, which in turn affects the photosynthetic machinery of *Synechocystis* PCC 6803 (Shunmugam *et al.*, 2014).

- The $^1O_2$ detection method was also used to study the role of flavodiiron proteins (FDPs) in PSII photoprotection. FDPs are specific electron transfer enzymes present mainly in anaerobic bacteria, and they are found also in cyanobacteria. Cyanobacterial FDPs constitute a specific group that evolved differently to cooperate with oxygenic photosynthesis. In *Synechocystis sp.* PCC 6803 there are four FDPs, Flv1-Flv4. Two of them, Flv2 and Flv4, are encoded by an operon together with a Sll0218 protein. We have shown that $^1O_2$ production is regulated by the amount of *flv2-4*, and also that photodamage was correlated with the rate of $^1O_2$ production (Bersanini *et al.*, 2014).

- The His-mediated $O_2$ uptake method was used to understand the role of the orange carotenoid protein (OCP) on photoprotection of *Synechocystis*. The photoactive OCP has been shown to be essential in photoprotection as energy quencher. When OCP is photoactivated by strong blue-green light, it is able to dissipate excess energy as heat by interacting with phycobilisomes. We demonstrated that OCP protects *Synechocystis* cells from photodamage also by red light. We showed that this photoprotection is related to a decrease of $^1O_2$ concentration due to a scavenging action of OCP. Our results also showed that an OCP overexpressing mutant shows higher resistance to photoinhibition and exhibits a decreased production of $^1O_2$. Altogether, the results showed that the OCP in *Synechocystis* is an efficient $^1O_2$ quencher and contributes to photoprotection (Sedoud *et al.*, 2014).

- The His-mediated $O_2$ uptake method was employed to investigate the growth and photosynthetic activity of PSI-less and PSI-less/ScpABCDE$^-$ strains of *Synechocystis* and to explore the effect of moderate salt stress conditions. Small CAB-like proteins (SCPs) are single-helix light-harvesting-like proteins found in all organisms performing oxygenic photosynthesis. We observed that the addition of 0.2 M NaCl to the growth medium in the PSI-less/ScpABCDE$^-$ mutant strain decreased $^1O_2$ formation and induced resistance to photoinhibition compared to control strain (Tibiletti *et al.*, 2016).

**Applications of His-mediated $O_2$ uptake method to study $^1O_2$ production in dinoflagellate cells**

- The His-mediated $O_2$ uptake method was also tested with cultured *Symbiodinium* cells. We observed oxygen uptake in intact *Symbiodinium* cells and intact corals during illumination in the presence of His, which demonstrated the production of $^1O_2$. We observed that the production of $^1O_2$ in *Symbiodinium* cells was enhanced during thermal and light stress conditions, which was accompanied with photo-inactivation of PSII and $^1O_2$ production, while His itself provides protection against PSII photo-inactivation and pigment bleaching. Therefore, the inactivation of PSII and enhanced production of $^1O_2$ by heat and light stresses promotes bleaching event in cultured *Symbiodinium* cells. We propose that $^1O_2$ induced inactivation of *Symbiodinium* cells may be involved in triggering the expulsion of *Symbiodinium* cells from the coral host, which leads to coral bleaching (Rehman *et al.*, 2016c).

- We have also investigated the inhibition of the Calvin-Benson cycle by glycolaldehyde (GA) and imulation cyanide (KCN) in cultured *Symbiodinium* cells to understand the role of Calvin-Benson cycle inhibition in coral bleaching. We showed that the inhibition of the Calvin-Benson cycle by GA did not induce coral bleaching without thermal stress, while KCN induce coral bleaching even without thermal stress and promote $^1O_2$ formation (Hill *et al.*, 2014).

- We have also shown the detection of $^1O_2$ production in cell free culture medium of *Symbiodinium* by His-mediated chemical trapping, demonstrating the excretion of $^1O_2$ sensitizing metabolite(s) from *Symbiodinium* cells (Rehman *et al.*, 2016c).

**CAP mediated damage of PSII via superoxide production in intact *Synechocystis* cells**

We tested the effect of CAP on the rate of photodamage in intact cyanobacterial cells. We showed that ΔOCP is more sensitive to high intensities of white light than wild type *Synechocystis* in the presence of lincomycin. However, in the presence of CAP the wild type and ΔOCP show no differences in high light sensitivity, which indicate that CAP induced PSII photoinhibiton by additional damage via the production of superoxide in intact *Synechocystis* cells (Rehman *et al.*, 2016a).

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## KIVONAT

**$^1O_2$ detektálása és a fénygátlásban játszott szerepének vizsgálata *Synechocystis* sp. PCC 6803 cianobaktériumban és *Symbiodinium* ostoros algában**

A szinglet oxigén ($^1O_2$) az egyik legjelentősebb reaktív oxigénforma a fotoszintetikus rendszerekben. A $^1O_2$ triplett alapállapotú molekuláris $O_2$ és triplett gerjesztett pigmentek, elsősorban a klorofill kölcsönhatása révén keletkezik a második fotokémiai rendszerben (PSII) és a fénygyűjtő komplexekben. Bár több módszer (úgy mint ESR és fluoreszcens spin csapdázás) is rendelkezésre áll a szinglet oxigén detektálására izolált fotoszintetikus rendszerekben, ezek nem alkalmazhatók élő cianobaktériumok és mikroalgák esetén, mivel a jelző molekulák nem képesek a sejtfalon áthatolni, ami hátráltatja a szinglet oxigén szerepének kutatását a fénygátlás folyamatában. Annak érdekében, hogy túllendüljünk ezen a nehézségen, kifejlesztettünk egy hisztidines kémiai csapdázáson alapuló módszert a $^1O_2$ detektálására intakt mikroalga sejtekben. A módszer lényege a $^1O_2$ kémiai megkötése hisztidin által, ami megvilágítás alatt, kereskedelmi forgalomban kapható oxigén elektródákkal detektálható $O_2$ felvételhez vezet.

Fő eredmények:

**Szinglet oxigén képződés és fénykárosítás *Synechocystis* 6803-ban**

- Kimutattuk, hogy megvilágítás hatására hisztidin jelenlétében $O_2$ felvétel figyelhető meg intakt *Synechocystis* sejtekben. Ez a jelenség felerősíthető a szinglet oxigén élettartamát megnövelő $D_2O$ jelenlétében, és lecsökkenthető a $^1O_2$ kioltó $NaN_3$ segítségével. A szinglet oxigén behatárolt mobilitása miatt ezek az adatok azt mutatják, hogy a külső eredetű hisztidin eléri a szinglet oxigén képződés helyeit a sejten belül, tehát a hisztidin által közvetített $O_2$ felvétel vizsgálat alkalmas élő sejtek szinglet oxigén termelésének detektálására.
- Korábbi vizsgálatok azt mutatták, hogy a második fotokémiai rendszer D1 reakciócentrum fehérjéit érintő D1-Q130E és D1-Q130L mutációk *Synechocystis* 6803-ban befolyásolják a PSII sugárzás nélküli töltésrekombinációs folyamatait. Kimutattuk, hogy a D1-Q130E törzs esetén a felgyorsult sugárzás nélküli töltésrekombináció csökkenti a $^3P680$ és ezáltal a szinglet oxigén kialakulását és ezáltal fénykárosítás elleni védelmet biztosít, a D1-Q130L törzs esetén pedig az ellentétes hatás, azaz megnövekedett szinglet oxigén termelés és fénykárosítás következik be. Eredményeink azt mutatják,

hogy a különböző mértékű fénykárosodás korrelációban van a Q130E, vad típus, és Q130L vonalakban termelődő szinglet oxigén mennyiségével.

- *A szinglet oxigén detektáló eljárást alkalmaztuk a* flavodiiron proteinek (FLV) szerepének felderítésére a cianobakteriális PSII fényvédelmében. A *Synechocystis*-ben négyféle FLV-t találunk, Flv1-Flv4, amelyek specifikus elektrontranszport fehérjék. Közülük az Flv2 és Flv4 a PSII-ből az FLV1 és FLV3 a PSI-ből csatolnak ki elektronokat. Az eredményeink azt mutatták, hogy a szinglet oxigén fejlődés sebességét az Flv2-4 fehérjék növekvő mennyisége csökkenti, ami fénykárosodás mértékét is csökkenti.
- A hisztidin csapdázásos szinglet oxigén detektálási módszert alkalmaztuk a *ΔsigCDE Synechocystis* mutáns fénykárosításának vizsgálatára. A mutáns vonal csak egyetlen kettes csoportba tartozó stressz reszponzív σ factort (SigB) tartalmaz. Kimutattuk, hogy a ΔsigCDE törzs fokozott fénytoleranciával rendelkezik, ami a magas karotinoid és Flv4-2 tartalomnak, illetve az ennek következtében hatékonyabb szinglet oxigén kioltásnak köszönhető.
- A hisztidin-közvetített $O_2$ felvétel módszerét alkalmaztuk a *Nostoc* XPORK14A egy másodlagos metabolitjának jellemzésére, amit M22-nek jelöltünk. Ez a vegyület növekedés és fotoszintézis gátlást okozott Synechocystis sejteken, mind fényben és sötétben. Kimutattuk, hogy az M22 fény hatására szinglet oxigént képez, ami viszont befolyásolja a *Synechocystis* fotoszintetikus rendszerét.
- A hisztidin csapdázásos szinglet oxigén detektálási módszert alkalmaztuk az OCP (orange carotenoid protein) fehérje szerepének vizsgálatára is a *Synechocystis* fényvédelemében. Kimutattuk, hogy az OCP jól ismert fényenergia disszipáló hatása mellett, nagyon hatékony szinglet oxigén kioltóként is működik. Ennek következtében az OCP fehérje mennyiségétől függő szintű fényvédelmet biztosít olyan körülmények között is (pl. vörös fényben), amikor a fényenergia disszipációs mechanizmus nem működik (amihez kék-zöld fény által kiváltott konformáció szükséges).
- A fenti módszert alkalmaztuk az ún. SCP klorofill kötő fehérjék szerepének vizsgálatában, amelyek minden oxigéntermelő fotoszintetikus szervezetben megtalálhatók. Korábbi eredmények azt mutatták, hogy az SCP fehérjék hiánya megnöveli a szinglet oxigén képződés ill. a fénykárosítás mértékét. Jelen munkánkban azt

mutattuk ki, hogy 0.2 M NaCl hatására PSI-mentes/ScpABCDE mutáns esetében lecsökkentett a szinglet oxigén fejlődés mértéke és egyúttal növekedett fénykárosodással szemben tanúsított tolerancia.

**Szinglet oxigén képződés szimbiotikus ostoros algákban**

- A szinglet oxigén detektálási módszert a korall szimbiózisban a fotobionta szerepet játszó *Symbiodinium* ostoros alga sejteken is vizsgáltuk. Megfigyeltük a hisztidin által közvetített $O_2$ felvételt megvilágítás alatt *Symbiodinium* sejteken és egész korallokon is, ami igazolta a szinglet oxigén fejlődés meglétét. A szinglet oxigén termelés sebessége felerősödött hő és fény stressz hatására, ami együtt járt a PSII fotoinaktivációjával. Ugyanakkor a hisztidin mint szinglet oxigén kioltó jelenléte részleges védelmet nyújtott a PSII fényindukált inaktiválása és a pigment károsodása ellen. Ezen eredmények alapján feltehető, hogy a szinglet oxigén által kiváltott sejtinaktiváció szerepet játszhat a *Symbiodinium* sejtek kilökődésében, ami korall pusztuláshoz vezet.
- Vizsgáltuk továbbá a Calvin-Benson ciklus glikolaldehiddel (GA) és kálium cianiddal (KCN) történő gátlásának hatását *Symbiodinium* sejteken, annak érdekében, hogy megértsük a Calvin-Benson ciklus szerepét a korall kifehéredésben. Az eredmények azt mutatták, hogy mind a GA mind a KCN jelenléte fokozza a szinglet oxigén termelést, de csak a KCN indukál korall kifehéredést.
- Igen lényeges eredményünk annak kimutatása is, hogy a *Symbiodinium* sejtek szinglet oxigén képződést érzékenyítő metabolitokat választanak ki a sejteken kívüli térbe, amelyek mennyisége megnő a korall pusztulást kiváltó fény- és hőstressz alatt. Ezek a metabolitok szerepet játszatnak a *Symbiodinium* sejtek és a korall polipok közötti szimbiózis szabályzásában.

**Kloramfenikol által indukált fénykárosítás *Synechocystis* sejtekben**

- Kimutattuk, hogy a protein szintézis gátlóként alkalmazott kloramfenikol szuperoxid képződéshez vezet és felerősíti a PSII fénygátlását *Synechocystis*-ban. Ez a hatás különböző mértékben érinti a vadtípusú és ΔOCP mutáns sejteket és eltorzítja a valódi fénykárosításból származó hatásokat. Ezért a kloramfenikol szintézis gátlóként történő alkalmazását kerülni kell fotoinhibíciós vizsgálatokban.

# SUMMARY

# DETECTION OF SINGLET OXYGEN PRODUCTION AND ITS RELATION TO PHOTOSYSTEM II PHOTODAMAGE IN INTACT CYANOBACTERIA AND MICROALGAE

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## INTRODUCTION

Reactive oxygen species (ROS) are produced primarily in the thylakoid bound photosynthetic complexes during the process of photosynthesis. Light energy absorbed by Chl molecules can 1) drive photosynthesis; 2) can be dissipated as heat, 3) re-emitted as red fluorescence. Light absorption by light harvesting chlorophylls in the light harvesting complex (LHCII) and in the PSII protein complex leads to excitation of the P680 reaction center Chl. The charge separation between the excited reaction center Chl assembly (P680*) and the pheophytin (Phe) molecule, i.e. the formation of the primary radical pair/charge separated state

($P680^{+\bullet}Phe^{-\bullet}$), is a primary event during photosystem II (PSII) electron transfer, which is further followed by rapid charge stabilization processes.

Excited Chls in LHCII can be converted to triplet state ($^3$Chl), while triplet P680 can be formed during recombination of charge separated states. The interaction of excited triplet $^3$P680 or $^3$Chl with ground state triplet oxygen leads to the formation of singlet oxygen ($^1O_2$), which is a highly reactive species and capable of unhindered oxidation of various cellular components such as lipids, proteins, and nucleic acids, which ultimately can lead to cell death. $^1O_2$ can also damage the photosynthetic apparatus during stress conditions. Previous studies have shown that increased production of $^1O_2$ seems to occur primarily under high light stress conditions, which will ultimately result in the inactivation of PSII and photoinhibition. Previously the $^1O_2$ detection methods, such as fluorescent and electron paramagnetic resonance (EPR) sensors were developed to study $^1O_2$ formation in isolated PSII membranes and plant leaves, but methods for detection of $^1O_2$ in intact cyanobacteria and microalgae cells were largely unknown.

## AIMS

The general aim of the PhD work was to gain knowledge on the mechanisms of photodamage and photoprotection of the PSII complex in cyanobacteria and in dinoflagellates with special emphasis on the role of charge recombination reactions. The specific aims of this study were:

1. To develop a method for the detection of singlet oxygen ($^1O_2$) in intact cyanobacterial cells and to investigate the correlation of photodamage and the extent of $^1O_2$ production in WT *Synechocystis* as well as in mutants in which Gln130 residue of the D1 reaction center subunit of PSII is replaced with Glu or Leu.
2. To investigate the roles of $^1O_2$ in photoinhibition of *Synechocystis ΔsigCDE*, which contains the stress responsive SigB as the only functional group 2 σ factor.
3. To understand the role of orange carotenoid protein (OCP) as $^1O_2$ quencher by using mutants lacking, or overexpressing the OCP gene in *Synechocystis.*
4. To characterize $^1O_2$ production by a secondary metabolite of the cyanobacterium *Nostoc* XPORK14A.
5. To investigate the role of flavodiiron proteins (FDPs) in protecting against $^1O_2$-dependent photodamage of PSII in *Synechocystis*.

6. To investigate the effect of moderate salt stress on $^1O_2$-dependent photodamage in *Synechocystis* mutants, which lack the Scp ABCDE small Chl binding proteins.
7. To develop a method for the detection of $^1O_2$ in the dinoflagellate *Symbiodinium* and to study the correlation of inhibition of Calvin-Benson cycle by GA and KCN on the extent of photodamage under heat and light stress.
8. To investigate the effect of CAP-induced $O_2^-$ production on the rate of photodamage in intact cyanobacterial cells.

## MATERIALS AND METHODS

WT and mutant *Synechocystis* cells were cultured in BG-11 growth medium in a rotary shaker at 30 °C under a 3% $CO_2$-enriched atmosphere. The light intensity during growth was set to 40 µmole photons $m^{-2}s^{-1}$. Cells in the exponential growth phase ($A_{580}$ of 0.8-1) were used. The Chl concentration was determined by a UV-1601 (SHIMADZU) spectrophotometer after extracting the pigments with 100% methanol. Cells were harvested by centrifugation at 6500 g for 5 min and re-suspended in 100 mL of fresh BG-11 medium at concentration of 5 µg of Chl per $mL^{-1}$. The cells were kept under normal conditions for one hour before measurements. The CS-156 S*ymbiodinium* cells were grown in artificial seawater at 26 °C, and growth light intensity was 20 µmole photons $m^{-2}s^{-1}$. Measurements were performed in cultures with approximately 10 µg Chl/mL.

$^1O_2$ production in intact cells was detected by measuring the rate of light induced oxygen uptake in the presence of 5 mM His. *Synechocystis* cells were centrifuged and re-suspended in fresh BG-11 medium before $O_2$ uptake measurements, which were performed by using a Hansatech DW2 $O_2$ electrode in the absence of artificial electron acceptors at 2300 µmole photons $m^{-2}\ s^{-1}$ light intensity. Oxygen evolution was measured in the presence and absence of 5 mM His and the difference between the oxygen rates was calculated. The rate of His-mediated oxygen uptake was used as a measure of $^1O_2$ production.

For photoinhibitory treatment of *Synechocystis* wild-type and mutants, the cell suspensions were illuminated at the irradiation of 500 µmole photons $m^{-2}\ s^{-1}$ in the presence of the protein synthesis inhibitors lincomycin (300 µg/ml) or chloramphenicol (200 µg/ml), which block the protein synthesis dependent repair of PSII and allows determination of the rate of

photodamage. Oxygen evolution was measured in the presence of 0.5 mM DMBQ, as an artificial electron acceptor. Cell suspension of 2 mL at 5 µg Chl/mL was used in each measurement, and three replicates were measured. Flash-induced increase and the subsequent decay of Chl fluorescence yield were measured by a double-modulation fluorometer (PSI Instruments, Brno) in the 150 µs to 100 s time range. The sample concentration was 5 µg Chl/mL.

Singlet Oxygen Sensor Green (SOSG) reagent was used for $^1O_2$ imaging in cultured *Symbiodinium* Cells. SOSG was added to a culture of re-suspended *Symbiodinium* cells and imaging was performed by a confocal laser scanning microscope using excitation at 504 nm and emission detection at 525 nm. The images were analyzed by using computer software from Leica. A Fluorescence Stereo Microscope (Leica Microsystem) was used for detection of $^1O_2$ production in cells free medium by using SOSG imaging.

## RESULTS

### Development of method for the detection of $^1O_2$ production in *Synechocystis* sp. PCC 6803

1) We have developed a method for $^1O_2$ detection in intact cyanobacteria by His-mediated chemical trapping (Rehman *et al*. 2013). The method is based on chemical trapping of $^1O_2$ by His, which leads to $O_2$ uptake during illumination that can be detected and quantified by commercial oxygen electrodes. We have observed oxygen uptake in intact *Synechocystis* cells, during illumination in the presence of His. The His-mediated oxygen uptake effect was enhanced when the water content of the BG-11 culture medium was partly replaced with $D_2O$, which enhances the lifetime of $^1O_2$ and therefore facilitates its interaction with His. In contrast, the His-mediated oxygen uptake effect was suppressed by $NaN_3$, which is $^1O_2$ quencher. Due to the limited mobility of $^1O_2$ these data demonstrate that exogenous His reaches close to the vicinity of $^1O_2$ production sites inside the cells, oxidized by $^1O_2$ and also that the His-mediated oxygen uptake assay can be used for $^1O_2$ detection in intact cells.

2) In a previous study the mechanism of charge recombination in PSII was investigated by using flash induced Chl fluorescence and thermoluminescence measurements in the D1-Q130E and D1-Q130L mutants of *Synechocystis* 6803 showing an accelerated non-

radiative recombination patway in the D1-Q130E mutant. We have found that this regulatory mechanism provides photo-tolerance in the Q130E mutant, which prevents the formation of triplet $^3$P680 and $^1O_2$. Our results suggest that the differential photodamage of Q130E, wild type, and Q130L is correlated with the extent of $^1O_2$ production in these strains (Rehman *et al.* 2013). We investigated the roles of $^1O_2$ production with relation to the mechanisms of PSII photodamage and photoprotection in cyanobacteria and dinoflagellate by employing His-mediated oxygen uptake method.

**Applications of His-mediated $O_2$ uptake method *in Synechocystis mutants***

3) The His-mediated $O_2$ uptake method was used to investigate the roles of oxidative stress and photoinhibition in high light acclimation using a regulatory mutant of the cyanobacterium *Synechocystis*. The mutant strain *ΔsigCDE* contains the stress responsive SigB as the only functional group 2 σ factor. We observed that ΔsigCDE suffers from oxidative stress in standard conditions. ΔsigCDE has high carotenoid and Flv4-2 contents and protected PSII against light induced damage, which is accompanied by more efficient $^1O_2$ quenching than in the control strain. Therefore, the decreased extent of photodamage in *ΔsigCDE* mutant is correlated with suppressed rate of $^1O_2$ production (Hakkila *et al.* 2014).
4) We have employed the His-mediated $O_2$ uptake method to characterize the properties of a secondary metabolite of *Nostoc* XPORK14A denoted as M22. This compound induced retardation of cell growth and inhibition of photosynthesis in *Synechocystis* cells, both in light and in darkness. We showed that M22 generates $^1O_2$ in the presence of light, which in turn affects the photosynthetic machinery of *Synechocystis* (Shunmugam *et al*. 2013).

5) The $^1O_2$ detection method was also used to study the role of flavodiiron proteins (FDPs) in PSII photoprotection. FDPs are specific electron transfer enzymes present mainly in anaerobic bacteria, and they are found also in cyanobacteria. Cyanobacterial FDPs constitute a specific group that evolved differently to cooperate with oxygenic photosynthesis. In *Synechocystis* there are four FDPs, Flv1-Flv4. Two of them, Flv2 and Flv4, are encoded by an operon together with the Sll0218 protein. We have shown that $^1O_2$ production is regulated by the amount of Flv2-4, and also that photodamage was correlated with the rate of $^1O_2$ production (Bersanini *et al*. 2014).

6) The His-mediated $O_2$ uptake method was used to understand the role of the orange carotenoid protein (OCP) in photoprotection of *Synechocystis*. The photoactive OCP has been shown to be essential in photoprotection as energy quencher. When OCP is photoactivated by strong blue-green light, it is able to dissipate excess energy as heat by interacting with phycobilisomes. We demonstrated that OCP protects *Synechocystis* cells from photodamage also by red light. We showed that this photoprotection is related to a decrease of $^1O_2$ concentration due to a scavenging action of OCP. Our results also showed that an OCP overexpressing mutant has higher resistance to photoinhibition and exhibits a decreased production of $^1O_2$. Altogether, the results showed that the OCP in *Synechocystis* is an efficient $^1O_2$ quencher and contributes to photoprotection (Sedoud *et al.* 2014).

7) The His-mediated $O_2$ uptake method was employed to investigate the growth and photosynthetic activity of PSI-less and PSI-less/ScpABCDE$^-$ strains of *Synechocystis* under moderate salt stress conditions. Small CAB-like proteins (SCPs) are single-helix light-harvesting-like proteins found in all organisms performing oxygenic photosynthesis. We observed that the addition of 0.2 M NaCl to the growth medium in the PSI-less/ScpABCDE$^-$ mutant strain decreased $^1O_2$ formation and induced resistance against photoinhibition compared to control strain (Tibiletti *et al*. 2016).

**Applications of the His-mediated $O_2$ uptake method in dinoflagellate cells**

8) The His-mediated $O_2$ uptake method was also employed with cultured *Symbiodinium* cells. We observed oxygen uptake in intact *Symbiodinium* cells and intact corals during illumination in the presence of His, which demonstrated the production of $^1O_2$. We observed that the production of $^1O_2$ *in Symbiodinium* cells was enhanced during thermal and light stress conditions, which was accompanied with photo-inactivation of PSII and $^1O_2$ production, while His itself provides protection against PSII photoinactivation and pigment bleaching. Therefore, the inactivation of PSII and enhanced production of $^1O_2$ by heat and light stresses promotes bleaching events in cultured *Symbiodinium* cells. We propose that $^1O_2$ induced inactivation of *Symbiodinium* cells may be involved in triggering the expulsion of *Symbiodinium* cells from the coral host, which leads to coral bleaching (Rehman *et al.* 2016c).

9) We have also investigated the inhibition of the Calvin-Benson cycle by glycolaldehyde (GA) and potassium cyanide (KCN) in cultured *Symbiodinium* cells to understand the role of Calvin-Benson cycle inhibition in coral bleaching. We showed that the inhibition of the Calvin-Benson cycle by GA did not induce coral bleaching without thermal stress, while KCN induce coral bleaching even without thermal stress and promote $^1O_2$ formation (Hill *et al.* 2014).

10) We have also detected $^1O_2$ production in cell free culture medium of *Symbiodinium* by His-mediated chemical trapping, demonstrating the excretion of $^1O_2$ sensitizing metabolite(s) from *Symbiodinium* cells (Rehman *et al*. 2016c).

**CAP mediated damage of Photosystem II via superoxide production in *Synechocystis* cells**

11) We tested the effect of CAP on the rate of photodamage in intact cyanobacterial cells. We have shown that ΔOCP is more sensitive to high intensities of white light than wild type *Synechocystis* in the presence of lincomycin. However, in the presence of chloramphenicol the wild type and ΔOCP show no differences in light sensitivity, which indicate that chloramphenicol induced PSII photoinhibiton by additional damage via the production of superoxide in intact *Synechocystis* cells (Rehman *et al.* 2016a).

His-mediated chemical trapping is a convenient and useful method for the detection of $^1O_2$ production *in vivo.* It has already been successfully applied in different cyanobacterial strains and dinoflagellate cells to characterize the intracellular production of $^1O_2$, and expected to find useful applications in other systems, as well.

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**LIST OF PUBLICATIONS (MTMT: 10037725)**

**Peer-reviewed publication, for PhD thesis:**

- **Rehman, AU., Cser, K., Sass, L. and Vass, I.** (2013) Characterization of singlet oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium *Synechocystis* PCC 6803 by histidine-mediated chemical trapping. Biochim Biophys Acta, 1827: 689-698. **IF:4.66**

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potential to interact with the animal host in coral symbiosis (Accepted in New Phytologist). **IF: 7.67**

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2. **Vajravel, S., Kovács, L., Kis, M., Rehman, AU., Vass, I., Gombos, Z. and Toth, T.** (2016) β-carotene influences the phycobilisome antenna of cyanobacterium Synechocystis sp. PCC 6803. Photosynth Res (in press). **IF: 3.5**

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i. **Rehman, AU., Kodru, S. and Vass, I.** (2016) Chloramphenicol induces photodamage of PII via superoxide production in intact *Synechocystis* cells (for *BBA*).

ii. **Tibiletti,T., Rehman, AU., Vass,I. and Funk, C.,** (2015) Salt stress reveals a function in energy dissipation of the cyanabacterial smal CAB like proteins. (for *BBA*).

iii. **Rehman, AU., Deák, Z. and Vass, I.** (2014) Characterization of Superoxide and singlet oxygen production in isolated PSII membrane particles (for *BBA*).

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2. Kodru S, **Rehman AU** and Vass I (2015) How does chloramphenicol affect PSII photoinhibition in isolated spinach thylakoid membranes? abstract of the Straub conference, 3 June- 4 June 2015, Biological Research Center, Szeged, Hungary.

3. Vajravel S, Kis M, **Rehman AU**, Kodru S, Vass I, Gombos Z and Tóth T (2015)Does ß-carotene stabilize light harvesting antenna complexes of Synechocystis PCC 6803? abstract of the Straub conference, 3 June- 4 June 2015, Biological Research Center, Szeged, Hungary

4. **Rehman AU**, Deák Z, Larkum A, Ralph P and Vass I (2014) Production of singlet oxygen in cultured *Symbiodinium* cells, the photosyenthetic partner of the coral *Pocillopora damicornis.* Abstract of theInternational conferenceon Photosynthesis Research for Sustainability, 2 June- 7 June 2014, Pushchino, Russia.

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- ✓ **Rehman AU**, Sass L and Vass I (2015). Detection of singlet oxygen production in intact microalgae. Abstract of oral presentation in a Straub conference.3 June- 4 June 2015, Biological Research Center, Szeged, Hungary

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- ✓ **Rehman AU** (2012) Singlet oxygen production in microalgae and its relation to PSII photodamage. Abstract of oral presentation in a Harvest network meeting, 06-08 June, 2012, Kronlund, Sweden.

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