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Faculty of Science

Jihočeská univerzita v Českých Budějovicích
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Characterization of fucoxanthin triplet-state in pyridine and photoprotection in the LHC of *Aureococcus anophagefferens*

Bachelor Thesis

Marco Trathnigg

Supervisor: RNDr. David Bína, Ph.D.

Co-Supervisor: RNDr. Radek Litvín, Ph.D.

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Annotation

Spectroscopic properties of the triplet state of the carotenoid fucoxanthin were studied in pyridine, thylakoid membranes and isolated light-harvesting complex of the alga *Aureococcus anophagefferens.* Extinction coefficient of fucoxanthin triplet absorption, and rate constant of triplet transfer from chlorophyll a to fucoxanthin in solution were estimated.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 07.08.2018

Marco Trathnigg

Acknowledgements

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Secondly, I would also like to thank RNDr. Radek Litvín for giving me a hearty welcome and getting me started in the lab. Without his presentation who knows where I might have ended up? I also have to thank both my supervisors again for ensuring my time spent working in the lab was accompanied with friendly conversation and exchange.

Finally, I would like to thank all those who put up with me during the writing of this thesis. Thank you.

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1 Preface

Photosynthesis is the process in which plants and algae capture the photons of sunlight and convert them into a wide variety of organic molecules. These molecules serve as the foundation of all living organisms on earth. Current estimates say that photosynthesis produces more than 100 billion tons of dry biomass every year (Barber, 2009). Due to the abundance of the necessary starting materials for photosynthesis in the form of sunlight, water and carbon dioxide this process has taken a most central role in the development of the earth. In the centre of the conversion stands the splitting of a water molecule into oxygen and 'hydrogen'. This can be seen as the reverse of respiration which goal is to carefully combine oxygen and hydrogen in order to generate metabolic energy (Barber, 2009). The summary equation of oxygenic photosynthesis can often be found in the following form[1]:

$$6\,CO_2 + 6\,H_2O \xrightarrow[\text{Chlorophyll}]{\text{Light}} C_6H_{12}O_6 + 6O_2$$

While it correctly describes the overall chemical process (Blankenship, 2014) it leaves the impression that the chemistry somehow occurs in illuminated chlorophyll solutions. Nothing could be further from the truth: illuminated chlorophyll solution under aerobic conditions is a generator of reactive singlet oxygen (Krieger-Liszkay, 2004), hence, any cell that would fill itself with chlorophyll and expose itself to light would ensure its quick demise.

To avoid this fate, all oxygenic phototrophs pair chlorophylls with carotenoids (Frank, et al., 2000) which are capable of preventing production of reactive oxygen species. This photoprotective role of carotenoid forms the topic of this thesis.

[1] https://pmgbiology.com/2014/12/03/factors-affecting-rates-of-photosynthesis-a-understanding-for-igcse-biology-part-1/, 07.08.2018, 16:32; http://biology-igcse.weebly.com/the-equation-for-photosynthesis.html, 07.08.2018, 16:38.

2 Introduction

2.1 Where does photosynthesis happen?

Between all organisms employing photosynthesis as a means of energy generation, a distinction can be made between prokaryotic organisms, bacteria, and eukaryotic organisms, plants and algae.

Photosynthetic bacteria can be divided into two groups, anoxygenic and oxygenic phototrophs. The former group is characterized by a simpler photosynthetic apparatus based on bacteriochlorophylls, cyclic tetrapyrroles of the bacteriochlorin group. At present, the known anoxygenic bacteria comprise six groups: purple bacteria, green sulphur bacteria, filamentous anoxygenic bacteria, heliobacteria, chloracidobacteria and gemmatimonadetes (Satoshi, 2016; Zeng, et al., 2014). Anoxygenic photosynthesis represents the ancient form of the light-driven metabolism, with geological record of anoxygenic phototrophs dating back ~3400 millions of years (Myrs, Hofmann, et al., 1999; Schopf, et al., 2017)

Oxygenic prokaryotes comprise only one group, namely the cyanobacteria. This group utilizes chlorophylls, instead of bacteriochlorophylls. Using chlorophyll allows for the development of a strong oxidizing species with a redox potential high enough to oxidize water, the reaction centre of photosystem II (Em > 1 V, Ishikita, et al., 2005). Furthermore, this allows using water as the electron donor and as a consequence oxygen is produced. Therefore, this type of photosynthesis is of great importance to all oxygen breathing species, such as us, humans. Oxygenic photosynthesis is attested in the geological record at least ~ 2400 Myr ago in a global rise of oxygen levels, the Great Oxidation Event (Farquhar, et al., 2011), although whether this event coincided with evolution of oxygenic cyanobacteria or this group is even older remains an open question (Soo, et al., 2017; Grettenberger, et al., 2018).

Photosynthetic eukaryotes appeared as a result of an endosymbiotic event, in which the ancestral eukaryotic host cell engulfed a cyanobacterium. Over time this cyanobacterium transformed into what is known today as the chloroplast, a cell organelle. This event occurred roughly 1.5 billion years ago (Yoon, et al., 2004). Extant descendants of this primary endosymbiotic event are plants, green algae, red algae, and the glaucophytes (Rodríguez-Ezpeleta, et al., 2005). More recently, some of these primary endosymbionts engaged in additional events that generated a wide variety of endosymbionts of higher order. Of these, the group harbouring plastids of red-algal origin is of particular importance, since it includes organisms with major impact on global carbon cycles such as diatoms and haptophytes, as well as species of economic

importance, such as dinoflagellates or pelagophytes causing harmful algal blooms (Sieburth, et al., 1988; Field, et al., 1998; Holligan, et al., 1993)

2.2 Taking a closer look at chloroplasts

As previously mentioned, the chloroplast is a cell organelle responsible for photosynthesis in plants and algae. Inside the chloroplast, we find the important membrane compartments known as thylakoids. It is the membrane of these thylakoids that hosts the protein complexes involved in photosynthetic energy conversion. These complexes can be divided into four main types. First, there are simply the light-harvesting complexes which collect light energy and transfer it towards the second type, the reaction centre (RC). There the energy is utilized to drive the charge separation, initiated by excitation of a chlorophyll molecule, the primary donor. The third type comprises the cytochrome b6/f complex which performs electron transfer reactions coupled to proton translocation across the membrane. This results in a proton gradient across the membrane that is used by ATP-synthase, the fourth type, to generate ATP. Electron transfer between reaction centres and cytochrome complexes is mediated by diffusible carriers, quinones and plastocyanin. Since the focus of the present work is on the functions of light-harvesting complexes, also called antennas, only these will be treated in more detail in the following sections.

2.3 The role of pigments

'Pigment' commonly refers to a substance capable of selective colour absorption, resulting in the substance having a visible colour. Photosynthetic pigments are pigments that drive the process of photosynthesis by capturing the necessary energy in the form of light. Photosynthetic pigments differ among each other in the wavelength range in which they absorb best. This means that photons of a certain energy will have a higher chance of being absorbed. Therefore, different photosynthetic organisms employ different photosynthetic pigments based on their surroundings and needs (Chen & Blankenship, 2011).

Pigments involved in photosynthetic energy conversion belong to two classes: tetrapyrroles and carotenoids. Tetrapyrroles are further divided into linear forms (bilins) and cyclic forms ((bacterio-)chlorophylls)). Linear tetrapyrroles are limited to cyanobacteria and some more primitive eukaryotes, such as red algae (Green & Parson, 2003). Cyclic tetrapyrroles comprise the different kinds of chlorophylls, all sharing porphyrin as their main body structure with an additional ring attached to one of the pyrroles as can be seen in Figure 1. Chlorophylls differ in the side-chains attached to the main body of the molecule and are distinguished by adding a lowercase letter as suffix, e.g. chlorophyll a, chlorophyll b, and so on.

Carotenoids are linear molecules whose main structural feature is a chain of alternating single and double carbon-carbon bonds. Carotenoids are differentiated by their molecular structure. Fucoxanthin, the carotenoid of interest in this work (Figure 1) contains oxygen and is therefore classified as a xanthophyll. Carotenes on the other hand are pure hydrocarbons.

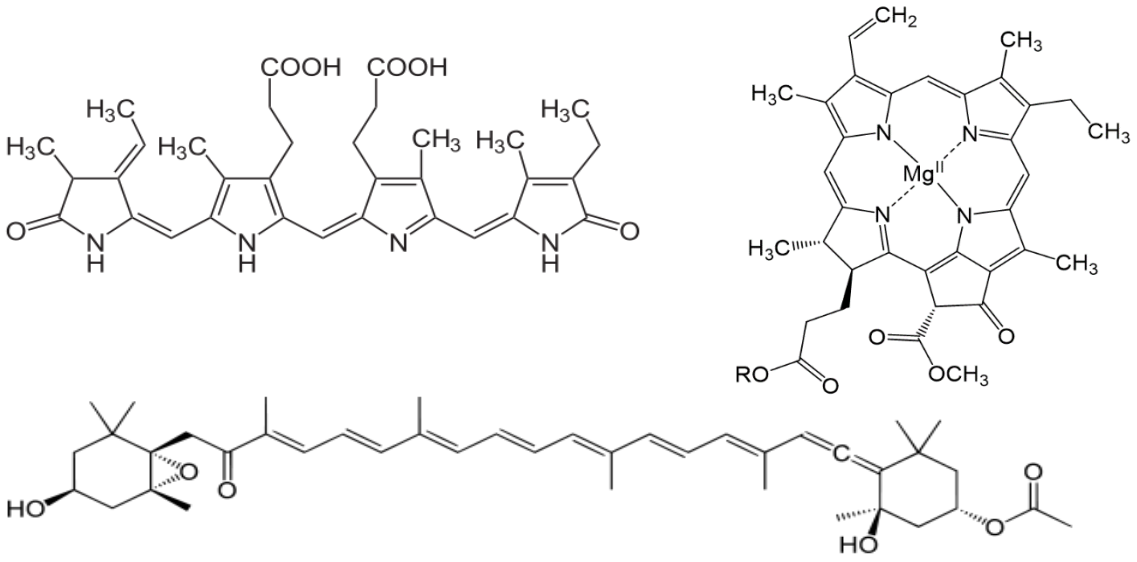


Figure 1 Phycocyanobilin (top left) a linear tetrapyrrole and chlorophyll a (top right) a cyclic tetrapyrrole. Fucoxanthin (bottom) a xanthophyll and the carotenoid of interest in this work.

2.4 Antenna complexes

The roots of the concept of a light-harvesting complex, or antenna, can be traced back to the experiments of (Emerson and Arnold (1932) as described in Blankenship, 2014) who observed that only one O_2 molecule was produced per ~2500 molecules of chlorophyll. Thus, it became apparent that the majority of the pigments present does not perform photochemistry but only captures light. The need for this can be rationalized by realizing that sunlight is a very dilute energy source: a single chlorophyll molecule very roughly absorbs only one photon every tenth of a second at the maximum of solar irradiance (Blankenship, 2014). On a molecular scale this timeframe would be much too long to sustain metabolism. Instead, pigments and proteins come together to form antenna complexes. If one of the pigments in these complexes absorbs light the energy is transferred to neighbouring pigments until it reaches a trap, the reaction centre in most cases. There the excited state is quenched by photochemistry with energy storage. The funnel concept provides an intuitive image to visualize how energy collection in antenna systems works (Figure 2).

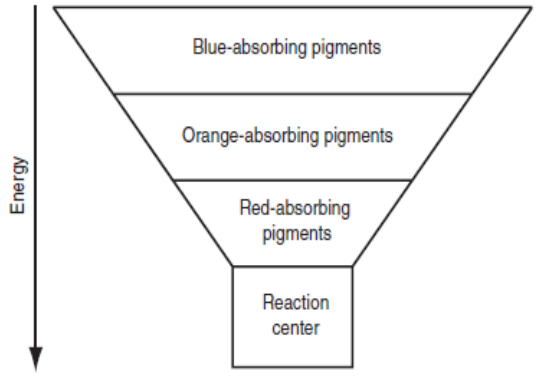


Figure 2 Visualization of the funnel concept (Blankenship, 2014).

Red absorbing pigments are placed in closer vicinity of the reaction centre compared to blue, and therefore higher energy, absorbing pigments. Since a bit of energy is lost with every transfer in the form of heat, the higher energy farther away results in a more stable energy supply. Since some degree of energy is lost with every step, this process tends to be irreversible (Blankenship, 2014). Contrary to reaction centre complexes, different antenna complexes can be found in remarkable diversity in nature. This could lead to the conclusion that these complexes were invented multiple times during the course of evolution. Antenna complexes can be divided into

integral and extrinsic antennas. Integral antennas have proteins that span the lipid bilayer with the pigments often being buried deep inside the membrane. Extrinsic antennas on the other hand are associated with components in the membrane but do not span it (Blankenship, 2014). An antenna complex consists of the protein part that serves as a scaffold holding pigment molecules in place. This is done in a very precise manner to ensure specific distances between pigments and mutual orientation that is essential for proper functioning. In eukaryotic organisms, the light-harvesting function is performed by arrays of proteins of the LHC family. The most studied of these is the LHCII antenna of plants (Figure 3).

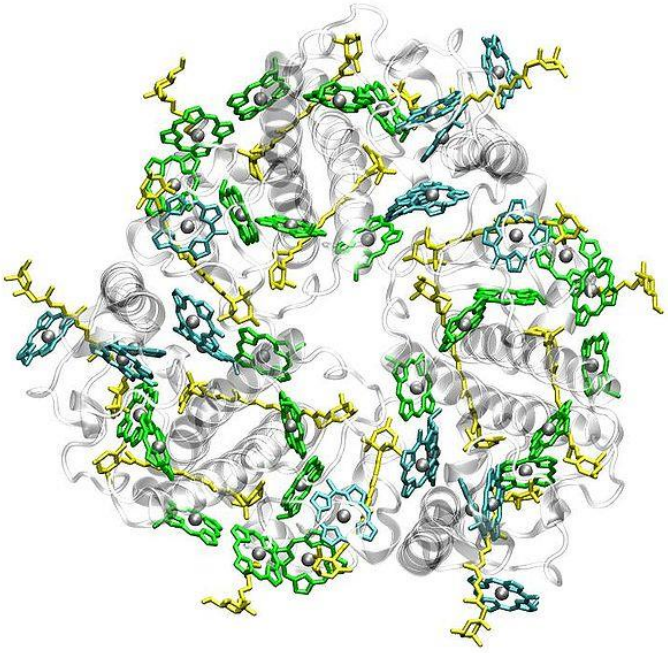


Figure 3 Structure of the light-harvesting complex (LHC II) of higher plants. Green structures are chlorophyll a, while blue structures are chlorophyll c. Yellow molecules are carotenoids.
By aegon – Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=1036077.

The structural blueprint of LHC proteins consists of three transmembrane helices connected with extramembrane loops. The LHCII of plants bind eight Chl a, six Chl b and four carotenoids (Standfuss, et al., 2005; Liu, et al., 2004). Pigment composition of LHC varies between organisms (Green & Parson, 2003), but it is always a combination of chlorophylls and carotenoids, with chlorophylls being the main light-harvesting pigments. Carotenoids, on the other hand, serve several purposes: Firstly, they aid in collection of light energy by absorbing in a range where chlorophylls do not (450-500 nm) and secondly, they also perform a photoprotective function (Blankenship, 2014).

2.5 The drawbacks of oxygen as electron donor

An environment with oxygen has certain risks attached to it that are due to the oxygen itself. While oxygen in its ground state is not much of a threat, this is not true for its intermediates such as peroxide, superoxide and hydroxyl radicals. In its relatively stable ground state oxygen (O_2) exists in a triplet state. This means that both unpaired electrons are occupying different anti-bonding (π^*) orbitals while having the same spin quantum number instead of following Hund's rule. In this form reactions are kinetically slowed due to spin restriction. By an input of energy, the very reactive singlet oxygen ($^1O_2$) can be created. Without the spin restriction the reactivity of the singlet oxygen is greatly increased. In plants, singlet oxygen is most prominently produced by the chlorophylls, acting as photosensitizers. On one hand chlorophylls are indispensable for photosynthesis but on the other hand they also bear the risk of generating reactive oxygen species (ROS) and singlet oxygen species. When the excitation energy of a chlorophyll molecule is not efficiently used, the spins of the electrons in the excited state can change to result in a lower energy excited state, known as the chlorophyll triplet state. This triplet state has quite a long lifetime of a few µs under oxygen saturated conditions and can react with (triplet) oxygen $^3O_2$ to produce the highly reactive singlet oxygen ($^1O_2$) (Krieger-Liszkay, 2004).

2.5.1. Photoprotection by carotenoids

Carotenoids are pigments that fulfil two very important roles in photosynthetic systems. Firstly, they absorb at different wavelengths than chlorophyll, broadening the range at which light can be harvested and secondly, they act in a process termed photoprotection. By rapidly quenching the triplet states of chlorophyll that are generated during photosynthesis, the carotenoids lessen the chance of oxygen and triplet chlorophyll reacting (Blankenship, 2014). This short-range energy process is called the Dexter energy transfer[2] (see Figure 4). An excited electron from a donor (^{3}Chl) is transferred to an acceptor (^{1}Car) by a non-radiative pathway. For this mechanism to occur both donor and acceptor have to be in close proximity, usually within 10 Å.

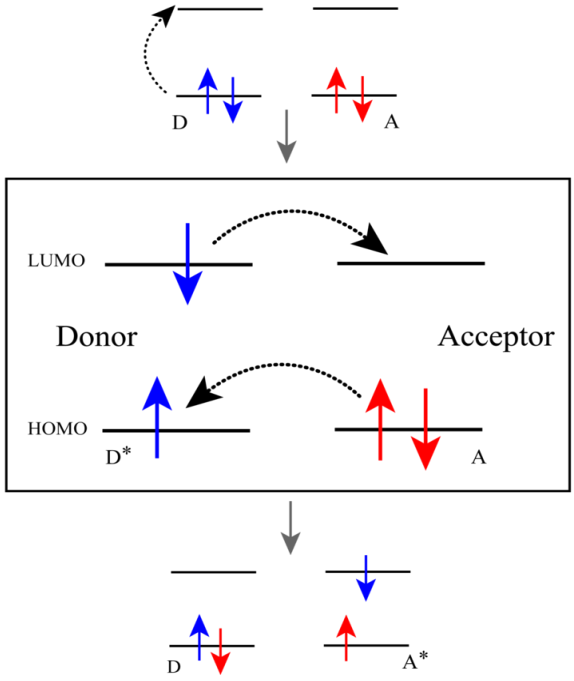


Figure 4: General visualization of Dexter energy transfer mechanism.
By Sobarwiki - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=34003111.

[2] IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http://goldbook.iupac.org (2006-) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook.D01654.

2.6 Methods to study triplet states

Studying the triplet state is efficiently performed using optical spectroscopy, more specifically absorption spectroscopy. In this spectroscopic technique the absorption of radiation is measured as a function of frequency or wavelength. The absorption spectrum of a given compound is the plot of attenuation of light as a function of wavelength. It allows for identification of the chemical species present and its concentration, using the Lambert-Beer law:

$$A = \varepsilon * c * d$$

Where A is the absorbance of a given compound, ε is its extinction coefficient, c is the concentration, and d is the optical pathlength.

This measuring technique can further be extended to allow for the observation of the time dependent development of chemical reactions. This method is called flash photolysis. A schematic overview of such a setup can be seen in Figure 5. A strong, so-called pump pulse is used to excite the sample and to populate higher energy levels. In this case the goal is to produce chlorophylls in the triplet state. The absorption of light of the sample is then recorded in short time intervals by a series of weaker, so-called probe pulses. The triplet state, being a different chemical species, absorbs light in a different manner than the original molecule in its singlet state, hence two effects can be observed: light-induced decrease in absorption of the singlet state of the parent compound, and the appearance of new absorption bands of the triplet state. With increasing delay after the pump pulse, the relaxation of the triplet state occurs, and the lifetime of the triplet state can be estimated. The factor complicating the analysis of this process in absorption changes, is the finite length of the instrument response, meaning the duration of both the pump and measuring pulse as well as the time it takes for the electronics to respond. Thus, the absorption signal is not generated instantaneously when starting the measurement but rises gradually during the pump pulse. To illustrate this, it can be viewed as if at each point in time of the pulse duration, a decay kinetics is initiated, and the resulting kinetics is the sum of these partial decays. Mathematically this can be expressed by a convolution of the instrument response, usually assumed to be a Gaussian function, and the decay function (van Stokkum, et al., 2004). Both the duration of the pump pulse and the time resolution of the instrument thus place limits on the maximum rate of the process that can be resolved: when the decay of the excited state is very fast, the measured kinetics can simply appear to be identical to the instrument response.

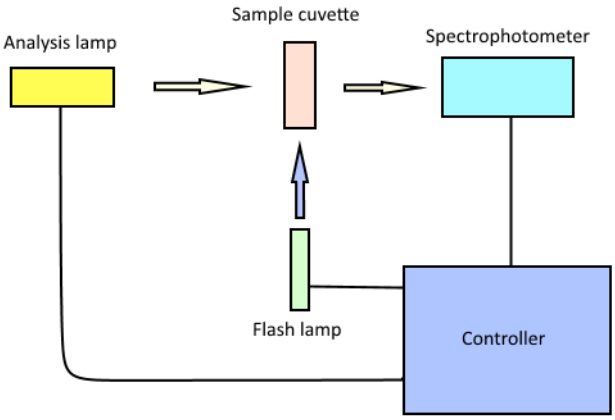


Figure 5 Schematic overview of an experimental flash photolysis setup.

2.7 Chromatography

To obtain the pigment and protein samples for the measurements and to analyse the pigment composition of the LHC sample, High Performance Liquid Chromatography (HPLC) was used. Under increased pressure a liquid solvent containing the sample mixture is pumped through a tight column made of an adsorbing material. Due to different interactions of the components of the sample mixture with the adsorbent material the flow rates of these components also differ. This leads to a separation of the components after flowing through the column.

Protein samples were prepared by combination of sucrose gradient and ion-exchange chromatography. This was done by my supervisor as part of another work and is not within the scope of this work.

3 Aims of thesis

One purpose of this bachelor thesis is to study the triplet photoprotection of chlorophyll in the antenna complex of the alga *Aureococcus anophagefferens*. This alga is a pelagophyte with the potential of causing harmful brown tide blooms (Sieburth, et al., 1988). The genome of *Aureococcus anophagefferens* includes all genes involved in photosynthesis, of which 62 genes encode light-harvesting complex (LHC) proteins, 1.5-3 times as many as other eukaryotic phytoplankton sequenced until 2011 (Gobler, et al., 2011). The pigment composition of this organism is complex, containing chlorophyll a and two types of chlorophyll c (c_2 and c_3). Carotenoids are represented mainly by fucoxanthin (fux), the 19'-butanoyl ester of fucoxanthin, diadinoxanthin and β-carotene (Alami, et al., 2012). The unique pigment composition makes *Aureococcus anophagefferens* a highly interesting specimen for this investigation. Given that *Aureococcus anophagefferens* is a heterokont alga and the presence of fucoxanthin (fux), the LHC complex of this organism can be classified as a form of fucoxanthin-chlorophyll-protein (FCP).

With a number of different carotenoids, the question which one performs photoprotection in *Aureococcus anophagefferens* is open, although fucoxanthin is the most likely candidate. To summarise the aims of this thesis: i) analysing the triplet quenching in the isolated LHC, ii) comparing the carotenoid triplet signal in isolated antenna and the intact thylakoid membrane and relating these to optical properties of fucoxanthin triplet in solution (iii).

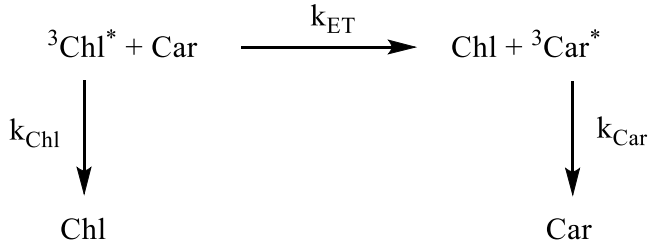
4 Material and Methods

4.1 Sample preparation

Photosynthetic pigments were extracted from organisms into organic solvents in the following fashion: The cells were collected via centrifugation and after the centrifugation was finished, the medium was discarded while the cell pellet was dissolved in methanol. By sonication of this suspension the cells were broken apart and centrifuged again. The resulting supernatant, which contained the pigments, was retained and the pellet was dissolved again. Then the process was repeated. For the third extraction the methanol was replaced with acetone. After the third extraction the pigment solution was dried, and the now colourless cells were discarded. The obtained pigments were analysed and purified using HPLC. The used system consisted of a Pump Controller Delta 600, a manual injection system and a PDA 2996 detector (Waters, USA). A reverse-phase Zorbax SB-C18 column (4.6 9 150 mm, 5 µm, silica-based, non-end capped; Agilent, USA) was used to separate the pigments. A linear gradient elution was used with a ternary solvent system of the following composition: solvent A (80:20 methanol:0.5 M ammonium acetate (aq., pH 7.2 v/v)), solvent B (90:10 acetonitrile:water), solvent C (100 % ethyl acetate) (Jeffrey, et al., 2005). The flow rate was 1 ml min^{-1}. The pigment molar ratios were estimated from the areas under the chromatograph peaks displayed at wavelengths corresponding to the respective extinction coefficients. The following molar extinction coefficients (in L $mmol^{-1}$ cm^{-1}) were used: Chl a: 79 at 662 nm (Litvin, et al., 2016) and fucoxanthin: 109 at 448 nm (Jeffrey, et al., 2005). Chlorophyll a was prepared from various organisms and fucoxanthin was prepared from *Aureococcus anophagefferens*. After purification the pigments were again dried and stored in the freezer at -20 °C.

4.2 Kinetic aspects of chlorophyll-carotenoid interaction

Inside the LHC carotenoids are bound closely to chlorophylls to ensure quick transfer of the triplet states. Thus, the triplet formation can be viewed as a first order reaction, depending only on the concentration of singlet-state chlorophyll. The rate of triplet-transfer is very fast in this case (Kvíčalová, et al., 2016; Khoroshyy, et al., 2018). The sample mixture behaves as if the carotenoid triplet-states are pumped directly, they are assumed to rise with the pump pulse and then decay.
Contrary to the situation in the LHC, the close contact between chlorophylls and carotenoids is not given in solution. The pigments are dispersed in the solvent and may only transfer triplet states upon entering the required range. This means the triplet formation is a second order reaction, depending on the collision of both molecules. The triplet transfer was studied using rate equations derived from the following scheme (Pendon, et al., 2006; Kaligotla, et al., 2010; Niedzwiedzki, et al., 2011):



where k_{ET} is the rate constant for the energy transfer between an excited triplet state chlorophyll a molecule (donor) and a carotenoid (acceptor). The rate constants k_{Chl} and k_{Car} represent the triplet state decay of chlorophyll a and the carotenoid respectively. The differential equation describing the change in population of chlorophyll a in triplet state is given by:

$$\frac{d[\,{}^3Chl^*]}{dt} = -k_{ET}[Car][\,{}^3Chl^*] - k_{Chl}[\,{}^3Chl^*] \quad (1)$$

Where [^{3}Chl*] is the concentration of the excited triplet states of chlorophyll a and [Car] is the concentration of carotenoids in the ground state. The solution to Eq. 1 is:

$$[\,{}^3Chl^*] = [\,{}^3Chl^*]_0 * e^{-(k_{ET}[Car]+k_{Chl})t} \quad (2)$$

Where [^{3}Chl*]$_0$ is the initial concentration of excited chlorophyll a right after the photoexcitation. The differential equation describing the change in population of carotenoid in triplet state is given by:

$$\frac{d[\,^3Car^*]}{dt} = k_{ET}[Car][\,^3Chl^*] - k_{Car}[\,^3Car^*] \quad (3)$$

Where [^{3}Car*] is the concentration of carotenoid in triplet state. The solution to Eq. 3 is then given by:

$$[\,^3Car^*] = \frac{k_{ET}[Car][\,^3Chl^*]_0}{k_{Car}-k_{ET}[Car]-k_{Chl}}\left[e^{-(k_{ET}[Car]+k_{Chl})t} - e^{-k_{Car}t}\right] \quad (4)$$

It can be assumed that only a small part of the total carotenoid concentration is converted to triplet, hence [Car] can be treated as a constant which can be determined from carotenoid absorbance. [^{3}Chl*] can be obtained as the decrease of population of the singlet state using the amplitude of Q_y band bleaching. Rate constants then can be extracted from fitting of the experimental kinetics of triplet concentration by the above given equations.

4.3 Spectroscopy

To perform optical spectroscopy on the pigments they were first dissolved in pyridine (Sigma-Aldrich, p.a.) and the steady-state absorption spectra were recorded using a Shimadzu UV-2600 spectrometer. The pigment solutions containing chlorophyll a were adjusted to OD_{671} 0.3-1.0. For recording the transient absorption spectra of the prepared solutions, a locally constructed spectrometer was used, described in detail in (Bína, et al., 2006). The scheme of the instrument is shown in Figure 6. The spectrometer utilises xenon (Xe) flashlamps (pulse width ~μs) as sources of both measuring and actinic pulses (FL1 and FL2, respectively in Figure 6). The spectra are recorded using arrays of photodiodes (PDA) having 38 elements. Combined with diffraction gratings of 600 and 1800 grooves/cm the resolution is 6 or 2 nm per spectrum point. The path of the measuring beam is split into sample and reference branches using a beam splitter, hence correction of variation of spectral shape of the Xe pulses can be done for each spectrum.

Due to the small number of detector elements, it might be necessary to combine several spectra to cover the whole spectral region of interest. In that case, it is possible to change the spectral resolution of different spectrum regions to minimize the time needed to acquire the whole spectral dataset. The Xe flashlamps are broadband light sources, if a spectrally narrow pump pulse is required, appropriate bandpass filters must be used. However, the spectra presented in this work were acquired using the broadband pulses.

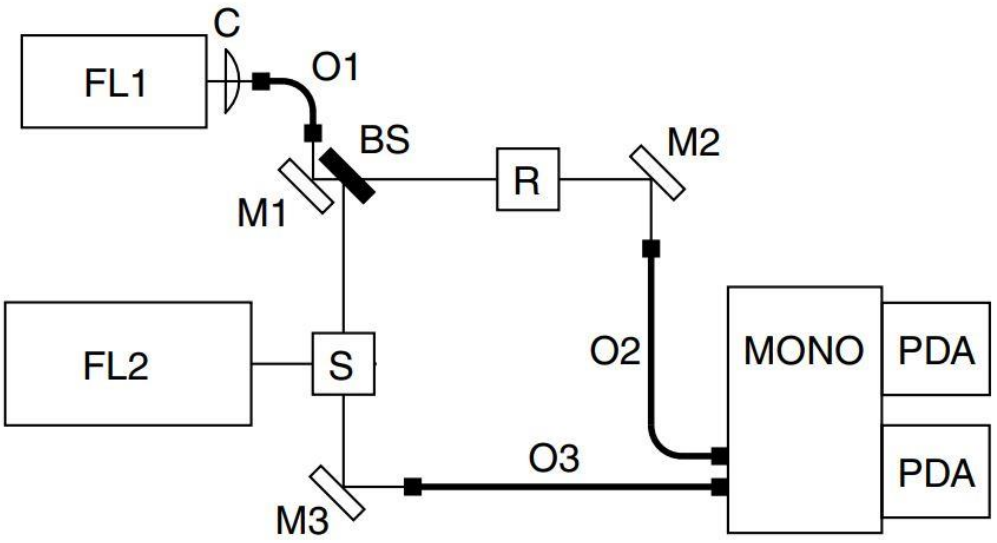


Figure 6 Scheme of the flash-photolysis instrument setup. FL1, FL2: Xenon flash lamps; M1, M2, M3: toroidal mirrors; O1: quartz rod, O2, O3: fibre optic bundles; C: condenser; BS: beam splitter; S, R: sample and reference cuvette holders; MONO: monochromator MS 247; PDA: photodiode arrays

4.4 Data processing and analysis

The analysis of the decay rates of the triplet states was performed using the Solver add-in utility of Microsoft Excel to fit the triplet decay kinetics at single wavelengths. In the case of the decay of triplet-state chlorophyll a (^{3}Chl a) in solution and kinetics of the triplet-state of carotenoid (^{3}Car) in the LHC, a single-exponential decay was assumed. To account for the finite duration of the instrument response, the kinetics were not fitted as simple decaying exponentials, but in the form of convolution of exponential decay describing the triplet kinetics and Gaussian function. Analytical solution of this convolution is given in Eq. 3 in van Stokkum et al. (2004). While the equation will not be reproduced in full here, we note that it can be expressed in a simple form as:

$$\text{Measured Kinetics} = \text{Decay}\,(k_{ET}, t) \times \text{sigmoid-function}\,(k_{ET}, t, \mu, \Delta), \quad (5)$$

where decay is in the form $\exp(-k_{ET}t)$, where k_{ET} is the rate constant (=1/time constant or lifetime) of the triplet decay and 'sigmoid' is a function that describes the rise of the signal during the pump pulse (using 'erf' function), where μ, Δ represent position and width of the instrument response, respectively.

For analysis of triplet transfer in pigment mixtures, kinetics of ^{3}Chl a were fitted in the above given form (5), and [^{3}Car] was fitted directly using Eq. 4 above and the ^{3}Chl a and ^{3}Car

kinetics were fitted simultaneously. The part of the ^{3}Car kinetics overlapping with the rise of the ^{3}Chl a signal was omitted from the analysis. Solver application was used to minimize the sum of squared differences between the computed kinetics and the measured data to obtain values of the rate constants.

For the purpose of extracting the value of the rate constant of the triplet transfer in solution, the concentration of ^{3}Chl a was computed from the amplitude of bleaching at 672 nm (Q_y maximum) using the published extinction coefficient for Chl a. Extinction coefficient of ^{3}Car at maximum was computed from the spectrum of mixture after subtraction of ^{3}Chl a contribution, normalized at Q_y, as described in Kaligotla (2011).

4.5 Nitrogen gas bubbling approach

Since both triplet-states of chlorophyll a and carotenoid interact with oxygen, their lifetimes are shorter under aerobic conditions (this effect will be projected into larger values of k_{car} and k_{Chl}), hence the triplet transfer is best studied under anaerobic conditions.

An excellent method of oxygen removal from organic solvents is by repeated cycles of freeze-thaw under vacuum as described in e.g. Niedzwiedzki & Blankenship (2010). However, since suitable equipment was unavailable, the simpler approach of bubbling the solution with N_2 gas was used, as seen in Figure 7. The gas was let into the solution through a syringe and another small syringe provided an exit for the excess gas. The cuvette was closed with a rubber stopper through which the syringes were pierced. All measurements were performed at room temperature.

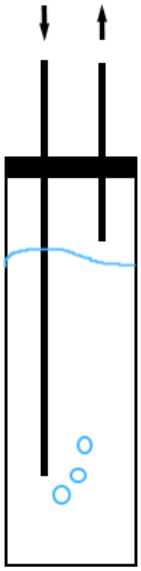


Figure 7: Schematic showing the strategy for bubbling the pigment solution. Arrows represent in- and outgoing gas.

5 Experimental and Results

5.1 Pigment composition of the Aureococcus antenna

A chromatogram (Figure 8) was recorded at 435 nm of the pigments extracted from purified LHC complex of *Aureococcus anophagefferens*. Pigments were identified using their absorption spectra given in Jeffrey et al. (2005) based on comparison to pigment analyses from this organism given by Alami et al. (2012). Pigment ratios of the major pigments were: 100 Chl a : 60 Chl c_2 : 55 Chl c_3 : 160 fux. In comparison, 19'-butanoyloxy-fux, diadinoxanthin, and β-carotene were all present in sub-stoichiometric amounts (< 5 / 100 Chl a). Total fucoxanthin was computed by pooling fux and its cis-isomer. In comparison to whole cell data from Alami et al. (2012), the composition of LHC was found to be much simpler. In particular, the absence of diadinoxanthin is striking, indicating that this carotenoid is not tightly bound to the antenna complex. However, this is similar to the situation in the diatom FCP (Herbstová, et al., 2017). Given that the amounts of carotenoids aside of fucoxanthin were negligible, this work focused on investigating fucoxanthin's triplet quenching properties.

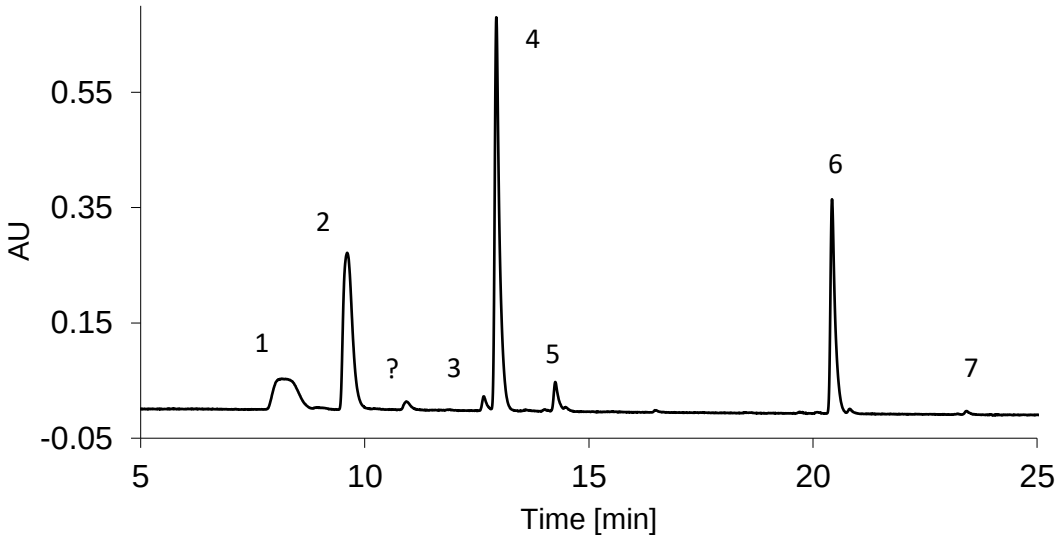


Figure 8 Chromatogram of the isolated LHC to determine its pigment composition. 1-Chlorophyll c_3; 2 - chlorophyll c_2; ? – unidentified carotenoid; 3 - 19'-butanoyloxyfucoxanthin; 4– Fucoxanthin; 5 – cis-Fucoxanthin/Diadinoxanthin; 6 – chlorophyll a; 7 – β-carotene.

5.2 Effect of N_2 gas bubbling

The bubbling experiment was performed on two different samples, both only containing chlorophyll a. The first experiment was conducted over a longer period of time, namely 93 minutes. The lifetimes showed a notable increase, especially after the 30 minutes mark (Figure 9). Figure 10 shows three different fits for the triplet-state decay with increasing bubbling time t_b. Comparing to the expected lifetimes of the Chl a triplet state under anoxic conditions, 413 ± 5 µs (Niedzwiedzki & Blankenship, 2010), the lifetime could only be increased to at most half of this by bubbling. A second sample was measured to determine how reliable the increase in lifetime was. As can be seen in Figure 9, after 30 minutes the increase in lifetime was considerably less than in the sample before. Since the observed results varied by a factor of 10, the method seemed to be unreliable and most of the experiments were conducted at atmospheric conditions.

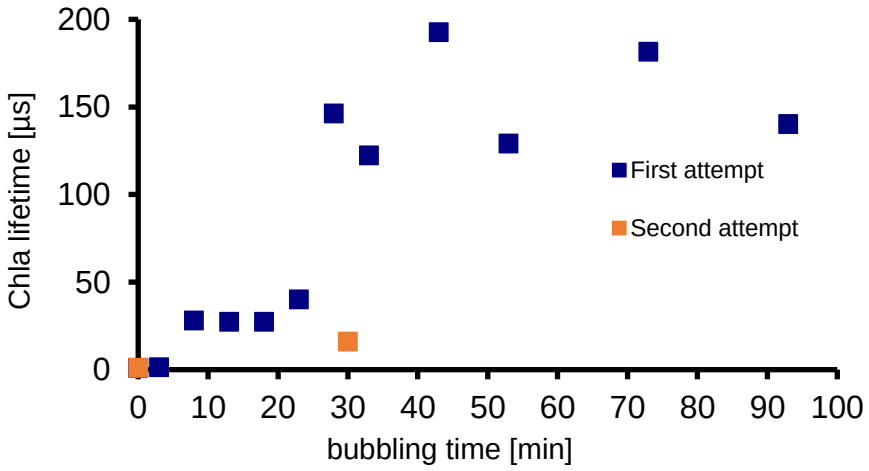


Figure 9: Extracted lifetime of Chl a triplet state vs. elapsed bubbling time t_b.

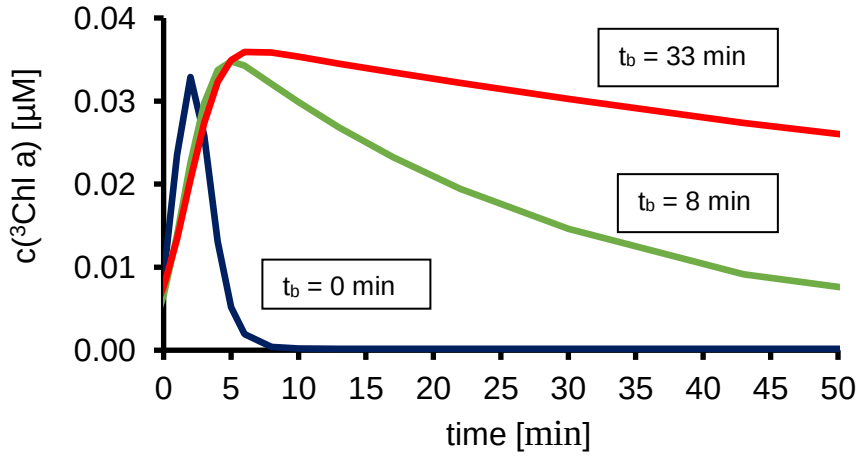


Figure 10: Prolonged triplet lifetime with increasing time of bubbling t_b. Decays were scaled to similar amplitude.

5.3 Measurements on triplet state properties

Prepared pigments were dissolved in pyridine and steady-state as well as transient absorption spectra were recorded. From the obtained spectra estimations of the extinction coefficient for the triplet absorption band and of the lifetime of the triplet states were made. Furthermore, the rate constant of triplet transfer between triplet state chlorophyll a, ^{3}Chl a, and a ground state carotenoid, ^{1}Car, were estimated.

5.4 Steady-state spectroscopy

Absorption spectra of the studied pigments in pyridine can be seen in Figure 11. Both the spectra containing chlorophyll a show the dominating contribution of its Q_y band maxima at 671 nm and Soret band maxima at 448 nm. The carotenoid absorbs strongly between 460 and 500 nm. In pyridine the carotenoid is lacking a well-resolved vibrational structure. For the mixture, the strong contribution of the carotenoid to the absorption can be seen between 470 and 490 nm, where the carotenoid absorption overlaps partly with the Soret band of chlorophyll a, whereas there is no change to the Q_y band.

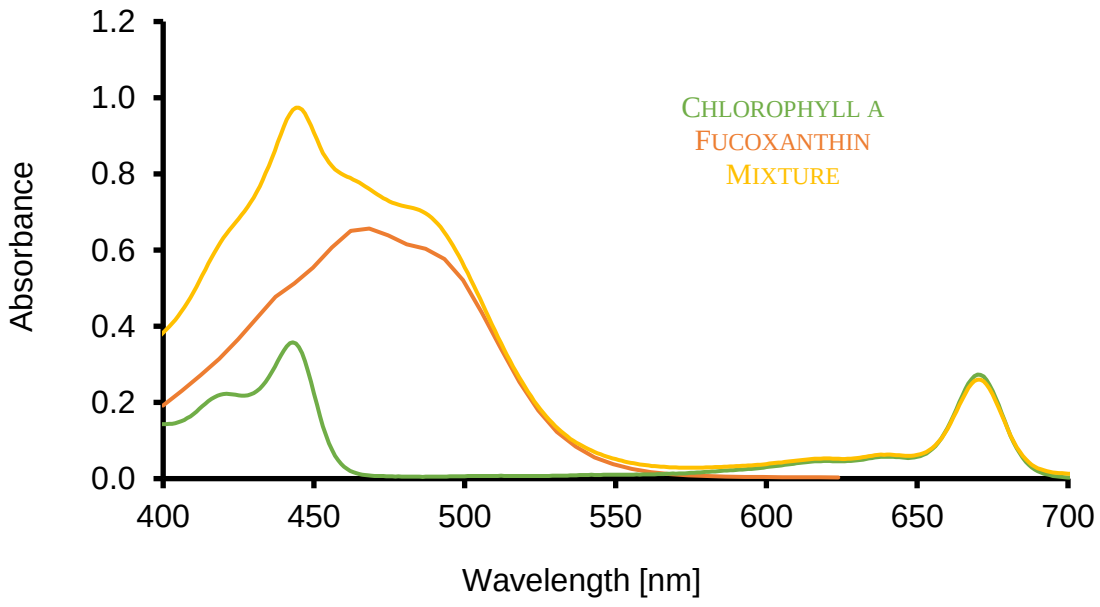


Figure 11: Steady-state absorption spectra of Chl a, Fucoxanthin and a mixture of both in pyridine.

5.5 Time-resolved spectroscopy of isolated pigments

The Triplet-minus-Singlet (TmS) absorption spectrum of chlorophyll a (Chl a) dissolved in pyridine was recorded (Figure 12, left). Corresponding to its former singlet-state absorption maxima, bleaching of these regions can be seen. Additionally, there is a new absorption band visible between 460 and 580 nm, the absorption of Chl a in triplet-state. Figure 12 also shows the decay of the triplet absorption in tandem with the decrease of the bleaching. The TmS spectrum of a mixture of Chl a and fucoxanthin (Figure 12, right) shows the aforementioned characteristics of Chl a, but in addition the carotenoid contributes in the region between 500 and 550 nm. To obtain only the TmS spectrum of fucoxanthin, the TmS spectrum of the mixture was subtracted by the TmS spectrum of pure Chl a. For this subtraction the TmS spectrum of Chl a was scaled to match the spectrum of the mixture in the Q_y band at 665 nm. The results can be seen in Figure 13. Bleaching of the ground-state carotenoid absorption can be seen between 450 and 490 nm, while triplet-state carotenoid absorption can be observed between 500 and 550 nm. Furthermore, the triplet absorption spectrum of fucoxanthin (T_1-T_N) in pyridine (Figure 14) was extracted by subtracting its ground-state absorption spectrum (S_0-S_2) from the obtained TmS spectrum of fucoxanthin (Kaligotla, 2011). From this spectrum an estimate of the extinction coefficient for triplet-state absorption of fucoxanthin was made. The calculated value for this extinction coefficient was 195 mM^{-1} cm^{-1} at 515 nm using the extinction coefficient in its ground-state at 109 mM^{-1} cm^{-1} at 462 nm (Jeffrey et al., 2005).

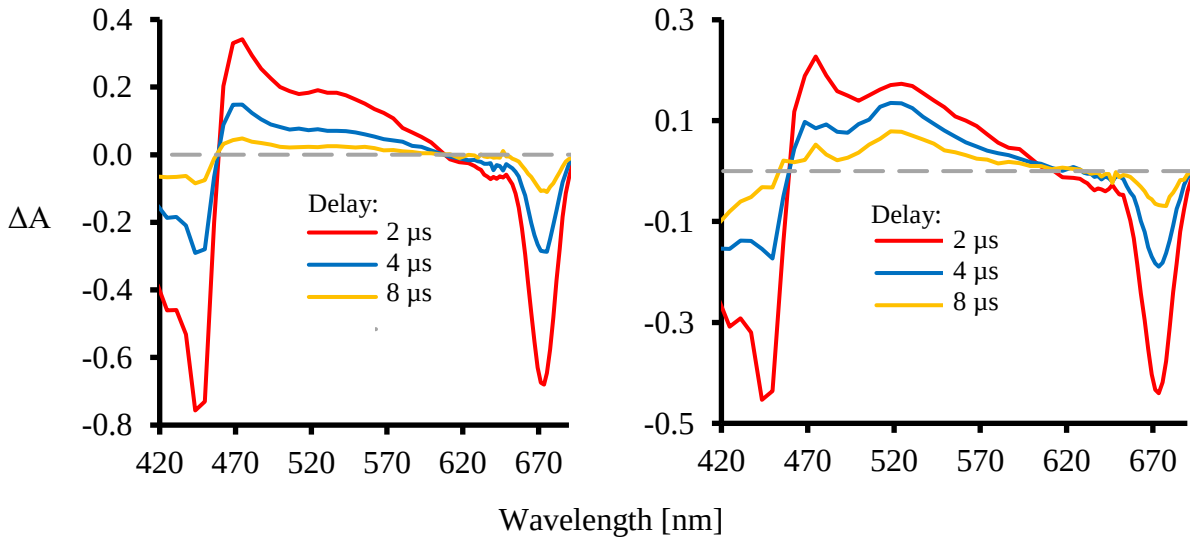


Figure 12 TmS spectrum of chlorophyll a in pyridine (left) and TmS spectrum of a mixture of chlorophyll a and fucoxanthin in pyridine.

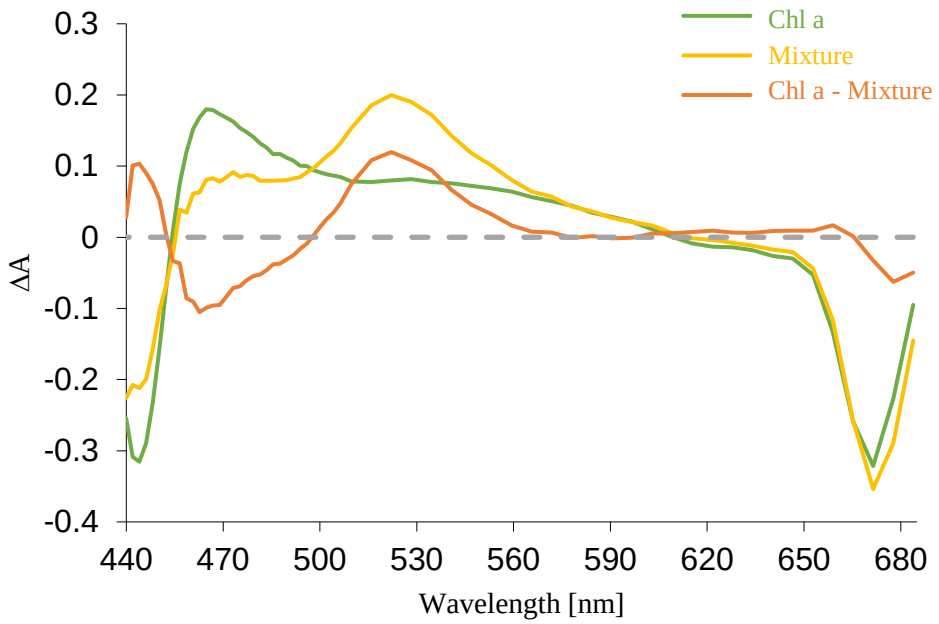


Figure 13 The TmS spectrum of fucoxanthin is obtained by subtracting the TmS spectrum of pure chlorophyll a from the TmS spectrum of a mixture of both, scaled to bleaching of Chl a band at 665 nm.

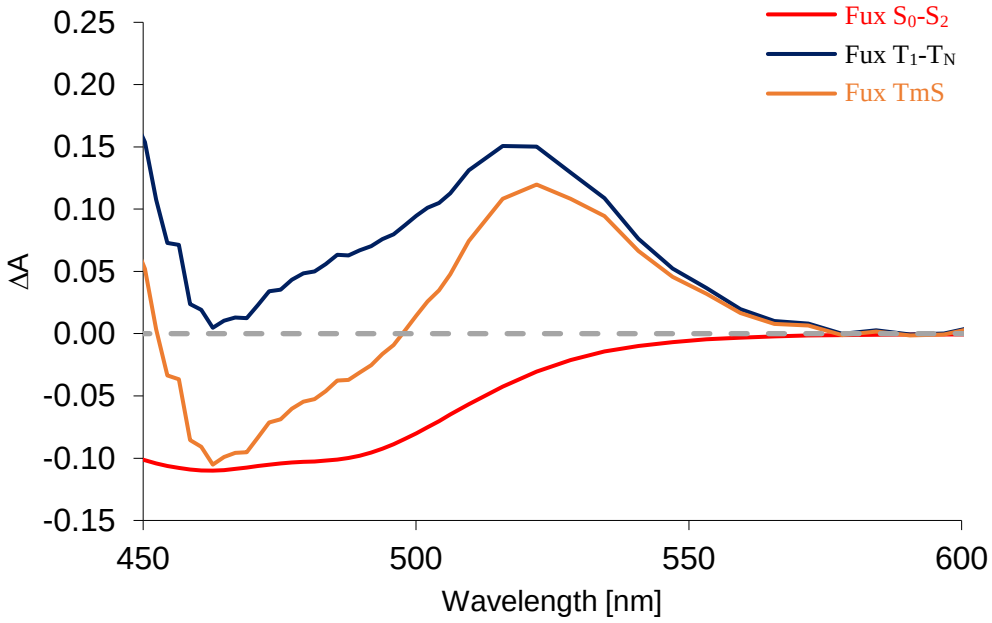


Figure 14 By subtraction of the absorption spectrum of ground-state carotenoid (S_0-S_2, red line) from the obtained TmS spectrum of fucoxanthin, the triplet-state absorption spectrum (T_1-T_N) of fucoxanthin can be extracted.

5.6 Estimation of rate of triplet transfer

With the TmS spectra of the mixture of chlorophyll a and fucoxanthin, the lifetimes of chlorophyll and fucoxanthin triplet-states were determined by fitting in MS Excel. The obtained triplet-state transfer rates k_{ET} can be found in Table 1. One measurement was performed on a sample after moderate bubbling with N_2, slowing the triplet-state decay rates. The fitting of the bubbled sample can be seen in Figure 15.

Table 1: Second-order rate constants (k_{ET}) for the quenching of chlorophyll triplet-state by carotenoid in pyridine.

	OD(Car) at 481 nm	**k_{ET} * 10^9 [M^{-1} s^{-1}]**
bubbled	0.48	3.96
not bubbled	0.80	4.75
not bubbled	0.62	9.07
not bubbled	0.39	7.21
not bubbled	0.28	3.95

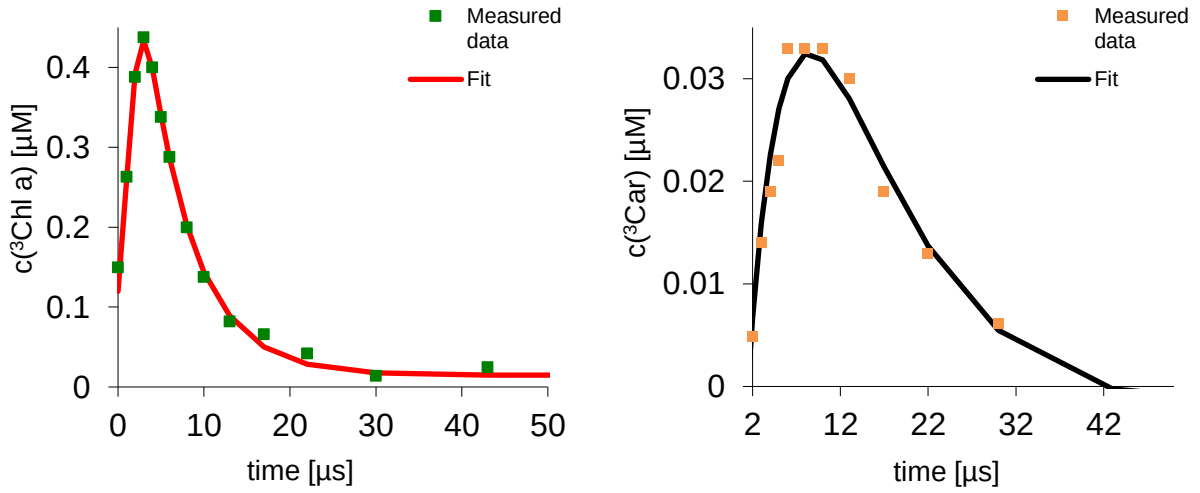


Figure 15 Example of the fitting done on the bubbled sample to calculate the triplet-transfer rate k_{ET}. Chlorophyll a singlet bleaching decay at 671 nm (left) and fucoxanthin triplet decay at 509 nm (right).

5.7 Comparison of LHC measurements

The absorption and Triplet-minus-Singlet (TmS) spectra of the LHC of *Aureococcus anophagefferens* inside the membrane (from here on referred to as "membrane") and LHC extracted into a HEPES buffer (from here on referred to as "LHC") can be seen in Figure 16 and 17. Additionally, the same spectra were recorded for the isolated LHC dissolved in pyridine (Figure 18). By dissolving the LHC in pyridine the pigments get released into the solution. By comparing Fig. 16 and 17, it can be seen that the spectrum of the membrane is mostly determined by the properties of the LHC. The most conspicuous difference was observed in the region between 430-460 nm of the Soret bands of chlorophylls. In the spectrum of the membrane, the dominant peak was at 438 nm (Chl a), whereas the maximum was at 463 nm for LHC (Chl c). This indicates higher proportion of Chl a in the membrane compared to the LHC. This is most likely due to the presence of reaction centres, which are assumed to bind Chl a but not Chl c similar to the situation in other Chl c containing organisms (Veith et al., 2009). In the absorption spectrum of Figure 17, very little carotenoid absorption above 500 nm can be observed, as expected in situations where the shift of the carotenoid absorption is due to specific pigment-pigment and pigment-protein interaction. On the contrary, the absorption maximum of Chl c is more red-shifted in solution than in the LHC, from 461 nm to 470 nm. The same behaviour is observed in the Soret band of Chl a that in protein absorbs below 440 nm but shifts by about 10 nm to lower energies in pyridine. Furthermore, when looking at the TmS spectra of Figure 16 to 18, the carotenoid triplet absorption is present in both the membrane and LHC in buffer measurements but disappears when the LHC is dissolved in pyridine. This can be explained by thinking about the pigment environment. As long as the pigments are neatly situated in the LHC, chlorophyll triplet-states get transferred rapidly and with high efficiency. In solution however, the rate of triplet-transfer is greatly reduced since it depends on the molecules coming in contact by chance. Therefore, almost no carotenoid triplets are generated. Although this is in contrast to the measurements above, it has to be considered that the triplet transfer experiment in solution aimed at determining Fux TmS spectra and transfer rates were conducted with much higher carotenoid concentrations.

The TmS spectra also reveal a difference in maximum carotenoid triplet absorption between the membrane and LHC and overall shape of the ^{3}Car band. This difference is illustrated in Figure 17.

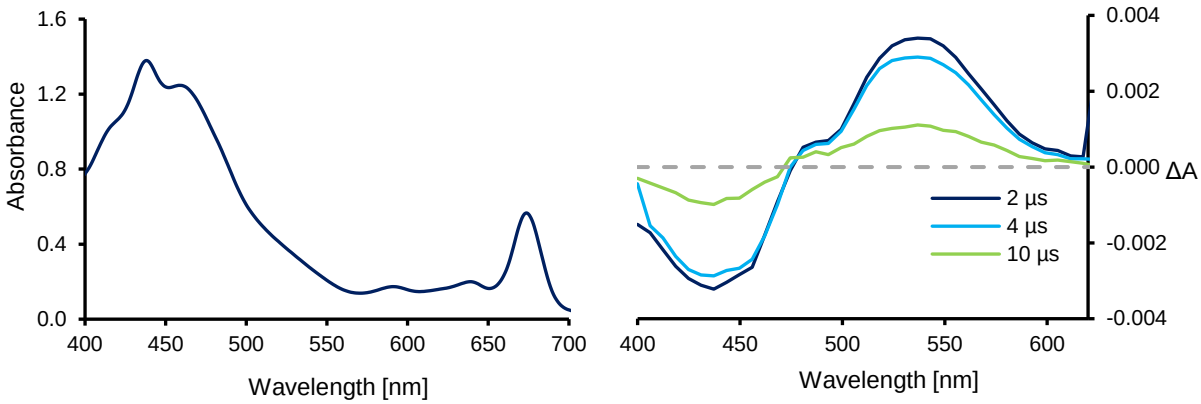


Figure 16 Steady-state absorption spectrum (left) and TmS spectrum (right) of the membrane of *Aureococcus anophagefferens.*

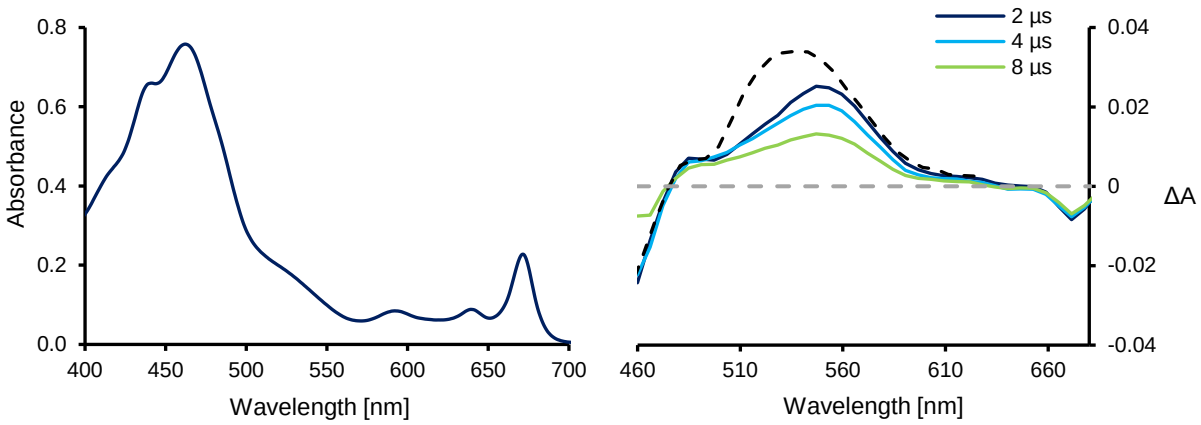


Figure 17 Steady-state absorption spectrum (left) and TmS spectrum (right) of the LHC of *Aureococcus anophagefferens* in HEPES buffer. For comparison the TmS spectrum at 2 µs delay of the LHC in the membrane was scaled and added (dashed line).

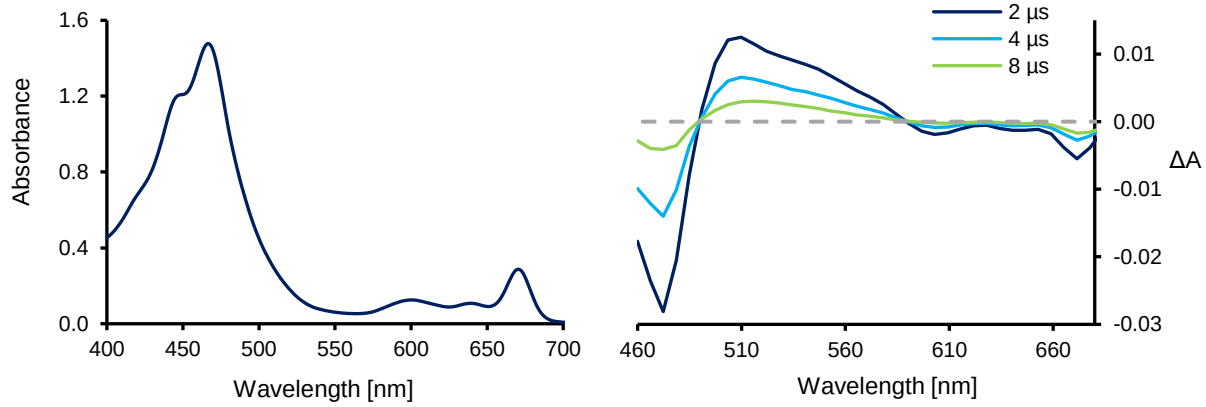


Figure 18 Steady-state absorption spectrum (left) and TmS spectrum (right) of the LHC of *Aureococcus anophagefferens* dissolved in pyridine.

Furthermore, the rate of decay for the carotenoid triplet-state in both the membrane and LHC were determined by fitting at maximum carotenoid triplet absorption (536 nm for membrane, 547 nm for LHC). For the LHC in the membrane the carotenoid triplet lifetime was determined to be 5.5 µs and for the LHC in buffer solution 8 µs. The corresponding data and fittings can be seen in Figure 19.

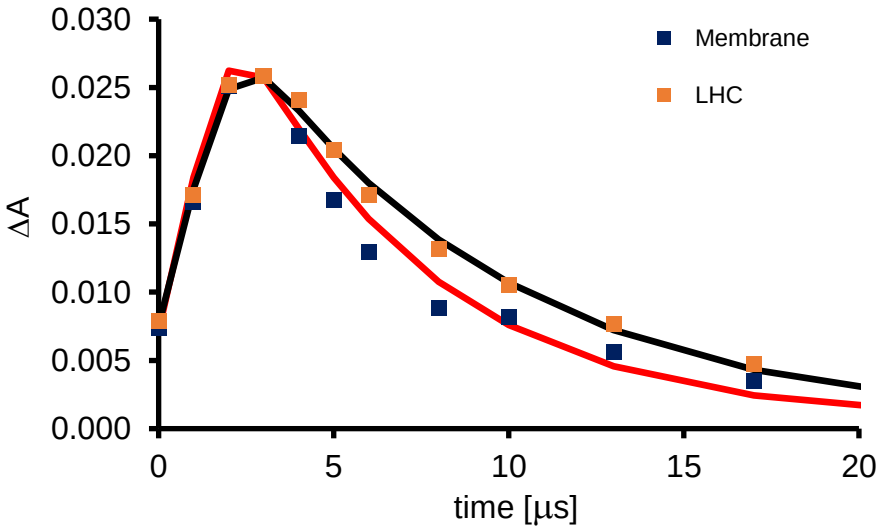


Figure 19 Carotenoid triplet-state decays in membrane and isolated LHC, scaled to the same amplitude.

6 Discussion

6.1 Extinction coefficient for T_1-T_N transition of fucoxanthin

The calculated extinction coefficient for the triplet-state of fucoxanthin is in a comparable range compared to those of other xanthophylls. However, it can be noted that other carotenoids showed a greater difference between their singlet state and triplet state extinction coefficients, whereas for fucoxanthin the change was less pronounced. However, difference in solvent must be noted. The extinction coefficient values shown in Tab. 2 correspond to carotenoids with well-resolved vibrational bands, unlike fux in pyridine. It is thus possible that performing the experiment in hexane, where the vibrational structure of fux is resolved (Zigmantas, et al., 2004) might yield larger value of triplet extinction coefficient.

Table 2: Molar extinction coefficients for singlet and triplet states of various carotenoids. All extinction coefficients are in M^{-1} cm^{-1}.

Xanthophyll	ε_S x 10^5	ε_T x 10^5	Solvent	ε_S / ε_T	Reference
Fucoxanthin	1.09 (462 nm)	1.95 (515 nm)	Pyridine	1.8	Jeffrey et al. (2005) + this work
Zeaxanthin	1.4 (450 nm)	3.6 (510 nm)	Ethanol	2.6	(Kaligotla, 2011)
Neoxanthin	1.3 (440 nm)	3.6 (485 nm)	Ethanol	2.8	
Violaxanthin	1.5 (440 nm)	4.2 (481 nm)	Ethanol	2.8	

6.2 Triplet transfer from chlorophyll to carotenoid

To further characterise the properties of fucoxanthin as a triplet quencher, we attempted to extract the rate constant of triplet energy transfer (k_{ET}) from ^{3}Chl a to ^{1}Fux in pigment mixtures. Initially, the experiments were performed on aerobic samples. However, Chl a triplet lifetime never exceeded 2 µs under these conditions. Given that even shorter ^{3}Chl a lifetimes in pyridine have been reported (Niedzwiedzki et al. 2014, ~350 ns) it was likely that the observed ^{3}Chl a lifetime was prone to a large error. As a consequence, the values for the rate of triplet-transfer found varied rather widely, although a clustering around ~4×10^9 M^{-1} s^{-1} could be observed. Thus, the pigment

mixture was bubbled with N_2 to remove oxygen at least partially and to extend the lifetime of the triplet-states. This increased the difference between the lifetime and instrument response, potentially resulting in a more accurate measurement of the lifetime. In this experiment, the ^{3}Chl a lifetime increased to roughly 5 µs. The rate constant for triplet transfer in this experiment was again close to 4×10^9 M^{-1} s^{-1}. Based on this the two outlying data points were removed from the analysis, yielding the average value of the rate constant of triplet-transfer between chlorophyll a and fucoxanthin, k_{ET}, of $4.2 \pm 0.4 \times 10^9$ M^{-1} s^{-1}.

In general, rate constants of triplet-state quenching by carotenoids do not seem to follow a trend concerning the number of conjugated double bonds. As such, the obtained values for the xanthophyll fucoxanthin are in the same range as carotenes such as neurosporene, spheroidene and β-carotene (Borland, et al., 1989). A comparison can be found in Table 3. The conclusion may be drawn that none of the functional groups of fucoxanthin (hydroxyl, carbonyl, acetyl) exert substantial steric effects on the Chl a – fux interaction compared to other carotenoids. Lack of dependence of k_{ET} was also noted for peridinin analogues (Kaligotla, et al., 2010). Over the conjugation length of 6 to 9, all molecules studied had k_{ET} between $4 - 6 \times 10^9$ in hexane; the value was around two times smaller in acetonitrile. The solvent effect was interpreted as difference in solvation structure giving rise to different distances between the carotenoid and Chl a. Clearly, the value for the triplet-transfer rate k_{ET} in this work is reasonable, despite the fact that our experiment was not performed in oxygen free conditions.

Table 3: Second-order rate constants (k_{ET}) for the quenching of chlorophyll triplet-state by various carotenoids.

Carotenoid	$10^9 k_{ET}$ [M^{-1} s^{-1}]	Number of double bonds	Reference	Solvent
Neurosporene	2.34	9	(Borland, et al., 1989)	Benzene
Spheroidene	5.11	10		
β-Carotene	0.9	11		
Fucoxanthin	4.2	8*	This work	Pyridine

*Conjugation extends to the carbonyl group

6.3 Membrane and LHC carotenoid pools

Absorption spectrum of *Aureococcus Anophagefferens* is characterized by a dominant contribution of Chl c and strongly red-shifted absorption band of fucoxanthin. Overall, the shape of the spectrum resembles the membrane antenna (acpPC) of the dinoflagellate *Amphidinium carterae* (Kvíčalová, et al., 2016). Since fucoxanthin is the only carotenoid present in the complex in substantial amount, it can be assumed that it is the triplet-quenching carotenoid. In accordance with the red shift of the carotenoid S_0-S_2 absorption band, the position of the triplet absorption band is also strongly red-shifted, compared to fux in pyridine, peaking at ~550 nm.

Another observation was made when comparing carotenoid triplet maxima in the membrane and LHC (Figure 20). Here, both spectra were normalized to match each other at 580 nm to emphasize the difference in the carotenoid triplet absorption band. Assuming that at least part of the membrane triplet signal must be due to the LHC, by subtracting the LHC spectrum from the membrane spectrum, we obtained the approximate spectrum of the additional absorbing species, peaking at ~520 nm. Comparison of this peak with a TmS spectrum of fucoxanthin in pyridine showed excellent agreement. With this peak identified as triplet-absorption of fucoxanthin, it can be assumed that there is some other pool of carotenoid in proximity to chlorophyll, capable of quenching the triplet, in addition to the fux pool bound to LHC. This additional carotenoid is removed during purification as it is not present anymore in the LHC spectrum. It might represent a triplet signal from a different antenna complex, such as antenna associated to PSI or it might represent a different pool of LHC, where the carotenoid spectrum is modified by conformational change of the protein complex. Another explanation could be that it represents a separate, loosely bound carotenoid that is nevertheless in close proximity to Chl a in the LHC, while the complex is in the membrane, but gets removed during LHC purification.

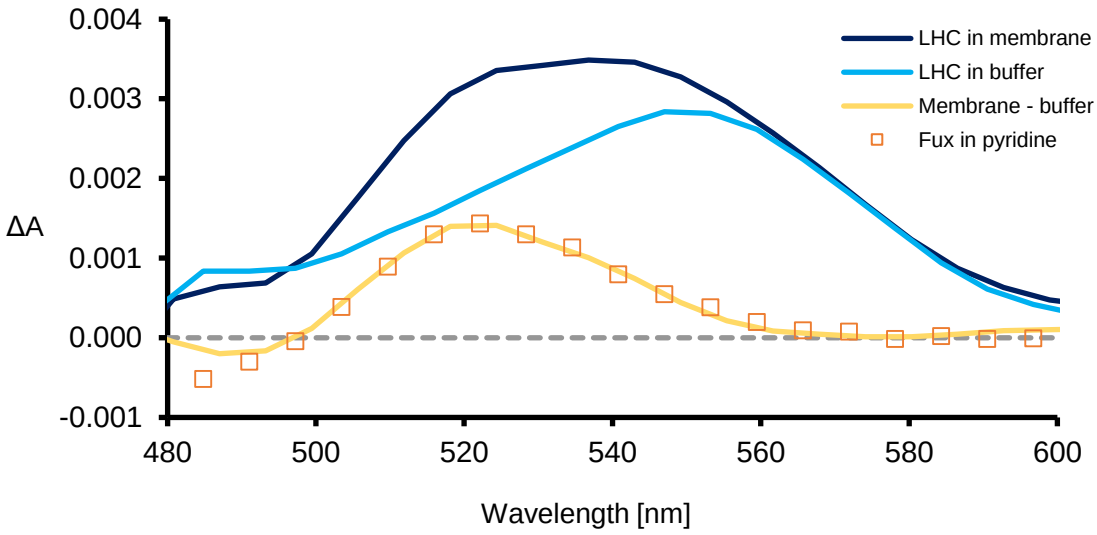


Figure 20 Comparison of TmS spectra of LHC in membrane and in buffer. By subtraction, an absorption band is obtained, matching fucoxanthin triplet absorption in pyridine.

7 Conclusions

The triplet-state absorption spectrum of fucoxanthin in pyridine was characterized and its extinction coefficient was estimated to be 1.95 x 10^5 M^{-1} cm^{-1} at 515. Furthermore, the rate constant of triplet-state transfer from chlorophyll a to fucoxanthin was found to be 4.2 x 10^9 M^{-1} s^{-1}. Fucoxanthin was also observed in the membrane of *Aureococcus anophagefferens* and in its isolated LHC. An additional pool of carotenoid seems to be bound in the membrane that gets removed during purification of the LHC.

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