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Pigment composition of photosynthetic light-harvesting complexes of eukaryotic alga *Emiliania huxleyi*

Bachelor thesis

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Annotation

Pigment composition of light-harvesting complexes from eukaryotic alga *Emiliania huxleyi* was analysed. Light-harvesting proteins were purified by sucrose gradient ultracentrifugation and ion exchange chromatography. Pigment content was analysed by HPLC. Effect of different growth light intensity on pigment composition was investigated.

Affirmation

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Abstract

Photosynthetic organisms developed mechanisms to dynamically and reversibly adapt to changing illumination. A maximum of energy should be gained at limiting light condition, but at excessive illumination mechanisms for photoprotection are required. The eukaryotic alga *Emiliania huxleyi* was studied to investigate the conversion of fucoxanthin to its major derivative by the addition of a six-carbon side chain as well as the basic pigment variation resulting from the xanthophyll cycle. Pigment analysis of *E. huxleyi* cells by HPLC supported previous results that a substituted fucoxanthin is a major pigment and that the cells contain three forms of chlorophyll c. The content of substituted fucoxanthin increases upon higher illumination while fucoxanthin itself decreases. Concerning the carotenoids from the xanthophyll cycle an increase in both was observed on high illumination intensity, however the ratios were significantly different in cells grown at different light conditions. Purification of the light- harvesting complexes suggests that the fucoxanthins and chlorophylls are bound to the antenna complexes, while the pigments from the xanthophyll cycle are not.

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Abbreviations

19´-BFx	19′-Butanoyloxyfucoxanthin
19´-HFx	19′-Hexanoyloxyfucoxanthin
Ax	Antheraxanthin
β-DM	n-Dodecyl-β-D-maltoside
Chl	Chlorophyll
Dd	Diadinoxanthin
DDE	Diadinoxanthin de-epoxidase
DEP	Diatoxanthin epoxidase
Dt	Diatoxanthin
Fx	Fucoxanthin
HL	High light
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
LHC	Light-harvesting complexes
LL	Low light
Lut	Lutein
MGDG	Monogalactosyldiacylglyceride
MV	Monovinyl
Neo	Neoxanthin
NPQ	Nonphotochemical quenching
PS	Photosystem
VDE	Violaxanthin de-epoxidase
Vx	Violaxanthin
ZEP	Zeaxanthin epoxidase
Zx	Zeaxanthin

1. Introduction

1.1. *Emiliania huxleyi*

The eukaryotic algae *Emiliania huxleyi* is a photosynthetic marine microorganism. It belongs to the group Haptophyta, more precisely to the coccolithophores. These are unicellular species forming coccoliths, which are cell coverings formed around the cells in sometimes multiple layers. At natural conditions a cell of *E. huxleyi* is covered with 10 – 15 coccoliths. Under optimum conditions 1 – 2 coccoliths are formed per hour, even if the cell itself is not growing. The coccoliths consist mainly of calcium carbonate but have an organic skeleton. Despite the fact that a lot is known about the coccoliths so far, their biological role is still a subject of debate [1; 2].

E. huxleyi is the main contributor to the worldwide calcite production, often found in sediments in the oceans. Also, it is the most widespread coccolithophore in the world's oceans. It can be found in any water with exception of the polar waters. Under certain conditions it forms blooms exceeding areas of 100,000 km^2 with cell densities reaching 10,000 cells mL^{-1} [3; 2].

Blooms of *E. huxleyi* form in rather eutrophic regions, but also in the subtropical gyres, which are mostly oligotrophic, the alga occurs in high numbers. Its cells have a diameter of only 5 µm, therefore it is not the major contributor to the overall biomass, even though it is the most frequent phytoplankton in surface layers of the oceans. Examples for blooms were observed in the Norwegian fjords in the 1950s with concentrations of $10 – 100 \cdot 10^6$ cells $litre^{-1}$ or in the Black Sea in 1992 with concentrations up to $10 \cdot 10^6$ cells $litre^{-1}$. The concentration and also the colour of the water differs depending on the stage of the bloom. Bright colours of the water, which can be detected by satellites, might also be caused by detached coccoliths from dying cells scattering the sun light. The environment is influenced by these blooms as they cause stronger reflectance of the water, a reduction in the calcium carbonate amount in surface waters and an altered CO_2 uptake. There are various factors causing the blooms, some of which seem to be high light and high carbonate concentrations but low amounts of silicate and dissolved carbon dioxide [4].

E. huxleyi can be grouped into two molecular clades based on genetics, which prefer different environmental conditions. Type I has preferences for warm-water from temperate/tropical areas, while type II is found mostly in cool-water from subarctic latitudes.

Moreover, five morphotypes (A, B, B=C, C and R) are distinguished based on differences of the coccoliths. These morphotypes randomly occur in both clades I and II [5].

The life-cycle of *E. huxleyi* is complex and not yet totally understood. It is known however that it includes different cell types, which can all exist independently of each other. C cells having coccoliths, nonmotile naked N cells and motile S cells with scales occur during the life-cycle. It could be observed that N and S cells form out of C cells. The C cells are diploid, while the S cells are haploid, which suggests that the S cells might be acting as gametes. [2].

Scientists are attracted by *E. huxleyi* because its coccoliths might be useful for various applications. To show how wide the scope application of coccoliths is, some examples are given here. Coccoliths may be used in the production of paper, colours and lacquers, special ceramics, but also for semiconductor and composite materials. They are resistant to high temperatures and may play a role in optical uses. Even for biomedical substances, transplant materials, drug delivery and maybe also as enzyme carriers they can be of use [2; 1].

However, *E. huxleyi* is also drawing the attention to itself concerning one of the major environmental problems of modern days. It plays a role in ocean acidification as a result of increasing atmospheric CO_2 partial pressures. Based on observations it is expected that the increase in CO_2 caused by humans will lead to a decrease in calcification by *E. huxleyi* and other marine species [6].

1.2. Photosynthesis

1.2.1. Basic principles

Photosynthesis is fundamental for life on Earth by supplying food and energy sources by using the energy from the sun. Higher plants who everyone is familiar with derive basically all their energy from the sun. Other organisms use photosynthesis only at certain conditions.

From the sun light reaching the earth most plants use radiation in the range of 400 – 700 nm. Some organisms can utilize light even at wavelengths up to 1000 nm. It is commonly known that plants need the pigment chlorophyll for photosynthesis. This is true for the majority of photosynthetic organisms, however not for all. The photosynthetic pigment of some bacteria and archaea is rhodopsin, others are not yet known to far extents. There is a difference in the mechanism of photosynthesis between organisms using chlorophyll and those needing other pigments. The chlorophyll-based type of photosynthesis works via a light-driven

energy transfer. The working principle will be described in greater detail in the next chapter (1.2.2). During this process some organisms generate oxygen others do not. The first group of organisms is referred to as oxygenic, the latter one is termed anoxygenic organisms [7].

1.2.2. Four phases of photosynthesis

Photosynthesis is often divided into four phases instead of distinguishing between light reactions and dark reactions. These terms might be misunderstood since all phases include light-driven reactions, but only the first phase is dependent on light. When using the terms light reactions and dark reactions, phases 1 to 3 were grouped as light reactions, while phase 4 was referred to as dark reaction.

Phase 1 is constituted of light absorption followed by the energy delivery by the antenna systems. In the next step, phase 2, the primary electron transfer takes place in the reaction centres. In phase 3 this energy is stabilized by secondary processes and finally used for synthesising of the products in phase 4.

The antenna systems play an important role, because they increase the amount of light collected. There are different pigments in the antennas often located in a way that the outer pigments absorb at shorter wavelengths and thus higher energies than the inner ones. The electronic excited states are then delivered to the reaction centre going from pigments with higher to those with lower energy. Depending on the environment of a plant the number and type of antennas bound to the reaction centre varies.

The chemical reactions resulting from the delivered energy happen in the reaction centre. This pigment-protein complex consists of multiple subunits and holds the pigments and cofactors required for the electron transfer processes. The antennas deliver the energy to a dimer of special chlorophyll-like pigments in the reaction centre, which acts as the primary electron donor. If one of these special chlorophyll pigments is transferred to an excited state, it becomes a strongly reducing species undergoing a redox reaction with an adjacent acceptor molecule. To avoid a backreaction this is followed by a second reaction separating the ions further apart.

Some species utilize a cyclic electron transfer chain to create a protonmotive force by moving protons across the membrane, which is then used to make ATP. The more common species have two reaction centres called photosystem I and II (PSI and PSII) which together operate in a non-cyclic electron transfer chain. Briefly described PSII oxidizes water to O_2, which is not required for further processes. Via a quinone and the cytochrome b_6f complex the generated electrons are transferred to PSI. There $NADP^+$ is reduced to the intermediate

NADPH. In the same way as in the process described before, protons are transported across the membrane and via the protonmotive force ATP is synthesised.

Finally, NADPH and ATP are utilized to reduce CO_2 from the air to sugars. These high-energy compounds are needed for cellular processes requiring energy. In eukaryotic photosynthetic species the phosphorylated sugars are transported out of the chloroplast und further used in the stroma [7].

1.2.3. Photosynthetic apparatus

In eukaryotes the main phases of photosynthesis take place in organelles called chloroplasts. Inside the chloroplasts a membrane system is found, which is called thylakoid membranes, or lamellae. The interior of the thylakoid, which is surrounded by the membranes, is calls thylakoid lumen. The chlorophylls and all other pigments inside the chloroplast are located within the membranes. Thylakoids stacked together are known as grana thylakoid membranes. In the chloroplasts of higher plants typically most of the thylakoids are arranged in that way. In contrast stroma thylakoid membranes or lamellae refer to those not associated in stacks. The thylakoid membranes of diatoms are not stacked so there is also no distinction between granal and stromal lamellae. These organisms comprise thylakoid membranes which are grouped into three loosely stacked lamellae. The enzymes containing fluid between the thylakoids is called stroma. Light is absorbed by the thylakoids and also the first reactions take place there. The final carbon products like sucrose are synthesized outside the chloroplasts [7; 8].

The two photosystems are located within the thylakoid membrane. These are multi-protein membrane complexes with chlorophylls and carotenoids. Each of them comprises a core and outer antennas. The core functions in charge separation and the transport of the electrons while the antennas increase the amount of absorbed light. In higher plants the light-harvesting complexes (LHC), which are part of the outer antenna, and the PS cores form supercomplexes. For example, around the core of the PSII supercomplex, one of each of the minor antennas (CP29, CP26 and CP24) and several LHCII trimers are arranged [8; 9].

The LHC proteins of higher plants differ from those in diatoms as well. The main difference is the pigmentation. Chlorophylls and fucoxanthin together form the fucoxanthin-chlorophyll protein (FCP) complexes of diatoms. It is however unknown which of the different FCPs is correlated with which of the two PS and/or what the supramolecular structure looks like [8]. The complexes consist of 18 and 19 kDa apoproteins and form oligomeric states. It was shown that in the diatom *Phaeodactylum tricornutum* the basic unit of FCP complexes is

the trimer. In the diatom *Cyclotella meneghiniana* a trimeric FCPa as well as a oligomeric FCPb-complex were observed. Higher plants and green algae have PSII–LHCII supercomplexes or large PSII–LHCII complexes, respectively [10; 11; 12].

The structure of no light-harvesting protein containing Chl c is completely resolved, but it can be assumed that there are similarities to the well-understood Chl a/b-binding LHCs of plants. The LHC seem to be constituted of three α-helices spanning across the membrane. It was suggested that there is a total of six to seven Chls bound to each complex. Based on the high sequence identity of the first and the third transmembrane helix to those of Chl a/b-binding proteins, it was suggested that this is where Chl a binding sites are located. Further, due to what was observed in higher plants, it was proposed that the Chl c binding sites are located on the second helix. In the LHCs of haptophyte algae Fx is the dominating carotenoid. However, the location of the carotenoids could not be determined. A similar number of Fx and Chl a was present per protein, so it was concluded that the carotenoids are positioned close to the Chls [13].

1.3. Photosynthetic pigments

Diatoms seem to possess similar pigments as *E. huxleyi*. Since there has been only little research of *E. huxleyi* it is useful to study the pigments found in diatoms.

The pigments can be grouped into those responsible for light-harvesting and those acting in photoprotection. As mentioned above in diatoms chlorophylls and fucoxanthins act in harvesting light. The carotenoids β-carotene, diadinoxanthin (Dd) and diatoxanthin (Dt) are needed in photoprotection. Violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx) might also play a role in diatoms, for sure they do in higher plants.

The level of each of the pigments is highly dependent on the illumination. Plants can do fast transformation of their pigments without changing gene expression, which only occurs in long-term adaption [8].

In *E. huxleyi* the photosynthetic pigments Chl a and various types Chl c were found. It is common for Haptophyta like *E. huxleyi* to have a high diversity of Chls c. Recently new Chls c were isolated in addition to the well-known Chl c_1, Chl c_2 and Chl c_3. Two of the new Chl c-like pigments were identified in *E. huxleyi*, namely monovinyl Chl c_3 and Chl c_2-monogalactosyldiacylglyceride ester [14].

In the majority of photosynthetic organisms Chl a is the most important pigment in photochemical energy conversion. Chl c acts as an accessory pigment, playing a similar role as Chl b does in higher plants [8].

The basic structure of chlorophyll are four pyrrole-like rings with Mg^{2+} in the centre and a fifth ring next to them. Most of the chlorophylls are classified as Mg-chlorins, but Chls c belong to the Mg-phytoporphyrins. The different types of chlorophylls differ in their side chains. At the C-17 postion where Chls a and b have a propanoic acid side chain, Chls c have a propenoic acid. The difference between Chl c_3 and Chl c_2 is a methoxycarbonyl group bound at the C-7, making it more polar [14].

1.3.1. Responses to changing light conditions

Plants use antenna systems with various pigments to collect light at different wavelengths. Thus, antennas are beneficial by increasing the amount of absorbed light and so the energy transferred to the reaction centre. Under conditions of too high light this might however carry the risk of damage, because the organism cannot utilize the excess energy. Together with molecular oxygen this energy might form reactive oxygen species which cause photodamage. PSII fulfils both of these essential roles, collecting light energy but also dissipating excess absorbed energy [7; 9].

Plants have developed various mechanisms to protect themselves from the effects of too high irradiances. This adaption to varying light conditions is called photoacclimation. Examples for this process include gene expression changes, state transitions and thermal dissipation of the excess energy, termed nonphotochemical quenching (NPQ). Algae also change the structure of the photosynthetic apparatus in a way that the size of PSII and/or the number of reaction centres (RCs) is adapted to the light conditions. For this work the focus lies on NPQ, which is the fastest regulation mechanism in the adaption to changing light conditions, and the xanthophyll cycle [7; 5; 9].

NPQ, the process of regulating the light-harvesting capacity by dissipation excess energy, is induced by an external signal and works via the xanthophyll cycle and the PsbS protein. It is called NPQ, because it is a quenching process where energy is lost by the deactivation of excited states in the chlorophylls of the antennas, and nonphotochemical since it differs from the quenching resulting from normal photochemistry [7; 15].

The protein PsbS is thought to be the site of NPQ. It is interacting with the protons and the epoxy-free xanthophyll as well as with a chlorophyll containing complex [16].

As described before during photosynthesis a proton gradient is established across the thylakoid membrane causing a drop in pH. This acidic pH in the thylakoid lumen is what triggers NPQ and thus induces the two following events. Within seconds the PsbS is activated. This is followed by the activation of the de-epoxidase enzymes which transform the pigments taking a few minutes to do so. This process will be discussed in more detail below. Various de-epoxidases exist, showing a different pH-optimum. In addition, for the activity of these enzymes ascorbate and the most abundant thylakoid membrane lipid monogalactosyldiacylglycerol (MGDG) are required. Ascorbate acts as a reductant, while MGDG is needed to solubilize the epoxy-free xanthophyll, which would form aggregates and could not be transformed by the enzyme. Due to the conformational change of the enzymes they bind to the thylakoid membrane, the location of Vx and Dd. However, the presence of the epoxy-free pigments does not itself cause quenching in the individual complexes, neither does the low pH. The mechanism behind triggering NPQ is not clearly understood [15; 9; 12].

It is known that in higher plants the xanthophyll Zx is required for NPQ, however the principle of action is still under debate. LHCs possess two carotenoid binding sites, called L1 and L2. Under normal light conditions these bind lutein (Lut) and Vx. Additionally, some complexes also have the sites N1 and V1 where neoxanthin (Neo) and Vx/Lut are bound. The state of knowledge was that Zx binds to the LHCs causing conformational changes which lead to a quenched state. Recent research by Xu et al. [9] disproves these assumptions. The internal binding sites of the LHCs are not specific for Zx and can bind almost any xanthophyll. Under excess light conditions however it seems unlikely that high numbers of Vx are released from the already folded proteins to be transformed to Zx and rebound to the complexes. This process might only take place under long term adaption. In the other approach the affinity of the LHCII for protons is changed upon binding of Zx to the complex. Zx was thought to act by shifting the pH-dependence of NPQ to higher pH values. The idea was that the protonated protein complexes exist in a quenched conformation when Zx is bound. But not even at low pH this could be observed. Due to new results it is now believed that Zx is present at the periphery of the LHCII, but also at the minor antennas. No specific binding-site is required but instead there is an interaction with the Zx, located at the interface between two proteins, and some chlorophylls on the surface. Together with PsbS this creates new quenching centres [9].

Until now six forms of xanthophyll cycles were found in photosynthetic organisms with the most common ones being the violaxanthin cycle and the diadinoxanthin cycle. Each of these cycles occurs in different organisms. All vascular plant and some algae utilize the Vx cycle. The Dd cycle is found in several algal groups like the diatoms, phaeophytes, dinophytes,

and haptophytes, thus also in *E. huxleyi*. Normally only one type of cycle occurs in each organism, however it could be shown that the diatom *P. tricornutum* utilizes both, the Vx and the Dd cycle [15; 8; 12].

The principle of de-epoxidation in high light and the epoxidation in low light is met in all xanthophyll cycles. The molecular structures of the two cycles are shown in Figure 1 and Figure 2.

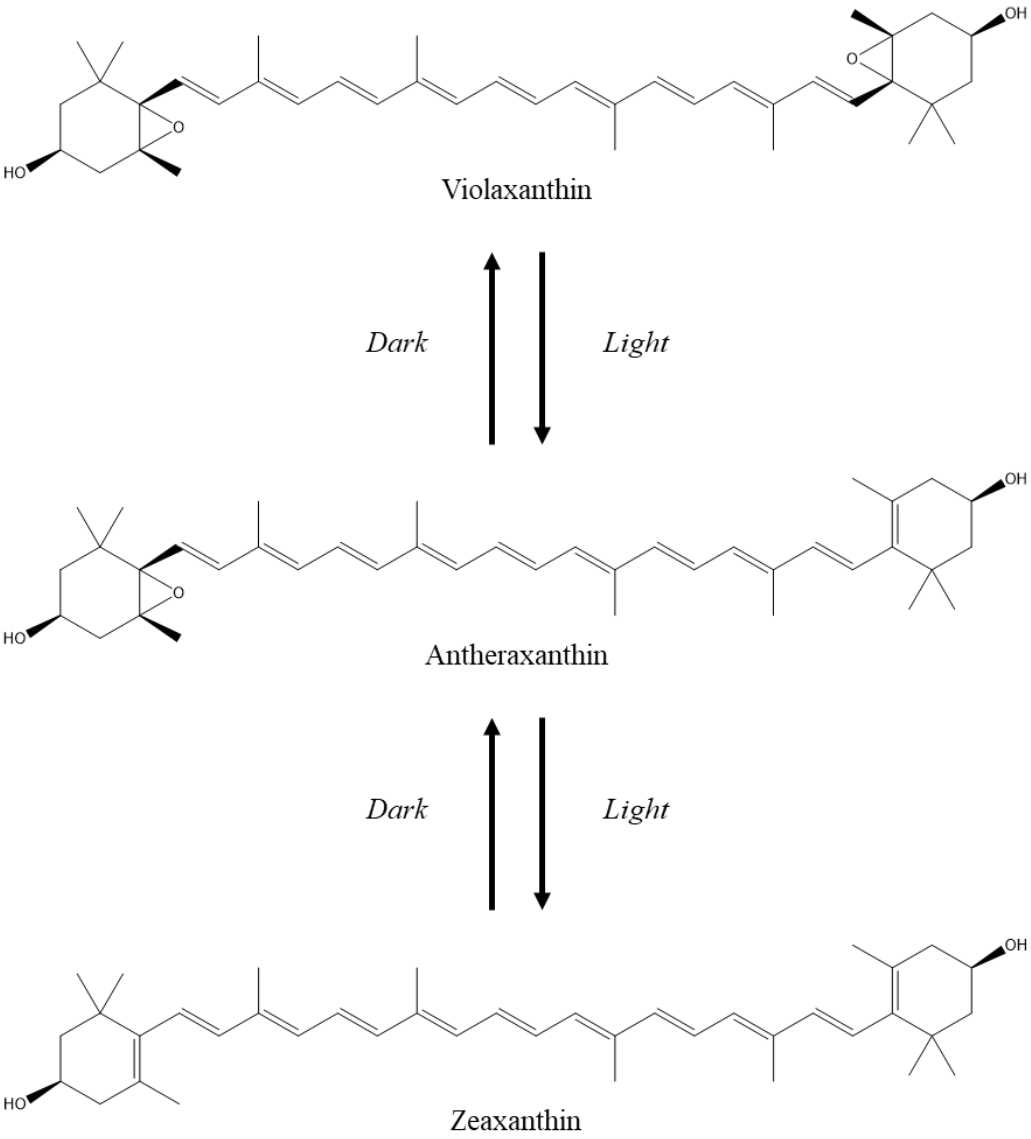


Figure 1: Chemical transformation of Violaxanthin to Zeaxanthin with the intermediate Antheraxanthin upon changing light conditions, named violaxanthin cycle [8; 17].

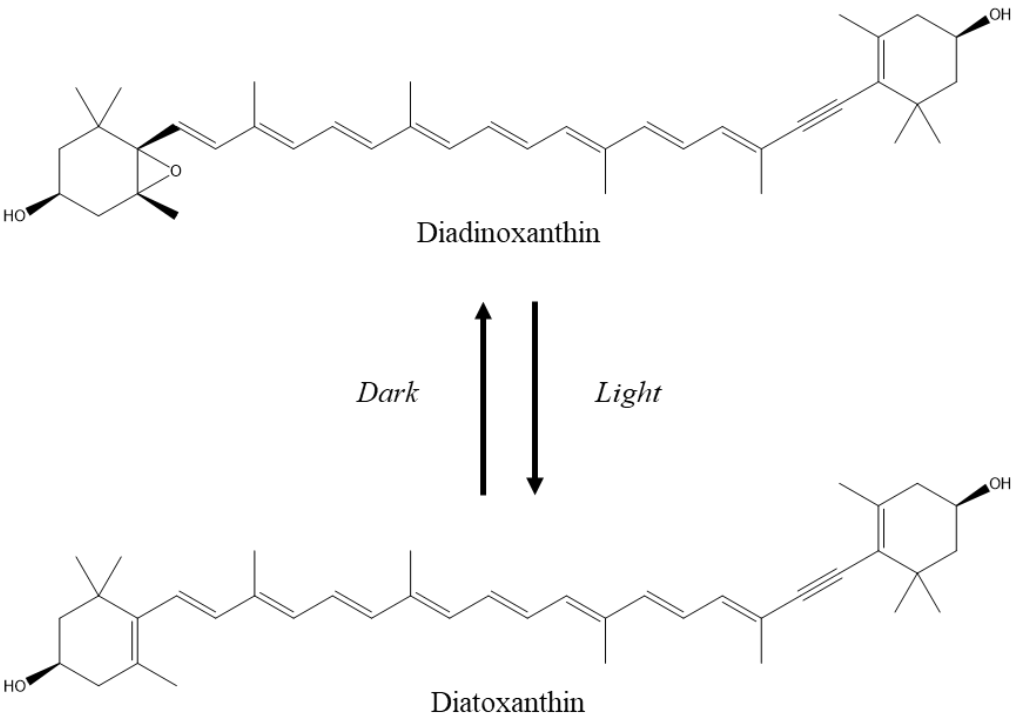


Figure 2: Chemical transformation of Diadinoxanthin to Diatoxanthin upon changing light conditions, named diadinoxanthin cycle [8; 17].

As mentioned above the de-epoxidized xanthophylls play a role in dissipating excess energy, protecting the organism from damage. The conversion into another xanthophyll is catalysed by special enzymes named epoxidases or de-epoxidases of the respective xanthophyll. These enzymes are water-soluble and can be found in the thylakoid lumen. Their site of action is the antenna system of PSII, as explained before [15; 8].

Upon high light conditions the di-epoxy Vx is transformed to the epoxy-free Zx. The intermediate in this reaction is the mono-epoxy Ax. The de-epoxidation of Vx is catalysed by Vx de-epoxidases (VDEs) and the reverse reaction by Zx epoxidases (ZEPs). In a similar way as described above, the monoepoxide Dd is converted to the epoxy-free Dt at higher light, however there is no di-epoxy form in this cycle and therefore also no intermediate. The cyclic conversion of Dd to Dt is catalysed by Dd de-epoxidase (DDE) and the epoxidation by Dt epoxidase (DEP) [15; 8].

1.3.2. Light acclimation in *E. huxleyi*

There are not yet research results on the detailed way of how the acclimation to higher light intensities works in *E. huxleyi*, but the principles and also the involved pigments seem to be similar to the process observed in *Aureococcus anophagefferens*. It should be noticed

however that while *A. anophagefferens* synthesizes 19′-butanoyloxyfucoxanthin (19´-BFx), 19′-hexanoyloxyfucoxanthin (19´-HFx) is mainly found in *E. huxleyi*. The difference between Fx and 19´-BFx or 19´-HFx is the butanoyloxy or hexanoyloxy group at the 19′ position, respectively, as it can be seen in the Figure 3.

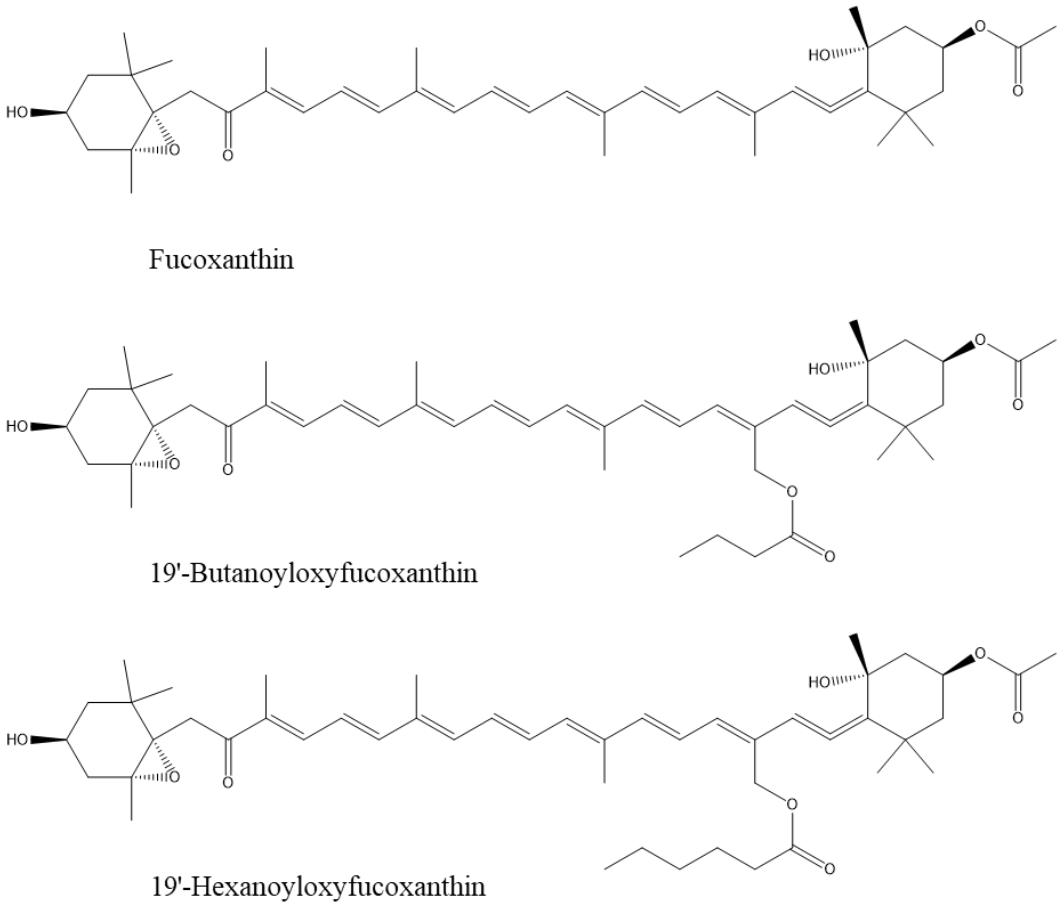


Figure 3: Structures of fucoxanthin and its derivatives [18].

The acclimation to higher light intensities takes place in three steps, of which the first one is the conversion of Dd to Dt. This happens in the first minutes of exposing the cells to higher light intensities and there is no net synthesis. This is followed by a de novo synthesis of both Dd and Dt during the first few hours. Also, the conversion of fucoxanthin (Fx) to 19′-butanoyloxyfucoxanthin starts. When transferring the cells back to lower light intensities the opposite conversion from 19´-BFx to Fx occurs, however it is slower than the other way around. The same is the case for the conversion of Dt back to Dd. In the final and slowest phase taking days to weeks, the increase in of 19´-BFx is continuously progressing. In addition, there are changes in the composition of the light-harvesting polypeptides and other physiological modifications to adjust to the new light conditions. Cells acclimated to higher light intensities were shown to possess light-harvesting proteins, which were not found, or

only in very low amounts, in cells growing at lower light intensities. To sum this up 19´-BFx seems to be formed only by transformation of Fx while there is a net synthesis of Dd and Dt during acclimating to higher light intensities [18].

The bulkier molecules 19´-BFx and 19´-HFx compared to Fx might act differently in binding and cause conformational changes of the protein, leading to larger distances and electrostatic changes and therefore reducing to efficiency of the energy transfer to the reaction centres. In cells exposed to higher light intensities the active antenna size was shown to be smaller with higher amounts of 19´-BFx and also the efficiency of PSII was reduced [18].
The light adaption of *E. huxleyi* also takes place in the antennas of PSII, which act in harvesting as well as dissipation of light energy. It was shown that these proteins respond to changes of the illumination the most [19].

Also, the conversion of Fx into 19´-HFx as a consequence of increasing irradiances occurs in *E. huxleyi*. In addition to that, a dependency of the pigments on the spectral conditions was described. The amount of pigments differs between red, blue, green and white light. However, this was not of importance for this work and therefore is not further explained here.

E. huxleyi utilizes the Dd cycle to adapt to changing illumination. Upon higher light conditions there is an increase in the amount of Dd and Dt, while these pigments are decreasing when transferring the cells back to low light conditions [5].

In photoacclimation of *E. huxleyi* the size and the number of photosynthetic units are adapted. Various Chl a binding proteins, belonging to the LHC, are involved in light-harvesting and NPQ. In cells acclimated to low light conditions a specific kind of Fx-Chl binding proteins (Lhcf) is more abundant, while another kind appeared in higher numbers at high light conditions (LI818-like) [19]. Equivalences to these proteins were also found in diatoms [10].

Due to all of these observations, the working principle of photoacclimation in *A. anophagefferens* as described above might describe the situation in *E. huxleyi* well.

2. Aims of the work

- To confirm the differing ratio of the two major fucoxanthins based on the cell growth illumination intensity in the alga *Emiliania huxleyi* on a cellular level.
- To investigate how this difference in the cellular pigment content is correlated with the pigment content of photosynthetic protein complexes.
- To investigate the content of xanthophyll cycle pigments in light-harvesting protein complexes.

3. Materials and methods

3.1. Chemicals

The chemicals used in this work are listed in Table 1. Common chemicals are not mentioned here for the sake of brevity.

Table 1: Chemicals used in the experiments.

Substance	Manufacturer	CAS Number
n-Dodecyl-β-D-maltoside (β-DM)	BioChemica PanReac AppliChem	69227-93-6
Protease Inhibitor Cocktail, EDTA-free	Sigma-Aldrich	-
Mobile phases for high performance liquid chromatography		
Water, HiPerSolv CHROMANORM	VWR Chemicals	7732-18-5
Methanol, HiPerSolv CHROMANORM	VWR Chemicals	67-56-1
Acetonitrile, far UV/gradient grade	J.T.Baker	75-05-8
Ethyl acetate, for HPLC-G	Chromservis	141-78-6
Stationary phase for ion exchange chromatography		
DEAE–Sepharose CL-6B	Sigma-Aldrich	57407-08-6

3.2. Cell growth and illumination

Cells of *Emiliania huxleyi* strain CCMP1516 were grown in 5 L Erlenmeyer flasks at 20 °C in artificial seawater f/2-Si medium [20], which was bubbled with filtered air and stirred. The illumination of the cells was performed using a metal-halide lamp with a daylight spectrum (OSRAM POWERSTAR HQI-E 70 W/NDL) with 15 h light and 9 h dark. The low and high-light cells were both grown under these same conditions with irradiances of 20 and 300 µmol photons m^{-2} s^{-1}, respectively. These growth conditions will be referred to as LL (cells grown at 20 µmol photons m^{-2} s^{-1}) and HL (cells grown at 300 µmol photons m^{-2} s^{-1}) in the following chapters. The cells were collected by centrifugation (7000 g for 5 minutes) and after washing with distilled water stored at -80 °C.

3.3. Thylakoid membrane isolation

3.3.1. Isolation of thylakoid membranes

The isolation buffer was prepared using the chemicals mentioned in Table 2 at the respective concentrations in water. Subsequently the pH was adjusted to 7.5 using 1 M NaOH.

Table 2: Chemicals and their concentrations for the preparation of the isolation buffer.

Substance	Molar mass / g mol^{-1}	Concentration / mM
HEPES	238.31	50
Potassium chloride	74.56	2
EDTA	372.2	10

The isolation was performed on ice in a dark room with dim green light. 10 mL of the buffer and EDTA-free Protease Inhibitor Cocktail (1 mL to 10 mL of buffer) were added to the sample. The cells were well resuspended before disrupting them using the EmulsiFlex-C5 high pressure cell disrupter (Avestin Inc., Canada) at 500 – 800 bar for 5 minutes. The broken cells were centrifuged for 5 minutes at 4000 g. Further, the supernatant was collected and centrifuged for another hour (Beckman Coulter, rotor 70 TI) at 45000 rpm. The pellet, which are the thylakoid membranes, obtained from the last centrifugation was resuspended in buffer to a chlorophyll concentration (Chl a) of 0.5 mg mL^{-1}. For the measurement using a spectrophotometer (UV 300 Spectronic Unicam, UK) a few microliters of the pellet were mixed with 80 % acetone and the chlorophyll concentration was determined according to Lichtenthaler [21].

To solubilize the thylakoid membranes, 1 % β-DM was added at a chlorophyll concentration of 1 mg mL^{-1}, so here it was 0.5 % β-DM as the concentration of chlorophyll is 0.5 mg mL^{-1}. The mixture was stirred in the dark on ice for 30 minutes. This was followed by centrifugation for 30 minutes at 15000 g, after which the supernatant was collected.

3.3.2. Sucrose density gradient

A buffer containing 0.02 % n-dodecyl-β-D-maltoside was prepared by adding the respective amount of β-DM to the isolation buffer prepared before (Table 2). In this buffer 19 g of sucrose were dissolved and the resulting 0.6 M sucrose solution was filled into centrifuge tubes. The tubes were frozen at -80 °C followed by thawing at 5 °C before loading the samples. This provided a 0 – 1.2 M continuous sucrose density gradient.

From the supernatant, which was gained as described above (3.3.1), 200 µL were now loaded on the sucrose gradient. Ultracentrifugation at 30000 rpm using a SW 40 TI swing-out rotor (Beckman Coulter) was performed overnight for 16 hours at 4 °C.

3.3.3. Gradient zone collection

Various zones, slightly different in colour, could be distinguished in the sucrose gradient. These zones were collected with a syringe and then concentrate by centrifuging using centrifugal filter units (Amicon Ultra, Merck). To get rid of excess sucrose the samples were washed with isolation buffer containing 0.02 % β-DM. The concentrated samples were kept in the freezer at -80 °C until needed for further analysis.

3.4. High performance liquid chromatography

For pigment analysis HPLC (Pump Controller Delta 600, a manual injection system and a PDA 2996 detector, Waters, USA) was applied using the method of Jeffery et al. [17]. The stationary phase was a reversed-phase column (Zorbax SB-C18, 4.6 × 150 mm, 5 µm, Agilent, USA). In the Table 3 the mobile phases are stated.

Table 3: Mobile phases composition.

Solvent A	MeOH:0.5M Ammonium acetate – 80:20, pH 7.2 v/v
Solvent C	Acetonitrile:Water – 90:10
Solvent D	Ethyl acetate 100%

A gradient of these solvents was used during the total run time of 40 minutes according to Table 4. The flow rate was constantly 1 mL min^{-1}.

Table 4: Gradient of mobile phases.

Time	% A	% C	% D
0.00	100.0	0.0	0.0
4.00	0.0	100.0	0.0
18.00	0.0	20.0	80.0
25.00	0.0	20.0	80.0
28.00	0.0	100.0	0.0
31.00	100.0	0.0	0.0
36.00	100.0	0.0	0.0

The thawed samples were suspended in methanol:acetone – 2:1 to reach optimal concentrations for detection of the pigments. The injection volume was 100 µL, which were injected manually. Samples containing thylakoid membranes, which were not further processed, were filtered using a syringe filter unit before injecting the sample into the HPLC.

The area under the peaks in the chromatogram was determined at the wavelength corresponding to the extinction coefficient of the respective pigment, which are given in Table 5.

Table 5: Extinction coefficients with wavelength of the pigments observed in the chromatogram.

Pigment	Extinction coefficients [L mol^{-1} cm^{-1}]	at wavelength [nm]
Chlorophyll c_3	$218.4 \cdot 10^3$	457
Chlorophyll c_2	$227.7 \cdot 10^3$	444
Fucoxanthin	$109.0 \cdot 10^3$	448
19'-hexafucoxanthin	$109.0 \cdot 10^3$	448
Diadinoxanthin	$130.0 \cdot 10^3$	448
Diatoxanthin	$119.0 \cdot 10^3$	444
Chlorophyll c_2-MGDG	$227.7 \cdot 10^3$	455
Chlorophyll a	$78.0 \cdot 10^3$	662
β-carotene	$134.0 \cdot 10^3$	453

Further processing of the data is described in the following chapters.

3.5. Ion exchange chromatography

The mobile phase was prepared by diluting NaCl in isolation buffer with β-DM (for preparation see 3.3.1) to a 1 M solution. This stock solution was then diluted with isolation buffer to 50 mL of a 300 mM and 50 mL of a 5 mM solution. As a stationary phase DEAE–Sepharose CL-6B was used.

The column with the stationary phase was washed for several minutes, then the sample was loaded. In the beginning the flow was set to 5 mL min^{-1} and the 5 mM solution was used. After 5 – 7 fractions were obtained the linear gradient (5 – 300 mM) was started at a flow of 2 mL min^{-1}. With these settings 14 – 18 fractions were collected. In the end the 300 mM solution was used for a few more minutes to elute any other material and to wash the column. During the whole run the apparatus was kept at 4 °C.

3.6. UV-Vis absorption

The absorbance of the samples obtained from ion exchange chromatography was measured using a UV-Vis spectrophotometer (SHIMADZU UV-2600). A fast scan was employed in the range of 350 – 750 nm. Based on the absorbance measured, 2 – 3 samples were chosen in a way that a new homogenous fraction was obtained. These new fractions were concentrated by centrifugation using centrifugal filter units (Amicon Ultra, Merck) and washed with the isolation buffer without NaCl once. The samples were frozen with liquid nitrogen and kept at -80 °C until analysed by HPLC.

3.7. Principles of used methods

Ultracentrifugation of the sucrose gradient is used to separate the analytes in the sample according to their density. The molecules to be separated are loaded on top of the continuous sucrose density gradient. Due to centrifugal forces the complexes will travel through the gradient until the surrounding gradient density equals their own. In this way, various bands are formed in the gradient, in each of which all the particles have the same density. The particles in different bands might be of the same size, since they are mainly separated on the basis of their density.

HPLC is a chromatographic method used to analyse non-volatile substances. Together with the mobile phase the analytes are pumped through a column, which is the stationary phase, at a high pressure. The mode used most frequently, and also for this work, is reversed-phase HPLC. A non-polar stationary and a polar mobile phase are used in contrast to normal-phase chromatography where the stationary phase is polar and the mobile phase non-polar. A common stationary phase used in RP-HPLC is C18, an octadecyl carbon chain bonded to silica. As mobile phase organic solvents and water, which is needed to increase retention times, are used. Examples for detectors are UV/Vis, photodiode array (PDA) or mass spectrometry.

In ion exchange chromatography analytes are separated based on their charge. The matrix of the stationary phase contains charged functional groups that have counterions reversibly bound. The analytes bind to the stationary phase in exchange for these counterions from the solution. There are two types of ion exchange, cation exchange and anion exchange. The stationary phase used during this work is a weak anion exchanger. Anion exchange chromatography is applied when the molecules of interest are anions. In the other case cation

exchange chromatography is used. Elution of the analytes bound to the column is achieved by using an eluant containing ions in higher concentration or by changing the pH.

UV/Vis absorption spectroscopy uses light in the ultraviolet-visible spectral region to obtain information about the molecule of interest. Analytes with π-electrons and/or non-bonding electrons can be detected since they absorb in this region. The absorption is caused because the excited electrons in the molecules undergo electronic transitions. Each wavelength of light corresponds to a certain energy. If the energy has the right amount to promote an electron from the HOMO to the LUMO this particular wavelength will be absorbed. Electrons which can be excited easily, due to a lower energy gap between the HOMO and the LUMO absorb at a longer wavelength.

4. Results

4.1. Thylakoid membranes

Some of the thylakoid membranes were not separated with a sucrose gradient but their pigment content was analysed by HPLC, as described in 3.4, right away. A typical chromatogram for cells grown at low and high light conditions is shown in the following (Figure 4, Figure 5). The peaks were identified by comparing their spectra (Figure 6) and retention times (Table 6), which are given below, to literature values [17].

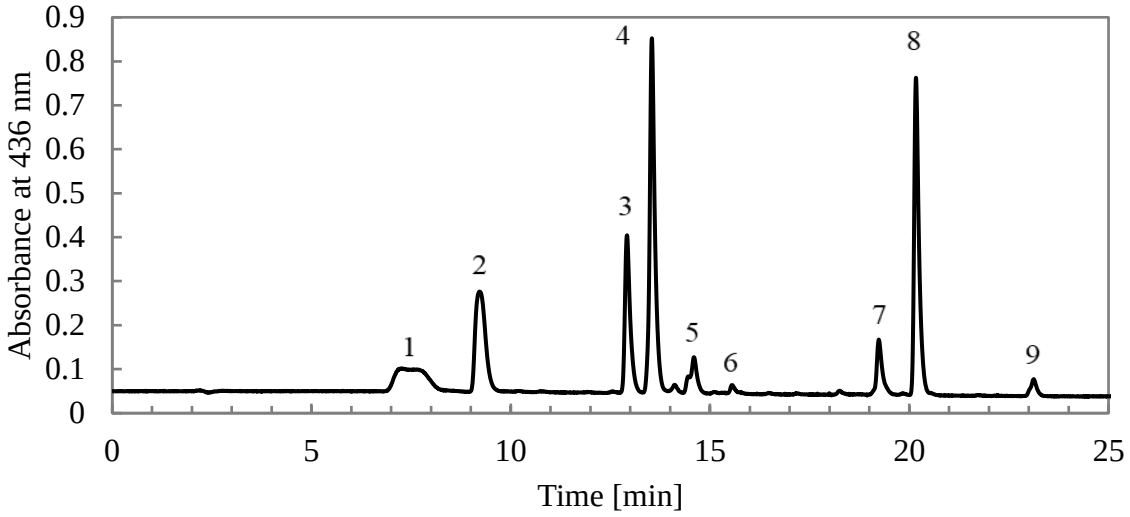


Figure 4: HPLC chromatogram of the thylakoid membranes of cells grown at LL conditions.

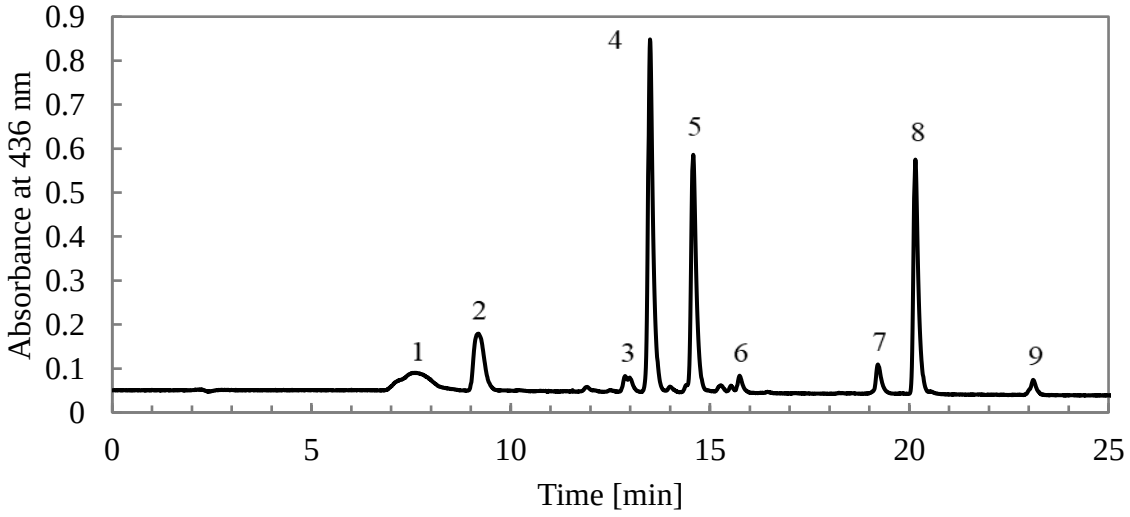
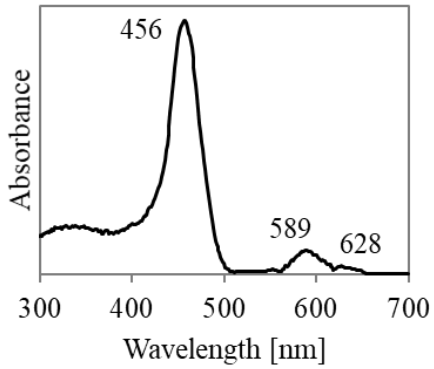
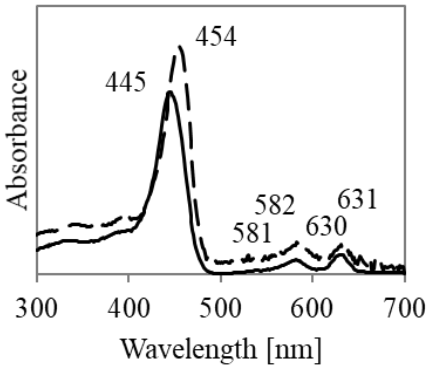


Figure 5: HPLC chromatogram of the thylakoid membranes of cells grown at HL conditions.

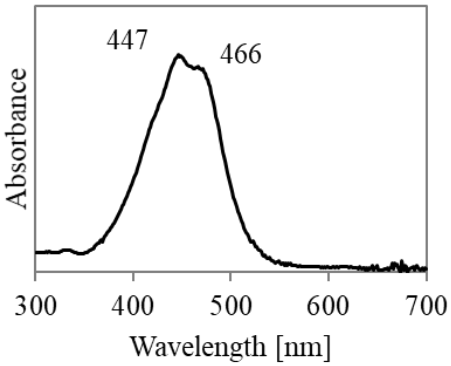
Chlorophyll c_3



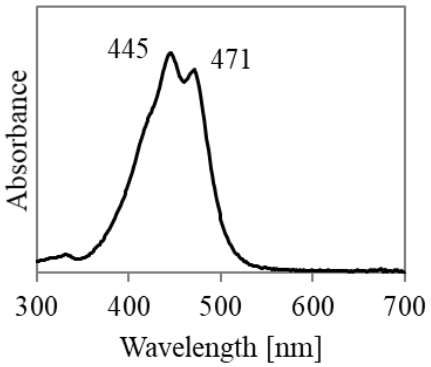
Chlorophyll c_2 + Chl c_2-MGDG (- -)



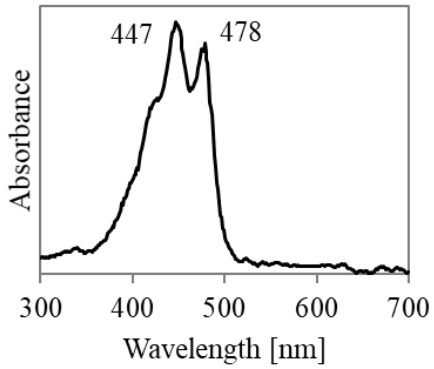
Fucoxanthin



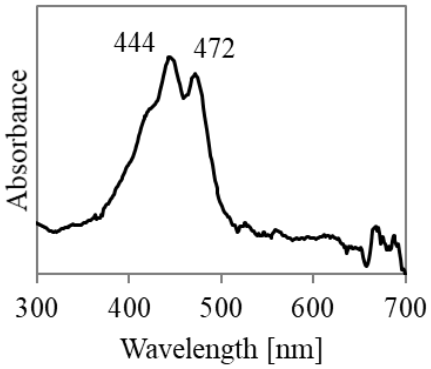
19′-Hexanoyloxyfucoxanthin



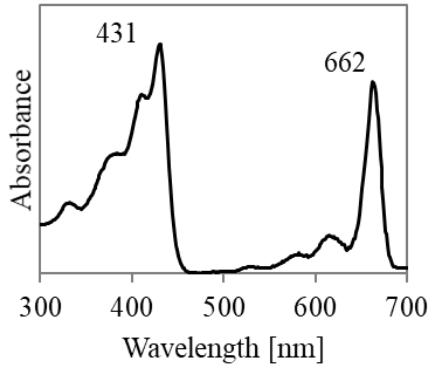
Diadinoxanthin



Diatoxanthin



Chlorophyll a



β-carotene

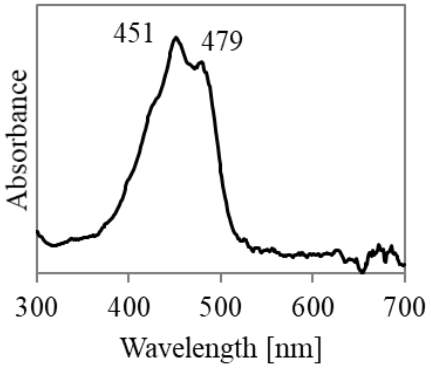


Figure 6: Spectra of the pigments from HPLC detector used to identify each in the chromatogram including the absorption maxima of each pigment.

In the chromatograms the numbers stand for the pigments in the samples as identified in Table 6. Also summarized in the following table are the respective retention times, which are mean values of the slightly varying times in different samples.

Table 6: Labelling of the peaks in the chromatograms (Figure 4, Figure 5) with corresponding retention times of each pigment.

Peak Number	Pigment	Retention time
1	Chlorophyll c_3	7.6
2	Chlorophyll c_2	9.2
3	Fucoxanthin	12.9
4	19′-Hexanoyloxyfucoxanthin	13.5
5	Diadinoxanthin	14.6
6	Diatoxanthin	15.6
7	Chlorophyll c_2-MGDG	19.2
8	Chlorophyll a	20.2
9	β-carotene	23.2

It should be noted that the samples do not have the same concentration and therefore the areas in the chromatogram must not be compared to each other directly. Only the ratios between peaks may be used for interpretation at this point. Two differences in the ratios can immediately be noticed. In the LL sample the ratio of 19´-HFx (4) to Fx (3) is smaller than in the HL cells. Also, the content of Dd (5) is significantly different with higher contents found in the HL cells. This trend is how it was expected from observations in other algae (1.3.1). At higher illumination of the cells 19´-HFx is made from Fx and more Dd is synthesised.

As mentioned above already (3.4), the peak areas were then divided by the corresponding extinction coefficients to end up with the respective quantities. These quantities were normalised to chlorophyll (100%). This way the chromatograms can be compared to each other without the need to know the concentration of the original sample.

The thylakoid membranes were analysed for two samples of cells grown at LL and two from HL conditions. The results after dividing the area under the chromatogram by the extinction coefficients and normalizing it to chlorophyll are summarized in the following. Since the results were similar, the mean and the standard deviation was calculated for the LL and the HL cells, respectively.

Three different types of Chl c were identified from the chromatograms, namely Chl c_3, Chl c_2 and Chl c_2-MGDG. Their contents in the samples are given in Table 7.

Table 7: Quantities of Chl c_3, Chl c_2 and Chl c_2-MGDG and the sum of them.

Cells	Chl c_3	Chl c_2	Chl c_2-MGDG	Total Chl c
LL	31.9 ± 0.5	23.8 ± 1.2	10.1 ± 0.6	65.8 ± 1.1
HL	27.2 ± 0.2	25.8 ± 1.5	10.4 ± 0.3	63.3 ± 1.6

Among the different Chl c pigments the variations between low and high light samples are too little to draw any conclusions from it. The total amount of all Chl c pigments stayed constant compared to Chl a.

Also, the trend for Fx and the modified 19´-HFx which could be observed from the chromatogram is supported by the obtained numbers (Table 8).

Table 8: Calculated quantities of Fx and 19´-HFx and corresponding percentage of the total amount of Fx + 19'-HFx.

Cells	Fx	19´-HFx	Fx %	19´-HFx %
LL	39.4 ± 0.6	87.9 ± 1.4	30.9 ± 0.0	69.1 ± 0.0
HL	10.4 ± 2.4	113.8 ± 3.4	8.3 ± 1.6	91.7 ± 1.6

In cells grown at LL 19´-HFx accounts for around 69% of the total amount of fucoxanthins, while it is more than 90% in cells growing at higher illumination. The sum of the fucoxanthins is similar in each sample (125.8 ± 4.6) which suggests no net-synthesis of either of the pigments.

The observation of the ratio of Dd to Dt in the chromatogram could be verified when calculating the values (Table 9).

Table 9: Quantities of Dd and Dt and the calculated ratio.

Cells	Dd	Dt	Ratio Dd / Dt	Ratio Fx + 19´-HFx / Dd + Dt
LL	10.0 ± 0.2	1.4 ± 0.1	7.1 ± 0.1	11.2 ± 0.4
HL	58.4 ± 5.4	4.0 ± 0.1	14.5 ± 1.5	2.0 ± 0.3

There seems to be especially a high accumulation of Dd when exposed to higher light therefore the ratio of Dd to Dt is higher in the illuminated cells. However, the quantities of Dt are small in each sample, thus there might be higher errors. Comparing the sums of Fx and 19´-HFx to the sum of Dd and Dt gives more reliable results. As mentioned before the total amount of the two Fxs does not change, but there is a net-synthesis of Dd. This is supported by the numbers where the ratio of Fx + 19´-HFx to Dd + Dt is higher in cells grown at LL conditions.

4.2. Sucrose gradient

The aim was now to further separate the proteins of the thylakoid membranes to obtain the photosynthetic proteins and analyse their pigment compositions. This separation was done by sucrose gradient ultracentrifugation. Four different samples of cells, two with cells grown at low and two at high light conditions, were processed as described in 3.3.

Below, one example of a gradient of cells grown at LL and one of HL conditions is shown. In addition, the zones as they were collected are depicted. The other gradients each looked similar to these.

Loading the sucrose gradient with 100 µL instead of 200 µL resulted in the same zones, however they were narrower which made sampling more difficult and gave only very little amounts for further analysis. Therefore, all the gradients used for analysis by HPLC were loaded with 200 µL of thylakoid membranes.

In the gradients of cells cultivated under LL there was a very narrow greenish band observed in zone 1, as it can be seen in Figure 7. In sucrose gradients of cells grown at HL there was no green band, but a uniformly yellowish coloured zone (see Figure 7).

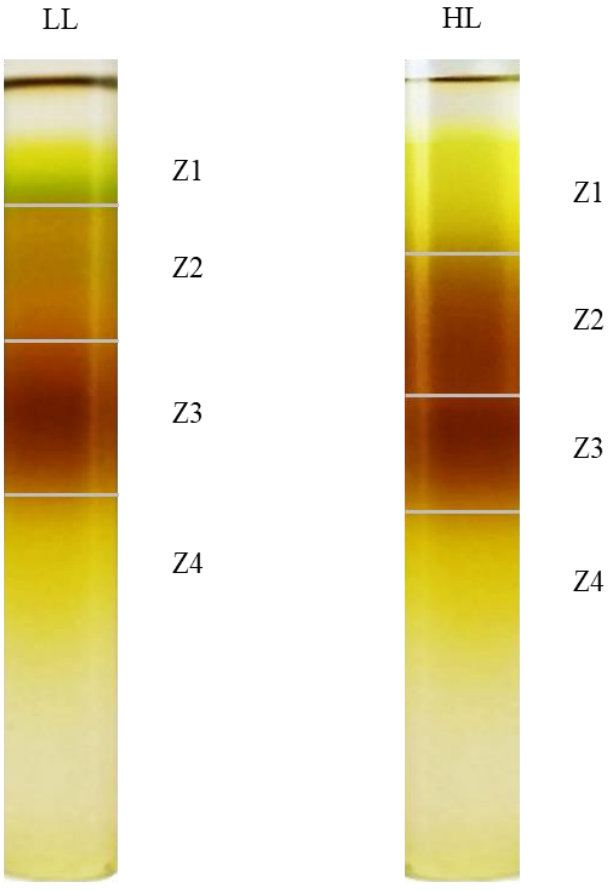


Figure 7: Representative sucrose gradients of solubilized thylakoid membranes from cells grown at LL (left) and HL (right).

Pigment analysis of zone 1, of cells from LL and from HL conditions, showed high concentrations of β-carotene. At the top of the gradient typically free pigments are found [22] and thus this band did not play a major role in further analysis.

Three fractions were found in all gradients independent of the illumination treatment, labelled Z2, Z3 and Z4. The pigment content in each of them was analysed by HPLC giving similar chromatograms as the thylakoid membranes (4.1). Therefore, the chromatograms are not shown again in this section. The results were similar for the two gradients of the same light conditions, each, so the mean was calculated for reasons of clarity and comprehensibility (Table 10). The results are presented as above, normalised to Chl a content.

Table 10: Calculated quantities for each of the pigments in the three zones.

Zone	Cells	Chl c_3	Chl c_2	Fx	19´-HFx	Dd	Dt	Chl c_2-MGDG	β-car.
2	LL	42.2 ± 3.2	29.8 ± 1.1	48.8 ± 1.2	104.6 ± 5.5	8.8 ± 0.7	1.2 ± 0.1	12.7 ± 1.0	2.6 ± 0.1
	HL	35.2 ± 2.6	29.4 ± 2.1	13.2 ± 1.7	144.4 ± 4.7	75.5 ± 5.5	4.7 ± 0.4	11.5 ± 0.4	2.5 ± 0.7
3	LL	42.2 ± 4.9	30.7 ± 4.6	52.9 ± 2.2	119.7 ± 4.6	11.7 ± 0.1	1.8 ± 0.3	14.3 ± 1.6	2.0 ± 0.1
	HL	34.8 ± 1.8	28.6 ± 1.2	13.9 ± 1.5	148.8 ± 3.5	65.1 ± 3.8	3.7 ± 0.2	11.7 ± 0.5	1.8 ± 0.1
4	LL	38.5 ± 3.4	20.1 ± 0.9	33.3 ± 2.7	78.7 ± 1.5	18.1 ± 1.8	4.9 ± 0.5	8.2 ± 2.9	3.1 ± 0.2
	HL	31.7 ± 3.2	31.7 ± 5.3	9.1 ± 0.3	96.7 ± 4.6	41.2 ± 3.7	2.0 ± 0.1	9.0 ± 0.1	3.2 ± 1.0

Independent of the light conditions in zone 2 and 3 the most abundant pigment was 19´-HFx. As expected it was present in higher amounts in the cells grown at higher light intensities. In zone 4 it is Chl a that is the most abundant. This could have already been guessed from the colour of the respective zone. Zones 2 and 3 are brownish, while zone 4 is yellow-greenish.

The difference between LL and HL cells in Fx and 19´-HFx as well as in Dd and Dt can be observed again in all three zones.

In the following graphs (Figure 8, Figure 9 and Figure 10) the results are visualised. One plot was used each for the comparison of the same zone of cells grown in low and high light conditions.

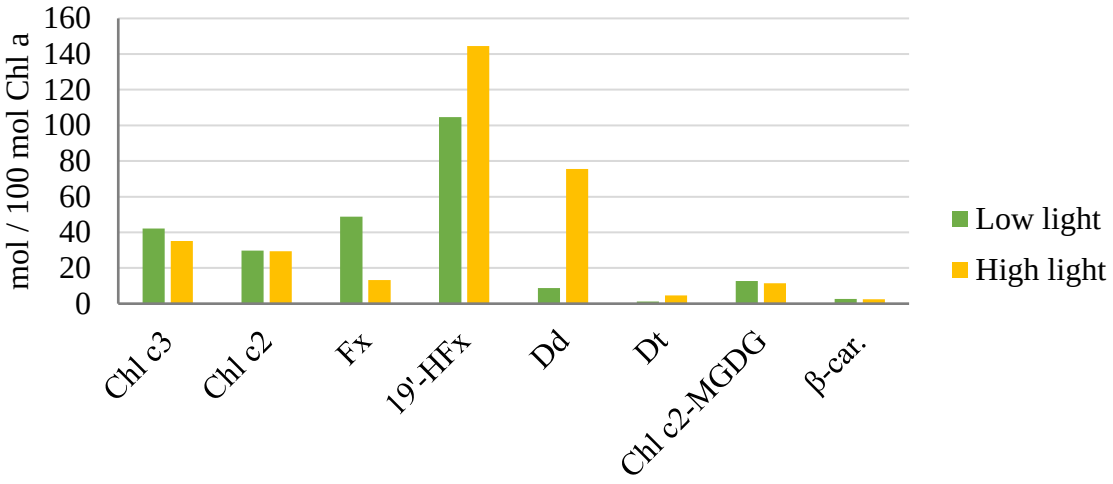


Figure 8: Comparison of zone 2 of cells grown at LL and HL.

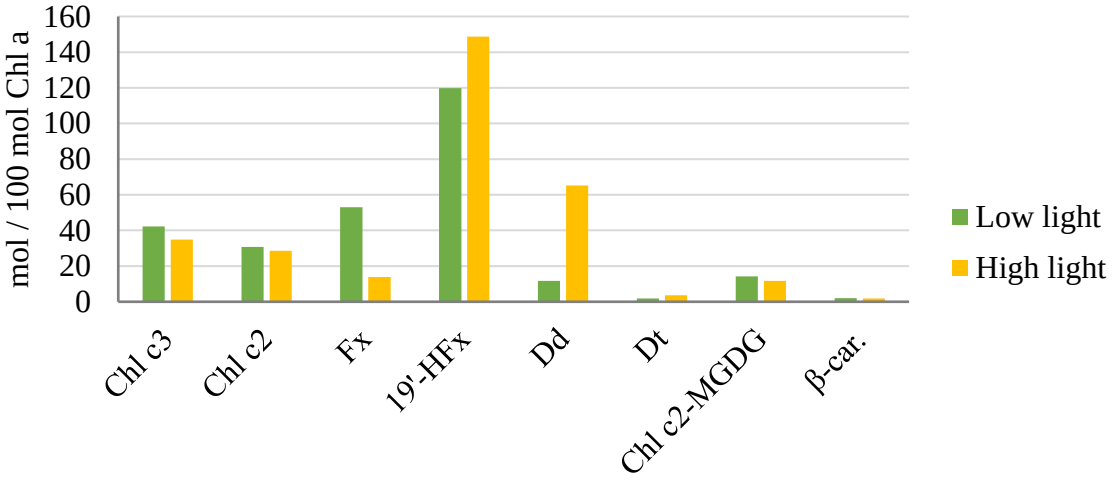


Figure 9: Comparison of zone 3 of cells grown at LL and HL.

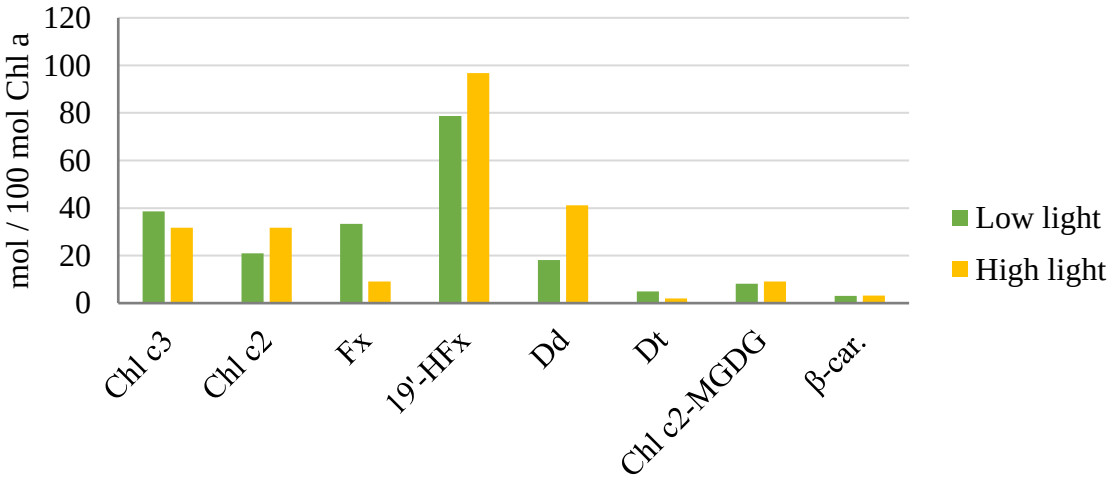


Figure 10: Comparison of zone 4 of cells grown at LL and HL.

The ratio of 19´-HFx to Fx is similar in each of the three zones, so no trend could be observed here. Concerning the ratio of Dd to Dt there might be a decrease in the ratio from the top to the bottom of the gradient, but the amounts of Dt were too small to be able to draw any conclusions from this.

4.3. Ion exchange chromatography

Based on the data obtained from the analysis of the zones of each gradient by HPLC, one zone was selected for further processing by ion exchange chromatography. For each of the four performed gradients it was zone 3 having the lowest content of β-carotene (see Table 10) and visually the highest concentration. Therefore, this zone was selected as a likely source of a major portion of the light-harvesting antennas from the thylakoid membranes. The ion exchange chromatography was carried out as described above (3.5) followed by measuring UV-Vis absorption spectra of the obtained fractions (3.6). The fractions to be pooled together were selected based on the ratio of their absorbances at 530 (fucoxanthins) and 672 nm (chlorophyll a). A representative chromatogram, used for the selection of the respective fractions, of one of the LL acclimated cells is shown below (Figure 11). The other three chromatograms looked very similar.

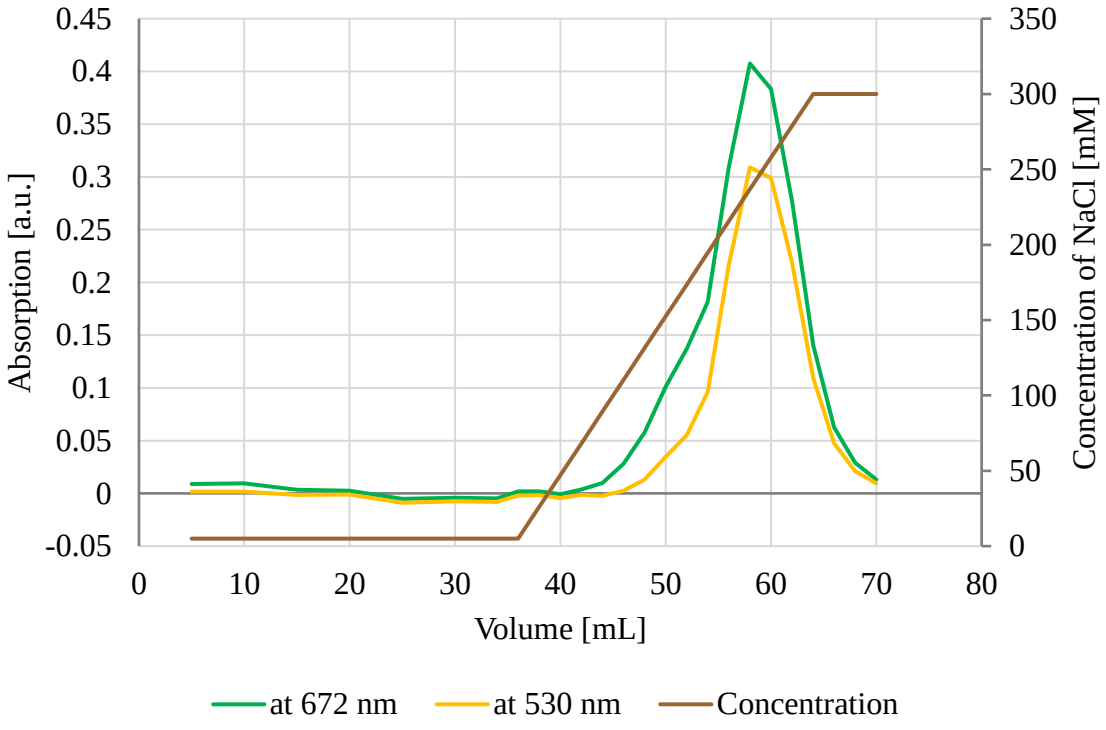


Figure 11: Chromatogram gained from ion exchange chromatography of LL cells plotted at 530 and 672 nm and the respective concentration of NaCl in the mobile phase.

The ratio of the absorbance at 530 nm to the absorbance 672 nm is plotted in Figure 12 to make it more visible. In addition, the fractions selected for pooling together are marked in the diagram. Of course, the selected fractions might be shifted slightly due to different times when fraction collector was started.

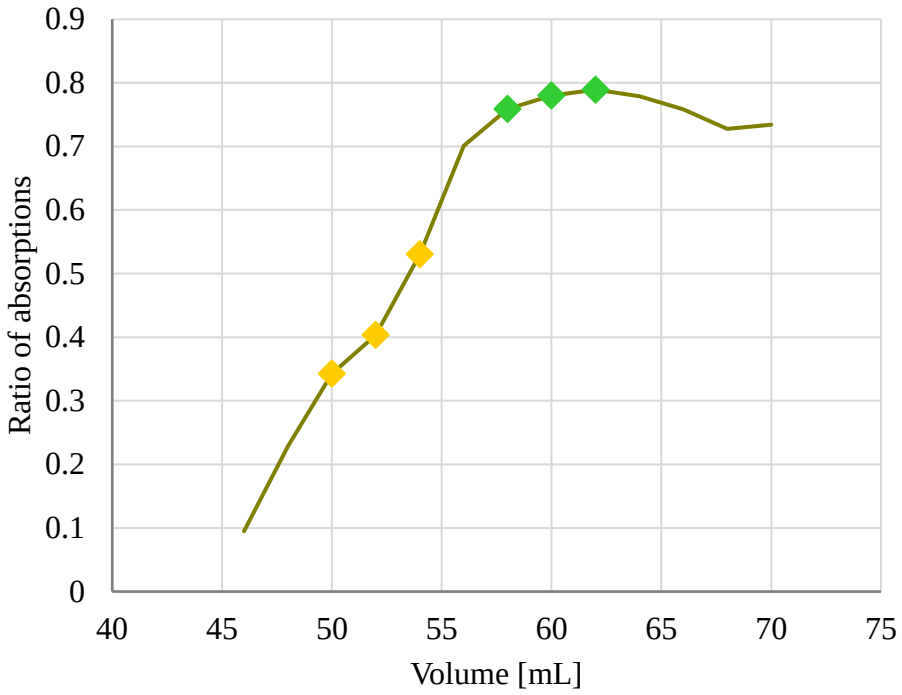


Figure 12: Ratio of the absorbance at 530 nm to the absorbance at 672 nm in the ion exchange chromatogram from Figure 11. The selected fractions are indicated by colored marks.

As shown in the Figure 12 fractions 8 – 10 (yellow marks) and fractions 12 – 14 (green marks) were pooled together to form two new fractions. The pigment content of the two resulting fractions was again analysed by HPLC. This was done for each of the salt gradients from the different cells. The second fraction (12 – 14) was having higher pigment content, as it can be seen from the figures above. This was the case for each of the samples. Therefore, only these results are shown here (Table 11). In Figure 13 are given the absorption spectra for the fractions 12 – 14 of one of the LL cells.

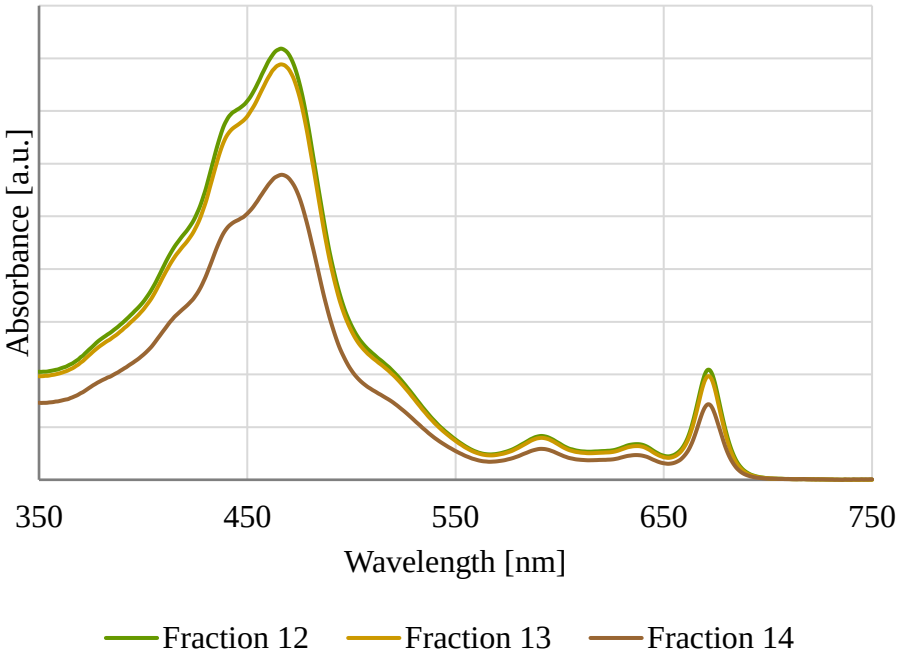


Figure 13: Absorption spectra of LL cells for the three major fractions from ion exchange chromatography which were pooled together and further analysed.

As mentioned above, also for HL cells the fractions with the highest pigment content were collected, following the same procedure as with the LL cells. In Figure 14 the absorption spectra for the selected fractions of the one of the samples of HL cells can be seen.

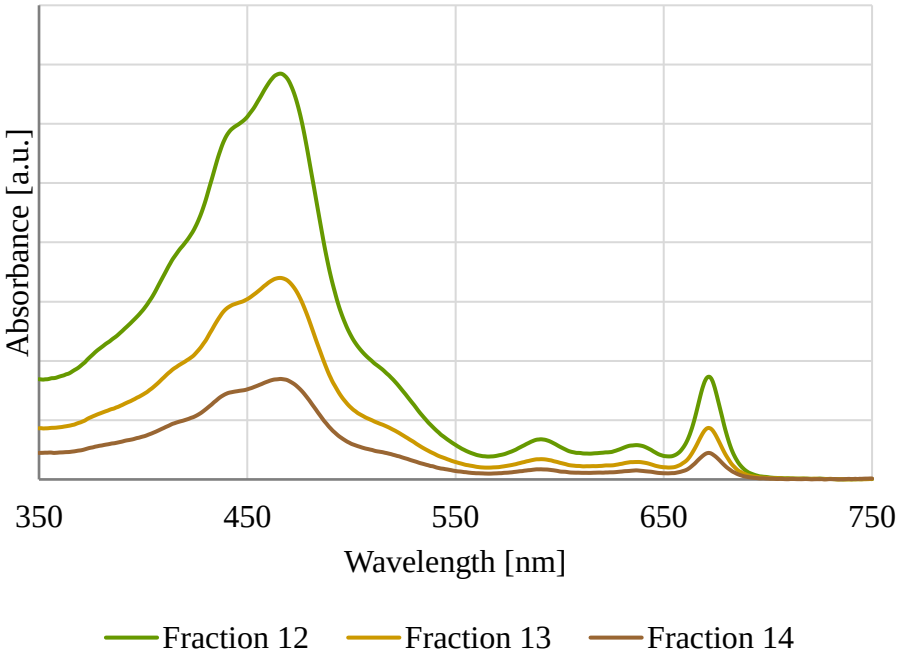


Figure 14: Absorption spectra of HL cells for the three major fractions from ion exchange chromatography which were pooled together and further analysed.

In both samples strong absorbance bands were observed from around 440 to 550 nm where chlorophylls and carotenoids, mainly Fx and Dd, have their absorption maxima. The absorbance of Chl a is visible at 440 nm and at 672 nm, the small peaks at 580 nm and at 636 nm are from Chl c. In the diatom *Cyclotella meneghiniana* a very similar spectra were observed from the sucrose gradient fractions. Here the absorbance between 475 and 565 nm was described to be carotenoids bound to the FCPs. Chl a and Chl c were observed at 672 and 636 nm, respectively [22].

The mean of the three fractions was calculated for the LL and the HL cells. To make them easily comparable, the data were normalized giving the maximum of the measurements the arbitrary value 100. The spectra are depicted in Figure 15.

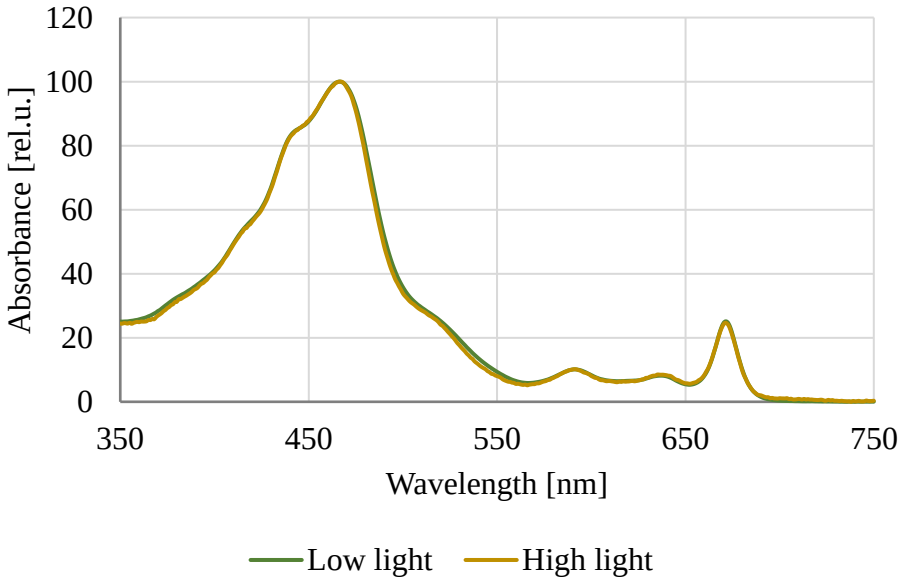


Figure 15: Comparison of the mean absorbance in the three selected fraction of LL and HL cells.

No significant differences between the spectra from LL and HL can be seen. This is likely because the sum of the Chls as well as of the Fxs does not vary significantly between the different light conditions. The difference in Dd and Dt is too small as to be noticeable.

As described before, the fractions with the highest pigment content were pooled together for further analysis. After dividing the areas under the peaks by the extinction coefficient, the values were normalized to Chl a. The mean of the results from the two LL and the two HL samples was calculated since the obtained values were similar, as it can be seen from the standard deviation. The data gained from pigment analysis of the new fractions are summarized in Table 11.

Table 11: Pigment content obtained from HPLC analysis of the second fraction from ion exchange chromatography of each sample of cells.

Cells	Chl c_3	Chl c_2	Fx	19´-HFx	Dd	Dt	Chl c_2-MGDG	β-car.
LL	69.5 ± 0.8	48.1 ± 1.1	66.6 ± 0.7	163.0 ± 1.1	3.7 ± 0.5	0.5 ± 0.1	22.2 ± 1.4	0.9 ± 0.1
HL	63.6 ± 0.7	44.6 ± 0.1	17.4 ± 1.0	234.4 ± 0.8	6.5 ± 0.4	0.6 ± 0.1	22.2 ± 0.2	1.0 ± 0.1

As mentioned before, the ion exchange chromatography was performed on zone 3 of each gradient. The results of the analysis of these zones by HPLC were already summarized in Table 10. The pigment content of zone 3 and corresponding samples purified by ion exchange chromatography are shown in Figure 16 and Figure 17.

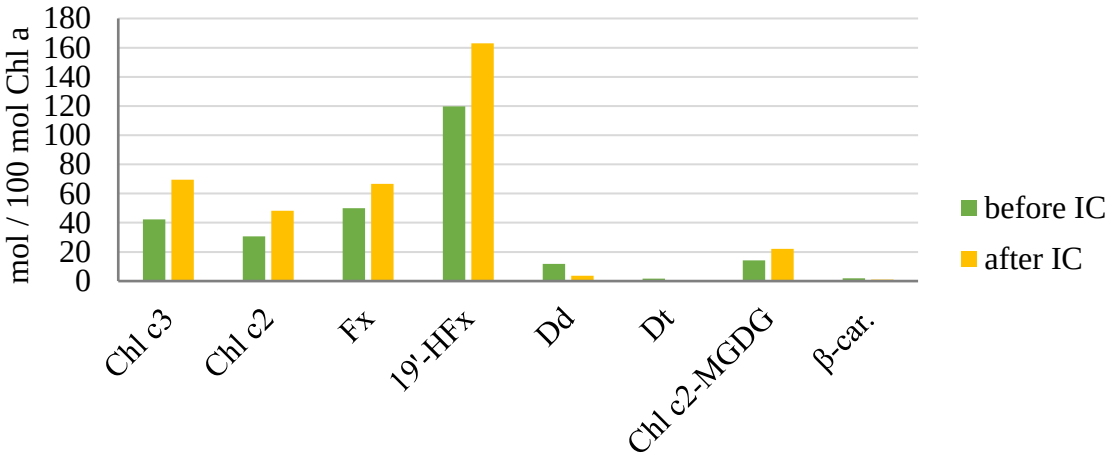


Figure 16: Comparison of the pigment composition of sucrose gradient zone 3 of LL samples before and after ion exchange chromatography.

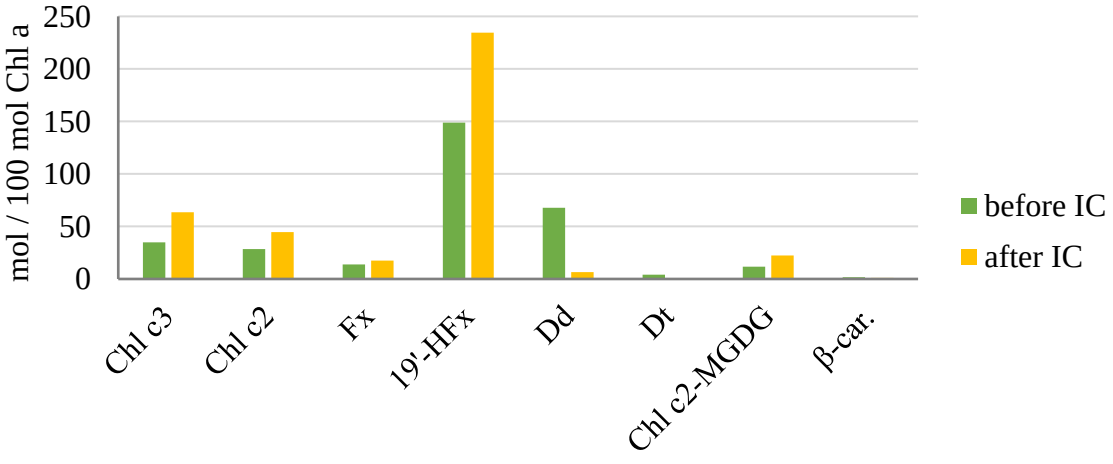


Figure 17: Comparison of the pigment composition of sucrose gradient zone 3 of HL samples before and after ion exchange chromatography.

For the samples of both light conditions the relative concentration after performing ion exchange chromatography was higher for all pigments except for Dd, Dt and β-carotene. These pigments were either not at all or very loosely bound to the analysed proteins or they were bound to co-purified proteins like photosystems which were eluted in other parts of the chromatograms than the major collected light-harvesting antennas. The ratio between 19´-HFx and Fx stayed the same or got even more pronounced in the high light cells (LL: 2.40 before IEC vs. 2.45 after IEC; HL: 10.73 before IEC vs. 13.47 after IEC). The concentration of Dd and especially Dt after ion exchange chromatography was too low for reliable quantification.

5. Discussion

In the first part of this work, pigment composition in cells grown at different illumination intensities was analysed. Pigment analysis of cells grown at LL showed higher relative quantities of Fx and Chl c_3 than in cells acclimated to HL. In diatoms and *A. anophagefferens*, the pigments mainly acting in light-harvesting are Fx and Chls c [8; 18]. The results of this work support the assumption that the same pigments are involved in light-harvesting of *E. huxleyi*. The occurrence of the Dd cycle in *E. huxleyi* is also supported by previous research. The xanthophyll carotenoids Dd and Dt are thus playing a role in photoprotection [5].

The decrease in Fx, comparing cells from LL to those from HL conditions, was correlated with an increase in 19´-HFx. Thus, the sum of the two Fxs was constant. This observation points to a conversion of Fx to 19´-HFx rather than to a de novo synthesis of 19´-HFx. In *E. huxleyi* the main Fx derivative seems to be 19´-HFx, but other have been reported as well. For example, at the C-19´position not only a hexanoyloxy can be bound but acyloxyfucoxanthins with four, five, seven and eight C-atoms were detected in *E. huxleyi* [23].

Chl c_3 seems to be present in higher amounts at LL [5; 18]. The results gained during this work do however not show significantly different quantities of Chl c_3. These discrepancies might be related to elution problems in HPLC and therefore insufficient peak separation. Comparing the obtained chromatograms to those presented by Garrido et al. [5] shows that there might be a problem of co-elution resulting in a broad peak. The peak assigned to Chl c_3 here seems to be not only Chl c_3 but also MV chl c_3. Due to the very similar polarities of the two pigments, the peaks merge. Further, these two different Chl c_3 pigments cannot be distinguished by absorption spectra [24]. MV chl c_3 is described as a HL pigment and might therefore counteract the decrease in Chl c_3 observed in HL cells [5].

The content of Chl c_2 as well as Chl c_2-MGDG does not strongly change depending on the light condition and no trend could be observed. Similar results were reported for *E. huxleyi* before [18]. The sum of the Chls c seems to be related to Chl a since the ratio of these is the same in LL and HL cells. This is in accordance to previous research [5].

Like in the diatom *P. tricornutum* at HL a sharp increase in the quantities of Dd and Dt was measured, while these pigments were strongly decreasing at LL. Dt is formed form Dd in the xanthophyll cycle, but in contrast to Fx and 19´-HFx the xanthophylls Dd and Dt are synthesized de novo [5; 18; 12].

Most of the pigments are bound to the protein complexes. This binding might however change when the xanthophyll is altered, e.g. by the addition of the butanoyloxy or hexanoyloxy group to Fx, resulting in new abundances and compositions of the proteins. As mentioned before Alami et. al [13] describe that the addition of the acyloxy chain to Fx causes the energy transfer from the antenna systems to the RCs to be less effective. Garrido et al. [5] hypothesize that the substituted forms of Fx are not only less efficient in energy transfer but also in harvesting light at different wavelength. The derivatives of Fx could result in conformational changes of the protein and its binding sites. Due to a new three-dimensional structure and altered interactions with the xanthophylls the organism might absorb at different wavelength [5]. In E. huxleyi Dd probably operates in a similar way in NPQ as Zx does in higher plants. Most likely Zx is needed to fully develop NPQ, because it acts between the complexes and in this way creates quenching sites. The exact principle of action is not yet fully understood [9].

Unfortunately, the separation was not very successful as the gradients were not of very good resolution compared to Litvin et al. [11], Gardian et al. [25] and Büchel [22]. Shaking of the centrifuge tubes holding the gradient or too fast melting might have negatively influenced the quality of the gradient. Nevertheless, four zones similar in each of the gradients were obtained. The greenish-yellow zone at the top of the gradient was identified as free pigments and therefore not of importance for the results. Zone 2 and 3 were of brownish colour, zone 4 was yellowish. High contents of β-carotene were found in zone 2 and 4, with slightly higher quantities in the latter one. Based on this, the colour and comparison to literature these two bands were identified to be rich in photosystems. Since zone 2 is closer to the top, it can be assumed that these are broken PS or PS cores. The highest quantities of Fx + 19´-HFx were measured in zone 3. Again, due to this observation but also the colour it was concluded that the major LHC were in this zone as LHCs are the most abundant Chl-binding proteins [22; 11; 25]. The ratio between Fx and 19´-HFx was stable in the gradient. This indicates that the protein complexes all bind these carotenoids in similar amounts and are not selective towards one or the other Fx. In gradients of LL cells, the content of Dd + Dt increased down the gradient. This might point to preference for Dd + Dt in LHCs attached to photosystems. The opposite trend was seen in cells grown at HL. The active antenna size is reduced when cells are adapting to higher light intensities [18], which could explain this observation.

The LHC purified by ion exchange chromatography contained high quantities of the light-harvesting pigments Chl c_3, Chl c_2 and Chl c_2-MGDG as well as of Fx and 19´-HFx. Chl c_2-MGDG is a quite bulky molecule and its incorporation into the LHC is unique among the known light-harvesting antenna systems. That said, the Chl c_2-MGDG content was only 1 out

of 6 Chl c pigments. Given that the expected stoichiometry in diatom FCP is 4 Chl a to 1 Chl c [26], this purification with 6 Chl c per 4 Chl a was very rich in Chl c. The general difference in the Fxs between LL and HL cells could be seen clearly and correlated well with the content of Fxs in thylakoid membranes. Thus, it appears that both Fxs are tightly bound to the LHCs and that the proteins can accommodate both. Whether this means that the peptides used in LHC assembly are different in HL and LL remains to be seen in the future.

Ratios between Dd and Dt could not be calculated because the amount of these pigments was too little. But the overall content of these pigments was diminished after ion exchange purification. This suggests that these xanthophylls are not binding to the antenna complexes. It was recently reported by Xu et al. [9] that Zx is located at the periphery but not bound to the plant antennas. Therefore, the pigments will be lost when performing ion exchange chromatography. In contrast the quantities of the Fxs did not decrease suggesting that these pigments are bound to the proteins and therefore not lost during purification.

6. Conclusions

Previous research showed that the photoprotective mechanisms utilized by higher plants, diatoms and other algae are present in *E. huxleyi* as well. Two of these mechanisms used for photoacclimation were observed during this work.

The two major Fxs found in *E. huxleyi* are Fx and 19´-HFx, which differ by the presence of a hexanoyloxy group. These two pigments, like the other main light-harvesting pigments Chl a and different Chls c, are bound to the antenna complexes. Upon higher illumination the hexanoyloxy group is added to Fx creating 19´-HFx. It is believed that the bulkier molecule induces conformational and spatial changes in the light-harvesting proteins and consequently reduces the efficiency of the energy transfer from the antennas to the RCs.

In HL conditions, approximately 92% of the total Fx pool is 19´-HFx. For comparison, 19´-HFx makes up about 69% at LL. The sum of the two main Fxs was constantly about 1.3 times higher than the content of Chl a.

Also involved in the adaption to changing light conditions is the xanthophyll cycle. *E. huxleyi* utilizes the Dd cycle. However, this pigment is not incorporated in the LHC antenna and it is presumably located in the lipid phase or on the periphery of the protein.

In HL cells the ratio of Dd to Dt was twice as high as in LL cells. The content of Dd was around 6 times higher in HL cells than in LL cells.

7. References

1. **I. Jakob, F.Weggenmann, C.Posten.** Cultivation of Emiliania huxleyi for coccolith production. *Algal Research.* 2018, 31, pp. 47-59.

2. **R. Laguna, J. Romo, B. A. Read, T. M. Wahlund.** Induction of phase variation events in the life cycle of the marine coccolithophorid Emiliania huxleyi. *Applied and Environmental Microbiology.* 09 2001, Vol. 67, 9, pp. 3824-3831.

3. **P. M. Holligan, E. Fernandez, J. Aiken, W. M. Balch, P. Boyd, P. H. Burkill, M. Finch, S. B. Groom, G. Malin, K. Muller, D. A. Purdie, C. Robinson, C. C. Trees, S. M. Turner, P v. d. Wal.** A biogeochemical study of the coccolithophore, Emiliania huxleyi, in the north atlantic. *Global Biogeochemical Cycles.* 12 1993, Vol. 7, 4, pp. 879-900.

4. **T. Tyrrell, A. Merico.** Emiliania huxleyi: bloom observations and the conditions that induce them. [ed.] Springer-Verlag Berlin Heidelberg. 2004.

5. **J. L. Garrido, C. Brunet, F. Rodriguez.** Pigment variations in Emiliania huxleyi (CCMP370) as a response to changes in light intensity or quality. *Environmental Microbiology.* 2016, Vol. 18, 12, pp. 4412–4425.

6. **M. D. Iglesias-Rodriguez, P. R. Halloran, R. E. M. Rickaby, I. R. Hall, E. Colmenero-Hidalgo, J. R. Gittins, D. R. H. Green, T. Tyrrell, S. J. Gibbs, P. v. Dassow, E. Rehm, E. V. Armbrust, K. P. Boessenkool.** Phytoplankton calcification in a high-CO2 world. *Science.* 18 04 2008, pp. 336-340.

7. **R. E. Blankenship.** *Molecular mechanisms of photosynthesis, 2nd edition.* s.l. : Wiley-Blackwell, 2014. ISBN: 978-1-405-18976-7.

8. **P. Kuczynska, M. Jemiola-Rzeminska, K. Strzalka.** Photosynthetic pigments in diatoms. *Marine Drugs.* 2015, 13, pp. 5847-5881.

9. **P. Xu, L. Tian, M. Kloz, R. Croce.** Molecular insights into Zeaxanthin-dependent quenching in higher plants. *Nature, Scientific Reports.* 2015, Vol. 5, 13679.

10. **B. Lepetit, R. Goss, T. Jakob, C. Wilhelm.** Molecular dynamics of the diatom thylakoid membrane under different light conditions. *Photosynthesis Research - Springer.* 2012, 111, pp. 245-257.

11. **R. Litvin, D. Bina, M. Herbstova, Z. Gardian.** Architecture of the light-harvesting apparatus of the eustigmatophyte alga Nannochloropsis oceanica. *Photosynthesis Research - Springer.* 2016.

12. **C. Wilhelm, A. Jungandreas, T. Jakob, R. Goss.** Light acclimation in diatoms: From phenomenology to mechanisms. *Marine Genomics.* 2014.

13. **A. N. Macpherson, R. G. Hiller.** Light-harvesting systems in chlorophyll c-containing algae. *Springer, Dordrecht.* 2003, 13, pp. 323-352.

14. **B. Grimm, R. J. Porra, W. Rüdiger, H. Scheer.** *An Overview of Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications.* s.l. : Springer, 2006.

15. **D. Latowski, S. Schaller, M. Olchawa-Pajor, R. Goss, and K. Strzalka.** Violaxanthin and diadinoxanthin cycles as an important photoprotective mechanism in photosynthesis. *Russian Journal of Plant Physiology.* 2011, Vol. 56, 6, pp. 952-964.

16. **P. Horton, A. Ruban.** Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. *Journal of Experimental Botany.* 2004, Vol. 56, 411, pp. 365–373.

17. **S.W. Jeffrey, R.F.C. Mantoura, S.W. Wright.** *Phytoplankton pigments in oceanography: guidelines to modern methods. Monographs on oceanographic methodology, vol 10, 2nd edition.* Pairs : UNESCO Publishing, 2005.

18. **M. Alami, D. Lazar, B. R. Green.** The harmful alga Aureococcus anophagefferens utilizes 19′-butanoyloxyfucoxanthin as well as xanthophyll cycle carotenoids in acclimating to higher light intensities. *Biochimica et Biophysica Acta.* 2012, pp. 1557-1564.

19. **B. A. McKew, P. Davey, S. J. Finch, J. Hopkins, S. C. Lefebvre, M. V. Metodiev, K. Oxborough, C. A. Raines, T. Lawson, R. J. Geider.** The trade-off between the light-harvesting and photoprotective functions of fucoxanthin-chlorophyll proteins dominates light acclimation in Emiliania huxleyi (clone CCMP 1516). *New Phytologist.* 2013, 200, pp. 74–85.

20. **R. R. L. Guillard, J. H. Ryther.** Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea (Cleve) Gran. *Canadian Journal of Microbiology.* 1962, Vol. 8, pp. 229-239.

21. **H. K. Lichtenthalter.** Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods in Enzymology.* 1987, Vol. 148, pp. 350-382.

22. **C. Büchel.** Fucoxanthin-chlorophyll proteins in diatoms: 18 and 19 kDa subunits assemble into different oligomeric states. *Biochemistry.* 2003, 42, pp. 13027-13034.

23. **R. L. Airs, C. A. Llewellyn.** Improved detection and characterization of fucoxanthin-type carotenoids: Novel pigments in Emiliania Huxleyi (Prymnesiophyceae). *Journal of Phycology.* 2005, 42, pp. 391-399.

24. **S. A. lvarez, M. Zapata, J. L. Garrido, B. Vaz.** Characterization of [8-ethyl]-chlorophyll c3 from Emiliania huxleyi. *Chemical Communications.* 2012, 48, pp. 5500–5502.

25. **Z. Gardian, R. Litvin, D. Bina, F. Vacha.** Supramolecular organization of fucoxanthin–chlorophyll proteins in centric and pennate diatoms. *Photosynthesis Research - Springer.* 2014.

26. **L. Premvardhana, B. Robert, A. Beer, C. Büchel.** Pigment organization in fucoxanthin chlorophyll a/c2 proteins (FCP) based on resonance Raman spectroscopy and sequence analysis. *Biochimica et Biophysica Acta.* 2010, Vol. 1797, 9, pp. 1647-1656.