Master's Thesis

Pigment analysis in *Sphingomonas glacialis* strain S2U11 and its light exposure effect

Katri Ketola

University of Jyväskylä

Department of Biological and Environmental Science

Cell and Molecular Biology

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UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science
Department of Biological and Environmental Science
Cell and Molecular Biology

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Aerobic anoxygenic photosynthetic (AAP) bacteria gain chemical energy from light through non-oxygen producing photosynthesis. The presence of the oxygen is required to produce pigments, bacteriochlorophyll a (BChla a) and carotenoids. During photosynthesis pigment constituting light harvesting (LH) complexes gather absorbed light and transfer it to reaction centre (RC) as excitation energy with process called singlet state energy transfer. BChl a is an essential pigment to both the LH complex and RC and its main light absorption area is in near infrared area. Photosynthetic carotenoids absorb light from 400 to 550 nm area and transfer it to the BChls. Carotenoids can act as photoprotectors and quench the excess light energy before it creates high energetic singlet oxygen, that is detrimental to BChl synthesis. The research of AAP bacteria, especially their pigment content, is scarce. This thesis works to find possible method for the pigment analysis and identification. The pigments were extracted from *Sphingomonas glacialis* and identified with high performance liquid chromatography retention times and absorption spectra. The presence of photosynthetic carotenoids was studied with fluorescence excitation. The light exposure examination addressed effect of light changes to the pigment composition. Pigment synthesis takes place in the darkness. Photosynthetic carotenoids were not present in *Sphingomonas glacialis,* but multiple carotenoids were found. Identification of the pigments is challenging but the method for pigment analysis is functional and can be utilized in other carotenoid containing AAP bacteria.

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Hapellisissa oloissa happea tuottamattomat fotosynteettiset (AAP-) bakteerit saavat kemiallista energiaa hyödyntämällä valoa happea tuottamattomassa fotosynteesissä. Hapellinen ympäristö on tarpeellinen bakteeriklorofyllien ja karotenoidien valmistukseen, jotka absorboivat valoa valonkeräyskomplekseissa, mistä valon virittämä energia siirretään reaktiokeskuksiin eksitaatioenergiansiirto-prosessilla. Bakteeriklorofylli absorboi valoa infrapuna-alueelta ja karotenoidit 400–550 nm alueelta. Karotenoidit voivat siirtää viritysenergian bakteeriklorofylleille. Toisaalta, karotenoidit suojelevat komplekseja reaktiiviselta hapelta poistamalla ylimääräisen viritysenergian. AAP-bakteereja sekä niiden pigmenttien tutkimus on vähäistä, tämä tutkimus kehittää menetelmää pigmenttien analysointiin. Pigmentit eluoitiin *Sphingomonas glacialis* -bakteereista. Identifiointi tehtiin korkean erottelykyvyn nestekromatografian retentioajoilla sekä absorptiospektreillä. Fotosynteettisten pigmenttien kartoitus tehtiin fluoresenssieksitaatiolla. Valo-olosuhteiden muutokset vaikuttivat pigmentteihin ja runsain kasvu tapahtui pimeässä. Pigmenttejä löytyi runsaasti, mutta yksikään ei ollut fotosynteettinen. Pigmenttien analysointimenetelmä on toimiva ja käytettävissä muihin AAP-bakteereihin, mutta karotenoidien tunnistamisessa on vielä käytännön haasteita.

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TERMS AND ABBREVIATIONS

TERMS

Anoxygenic photosynthesis	Non-oxygen releasing photosynthesis
Bacteriochlorophyll	Photosynthetic pigment in bacteria
Hydrophobic	Repellent of water
In vivo	Study taking place in living organism
Light harvesting complex	Pigment-protein complex, where absorption of light takes place
Oxidative stress	Damage caused by singlet oxygen
Oxygenic photosynthesis	Oxygen releasing photosynthesis
Photoprotection	Removal of excess light energy
Photosynthesis	Process to convert light to chemical energy
Photosynthetic apparatus	Complex where photosynthesis takes place
Polyene	Saturated organic compound with altering double and single carbon-carbon bonds
Quenching	Process to remove excess light energy
Reaction centre	Complex where light energy is converted to chemical energy
Single state energy transfer	Process which transfers excitation energy between pigments

ABBREVIATIONS

AAP	Aerobic anoxygenic photosynthetic
BChl	Bacteriochlorophyll
Chl	Chlorophyll

FR	Far red
HPLC	High performance liquid chromatography
ISTD	Internal standard
LH	Light harvesting
NM	Nanometers
NIR	Near Infrared
RC	Reaction centre
UV	Ultraviolet
S2U11	*Sphingomonas glacialis* bacteria strain used in this study

1 INTRODUCTION

1.1 Photosynthesis

Photosynthesis is a series of redox reactions where light energy is converted into chemical energy which is used by phototrophs for growth and survival (Bryant and Frigaard 2006). In the early years of life, photosynthesis was a large step forward for energy production and has made it possible to produce large amounts of carbon products compared to the previous energy source, hydrothermal sources (Des Marais 2000). Based on the reaction products, photosynthesis can be divided into two categories, oxygenic photosynthesis and anoxygenic photosynthesis (Björn and Govindjee 2009). The more known photosynthesis, oxygenic photosynthesis, is carried out in green plants, algae and cyanobacteria, which release oxygen along with carbon products (Bryant and Frigaard 2006). Anoxygenic photosynthesis takes place in bacteria, such as purple bacteria or heliobacteria, but instead of releasing oxygen the reaction products are sulphur and carbohydrates (Blankenship, 2002; Nelson and Cox, 2017). Based on environmental factors, anoxygenic photosynthesis can be divided into aerobic or anaerobic whether oxygen is present in the process (Hanada, 2016). Presence of oxygen is needed in some anoxygenic bacteria, even if they do not produce oxygen themselves, because it is used in bacteriochlorophyll (BChl) pigment synthetisation (Shimada, 1995). Aerobic anoxygenic bacteria have operating electron transport chain only when oxygen is present, and the photosynthesis slows down when oxygen levels diminish (Yurkov and Beatty, 1998). In anaerobic anoxygenic bacteria oxygen has an inhibiting impact on the photosynthesis and the formation of photosynthetic pigments (George et al., 2020).

Bacteria based photosynthesis takes place in their photosynthetic apparatus which consist of reaction center (RC) and light harvesting systems (LHs) with BChl and other pigments (Yurkov and Csotonyi, 2009). Carotenoids are important pigments used in photosynthesis for light absorption because they absorb light at different

wavelengths compared to bacteriochlorophyll (Yurkov and Csotonyi 2009; George et al., 2020). This mechanism expands the spectrum of the absorbed light. Carotenoids are found in many organisms and are the reason behind red, orange, and yellow colors in them (Britton 1995). It has been discovered that among the absorption and color function, carotenoids play an important role in photoprotection and cold resistance (Dieser et al. 2010). When comparing bacteria from different climates, bacteria from colder environments have higher carotenoid abundance due to adaptation to more challenging environments (Zhang et al. 2006). Carotenoids β-carotene and zeaxanthin are linked with oxygenic photosynthesis (Polívika et al., 2009), and since these are found in aerobic anoxygenic phototrophs (Yurkov and Beatty, 1998) it is a possibility these are linked with aerobic anoxygenic photosynthesis as well.

1.1.1 Anoxygenic photosynthesis and the photosynthetic apparatus

Whether anoxygenic photosynthesis is important to nature is a debated issue since it does not release oxygen into the atmosphere as a reaction product but is still common among bacteria (Hanada, 2016). Anoxygenic bacteria groups are purple bacteria, purple sulphur bacteria, green sulphur bacteria and heliobacteria (George et al., 2020). In anoxygenic photosynthesis, the main pigments used are BChls instead of chlorophylls (Chl), which is the significant difference between oxygenic and anoxygenic photosynthesis (Yurkov and Beatty, 1998; Allen and Williams, 2011; George et al., 2020). In both versions the light energy is harvested by the chlorophylls, along with other pigments, and transferred into RC (George et al., 2020; Hanada, 2016). The photosynthetic reaction is an oxidation-reduction process fuelled by the harvested light energy. The reaction consists of reactants; an electron donor and carbon base, and reaction products; carbohydrates and sulphur or oxygen. The electron donor oxidation produces electrons, hydrogens, and oxygen or sulphur gas. The hydrogens are transferred into the carbon base to form carbohydrates and the produced gas is reaction product. (Campbell et al. 2018**).** The electrons are excited with light energy and moved from electron donors into electron transport chain (George et al., 2020; Martin et al., 2018; Ohlson, 1970). In

anoxygenic photosynthesis, chemical compounds used as electron donors are sulphur, hydrogen, or organic compounds, because water oxidation is not possible (Hanada, 2016; Ozaki et al., 2019).

The photosynthetic apparatus consists of different pigments and molecules where the excitation energy moves through in singlet state energy transfer (Nelson and Cox, 2018). The apparatus is coded by a photosynthetic gene cluster which biosynthesises photosynthetic pigments and manufactures the machinery structure of LH antennae and one RC (Alberti et al., 1995; George et al., 2020). LH complexes consist of BChl and carotenoid pigments in different ratios (Lang and Hunter, 1994). They are bound within short distance from each other and are located around the RC (Frank and Cogdell, 1996; Yurkov and Csotonyu, 1993). LH structures absorb light and transfer the excitation energy it to the RC (Yurkov and Csotonyu, 1993). The absorbed wavelengths are dependant of the pigments within the LH complex. The carotenoids absorb in 400—550 nm area, and the specific absorption area changes with different the carotenoid distribution in all LH complexes. The closest to RC is the core antenna, LH1, that has absorbance wavelength at 870 nm due to BChl pigment B870. Further from RC are peripheral LH2 antennas with B800 - B850 pigments, with absorbance at 800–850 nm (Yurkov and Beatty, 1998; Yurkov and Csotonyu, 1993).

The RC is a membrane pigment-protein complex that converts the transferred excitation energy into chemical energy, which is used for ATP production and energy consuming processes (Yurkov and Beatty, 1998). Two different RC types are found in phototropic bacteria, type I and type II reaction centre. (George et al., 2020). Type I RC is found in cyanobacteria, green sulphur bacteria and heliobacteria, whereas type II RC is found in purple bacteria (Bryant and Frigaard, 2006; George et al., 2020). RCs are linked with the LH complexes and are dependent of BChl that has an important role in the RC structure functions (George et al., 2020; Kühlbrandt, 1995). The RCs have BChl pigments which collect the excitation energy from LH complexes. Type I RC has P840 pigment, which absorbs light at 840 nm and type II RC P870 pigment absorbs at 870 nm (George et al., 2020). Excited P870 or P840

pigments transfer electrons into electron acceptors in cyclic electron transport chain, where BChl are the primary acceptors (George et al., 2020; Yurkov and Beatty, 1998). Both aerobic and anaerobic anoxygenic photosynthetic bacteria contain the type II reaction centre (Tang et al., 2011).

Light energy excites the electrons within the photosynthetic complex and the excitation energy is transferred within the complex between pigments until it can be utilized (Koyama et al. 1996). This transportation method is called singlet state energy transfer. The process is based on the excitation energy levels within the pigments (Polívka et al. 2009). Excitation takes place when the electrons rise from the ground state to the higher energy level, the excited state. When the excitation states between two pigments overlap the energy is transferred from the donor pigment to the acceptor (Thorsten et al. 2001). For the singlet state energy transfer to take place, the pigments require to locate close within the membrane because the energy transfer between pigments takes place in picoseconds (Ihalainen et al. 2001, Sundström and Pullerits 1999). Photoprotection is based on the energy transfer. Carotenoids quench excess energy from the BChls by transferring it from them. The excess light creates free radicals which are highly reactive and detrimental to the BChl and LH complex and cause degrading of the pigment (Young and Frank 1996).

1.1.2 BChl in photosynthesis

BChl works as an important pigment in photosynthetic bacteria (Yurkov and Beatty, 1998). Many BChls have been found, but only BChl a is present in AAP bacteria due to most stable chemical formula compared to others (Shimada, 1995). The role of BChl as a light absorber in photosynthesis is based on its long excitation states and capability to transfer energy and electrons in light harvesting system due to oxidation-reduction potentials (Yurkov and Csotonyi, 2009; Yurkov and Beatty, 1998). BChl a in protein can absorb maximally at 800 to 870 nm area, in NIR region (Yurkov and Beatty, 1998). The absorption peak shifts to 770 nm when treated with organic solvent, which tells that the NIR absorption comes from being incorporated with protein-pigment complexes *in vivo* conditions (Yurkov and Beatty, 1998). When comparing ratios between carotenoids and BChl, the amount of BChl is

significantly smaller than the number of carotenoids in AAP bacteria, 1:9 (Yurko and Beatty, 1998).

1.2 Carotenoids

Carotenoids are hydrophobic fat-soluble isoprenoid polyenes with conjugated C_{40} carbon base structure (Britton, 1995; Melénez-Martínez et a., 2006; Shadidi et al., 1998). The carotenoid molecular structures differ from each other by (i) the length of the polyene chain, (ii) structures of terminal ends, (iii) not-conjugated double bond placement and (iv) placement of oxygenated groups such as hydroxyl or epoxy groups (Melénez-Martínez et a., 2006; Shahidi et al., 1998). The double bonds form carotenoids into cis- or trans-isomers, which can possess different biological functions and levels of efficiency (Britton, 1995; Stahl and Sies, 1993). Division of carotenoid into two groups is based on the oxygenated functional groups (Shahidi et al., 1998). Highly saturated hydrocarbons, which are known as carotenes such as α and β carotenes are formed from isoprene groups only and have no oxygenated functional groups. Xanthophylls contain one or more oxygenated functional groups and are classified based on the location and functional group type of them (Shahidi et al., 1998).

Over 600 different carotenoids are found in bacteria, algae, plants, and animals (Bendich and Olson, 1989; Shahidi et al., 1998). The diversity and distribution vary on different kingdoms depending on if the organism can biosynthesise carotenoids or how well carotenoids are absorbed and metabolized (Shadidi and Brown, 1998). Animals cannot biosynthesize carotenoids and are dependent on the nutritional supply from food alone, which is why the distribution is smaller compared to other kingdoms (Shadidi and Brown, 1998; Khachik et al., 1991). Most common carotenoids found in humans are β-carotene, α-carotene, cryptoxanthin, lycopene and lutein (Bendich and Olson, 1989). High variety of carotenoids are found in plant and bacteria kingdoms, but the most common ones are β-carotene, lutein, spheroidene, xanthophyll, zeaxanthin and derivates of β-carotene (Demmig-Adams et al., 1996; Khachik et al., 1991; Frank and Brudvig, 2004; Shimada, 1995).

Carotenoids in AAP bacteria are responsible for red, orange, yellow, pink, and purple colours (Yurkov and Csotonyi, 2009).

Carotenoid absorption peaks range from 400 nm to 550 nm depending on the molecule (Britton, 1995; Dieser et al., 2010). The formed carotenoid absorption spectrum depends on the length of the carotenoid and cyclic carotenoids absorb at lower wavelengths compared to acyclic (Melénez-Martínez et a., 2007). Colour of the carotenoids are linked with the structure, which is why different coloured carotenoids absorb at specific wavelengths (Melénez-Martínez et al., 2007). By changing the carotenoid composition, photosynthetic bacteria can alter the absorption properties as an adaptation mechanism to changing light conditions (Magdaong et al., 2014).

1.2.1 Properties and functions of carotenoids

Function of carotenoids in photosynthesis is three ways, the light capture properties, structural properties and protective functions. The light capture ability is based on structural formation of chromophore in the carotenoid (Young & Britton, 1993). The chromophore is an area in the polyene chain what consists of 9 to 13 double bonds which absorbs the light energy (Young & Britton, 1993). The absorption is based on excitation states of carotenoid within the double bonds which is activated when light with correct frequency reaches the carotenoid (Britton, 1995; Young & Britton, 1993). In excitation, the electron state transition happens between ground state and S_2 excitation state where the electronic double bond orbitals transit from π to π^* (Polívika and Frank, 2010; Melénez-Martínez et a., 2006; Britton, 1995). Excitation between ground state and S_1 state happens only if the energy acceptor has low energy (Polívika and Frank, 2010). Carotenoids have shorter excitation lifetimes compared to bacteriochlorophylls but are still used as collectors within photosynthesis (Polívika and Frank, 2010). Excitation energy donor function is achieved with short distances between BChl and carotenoids in the LH complexes (Frank and Cogdell, 1996). The absorbed light is transferred from carotenoids into BChls or chlorophylls (Chl) with singlet state energy transfer (Frank and Cogdell, 1996; Polívika and Frank, 2010). BChls can utilize this energy.

The transfer process can be detected for example with fluorescence excitation spectroscopy (Frank and Cogdell, 1996). The excitation energy transfer can alter depending on the interactions with proteins (Polívika et al., 2009).

Not all carotenoids are capable of photosynthesis. From all naturally occurring carotenoids, only 50 of them are linked photosynthesis (Polívika and Frank, 2010). This includes carotenes in both oxygenic and anoxygenic photosynthesis. The most common carotenoids linked with photosynthesis are β-carotene, zeaxanthin, fucoxanthin, salinixanthin and lutein (Frank and Cogdell, 1996; Polívika et al., 2009; Demmig-Adams et al., 1996; Yurkov and Beatty, 1998).

Carotenoids are most likely considered as being colour pigments or help with photosynthesis. When determining carotenoid composition in aerobic anoxygenic bacteria, most carotenoids do not have light absorbing functions, but their functions go further in wellbeing of the bacteria (Shimada, 1995). This function diversity of carotenoids is not only linked with AAP bacteria but can also be seen in other organisms (Rock, 1997; Stahl and Sies, 1993; Britton, 1995). Broad light spectrum is needed in photosynthesis as an energy source but when the wavelength shortens into UV (ultraviolet) spectrum, it begins to create harmful effects (Dieser et al., 2010). Organisms have developed mechanisms to fight this, and carotenoids are one of them (Dieser et al., 2010). In photosynthesis, Chl and BChl form exited triplet, which can create singlet oxygen, which is an excited state of O_2, a free radical (George et al., 2020; Stahl and Sies, 1993). Reactive singlet oxygen can react with other components within organism to create oxidative stress that damages DNA, proteins, and lipids (George et al., 2020; Cabiscol et al., 1999). Carotenoids are capable of inhibiting the formation of singlet oxygen by quenching excess Chl or BChl triplet excitation state formation or transfer the energy from singlet oxygen to itself (Stahl and Sies, 1993, 2003; Young and Frank, 1996). This oxidation reaction works as a barrier between the vulnerable compound and the singlet oxygen and give carotenoids their properties as an antioxidant (Woodall et al., 1997). Carotenoids are affected in the oxidation process; the chromophore is damaged which causes loss of colour and eventually destruction of the carotenoid (Britton

1995). One of the most researched carotenoids against oxidative stress and singlet oxygen is β-carotene (Woodall, 1997 and Britton, 1995). Photoprotection in other larger organisms than bacteria were investigated by Hairston Jr. (1976) study where astaxanthin and astaxanthin ester pigmented *Diaptomus nevadensis* was exposed do excess light over days. When they compared less pigmented and higher pigmented individuals' survival, it could be noted that the pigmentation prolonged the survival. This gives evidence that carotenoids have photoprotection functions in organisms.

Arctic bacteria are adapted to survive in cold and high solar radiation environments (Dieser et al., 2010). One possible adaptation technique is a broad range of different pigments, carotenoids. Carotenoid range comparison between arctic bacteria and warmer environment bacteria shows that the arctic bacteria have higher carotenoid distribution (Dieser et al., 2010; Mueller et al., 2004). Carotenoids in arctic bacteria are divided into light harvesting, photoprotection, antioxidant carotenoids, and they can control the photosynthesis process to maximise efficiency (Mueller et al., 2004). Dieser et al. (2010) showed in their study that bacteria collected from Antarctica tolerated colder environments more and could recovered better from UV radiation caused DNA damages if the carotenoids were more abundant.

Carotenoids have also biological functions along with the protective properties. They play a part in the assembly of the photosynthetic LH structures by stabilizing BChl into the system (Lang and Hunter, 1994). If carotenoids are not integrated into the LH complexes, another location is within membranes. Membrane fluidity is important function within organisms and carotenoids regulate it by increasing thickness, rigidity, and mechanical strength (Britton, 1995).

1.3 Aerobic anoxygenic photosynthetic bacteria

AAP bacteria are non-oxygen producing phototrophs, which still need oxygen for BChl synthetisation and can only survive in aerobic conditions (Shimada, 1995). In laboratory conditions, these bacteria cannot use photosynthesis as their sole energy

source and are classified as heterotrophs (Yurkov and Beatty, 1998). The AAP bacteria are heterogenous group in taxonomical, phylogenetical, and ecological ways, but do still share some similarities in characteristics (Hirose et al., 2016; Yutin and Béjà, 2005). They all utilise BChl in the RCs and LHs, have diverse carotenoid compositions and can grow photoheterotrophically in aerobic conditions (Yurkov and Beatty, 1998; Yutin and Béjà, 2005). This last part separates them from purple bacteria, anaerobic phototropic bacteria, which require anaerobic conditions for growth (Geroge et al., 2020). Even if the photosynthetic properties are investigated in AAP bacteria, diversity within them separates into photosynthetic and non-photosynthetic species (Yurkov and Beatty, 1998).

Most of the studies with AAP bacteria have been done in aquatic environments (Yurkov and Csotonyi, 2009), and in ocean surface water ecosystems they play important part in carbon cycling (Zheng et al., 2011). Interestingly, AAP bacteria have been found to be durable in extreme environments, such as hypersaline springs and hydrothermal vents (Yurkov and Csotonyi, 2009). In terrestrial environments, AAP bacteria have been found to be present in biological soil crust where they accelerate carbon cycle in nutrient poor soil (Tang et al., 2018). AAP bacteria have also been found to have a connection with different plant species and have been extracted from plant phyllosphere; the surface of the plant, and from the internal plant tissues (Nissinen et al., 2012).

1.4 Aims of the study

Studies considering AAP bacteria are quite scarce and more abundant investigations are needed, especially within the photosynthetic properties (Rathgeber et al., 2004; Shimada, 1995). One aspect is to chart pigments found from these bacteria. Carotenoids have been found to have importance to both anoxygenic and oxygenic photosynthesis (Britton, 1995) while holding functions which benefit the organisms' wellbeing (Dieser et al., 2010; Stahl and Sies, 1993 and 2003). Light harvesting pigment bacteriochlorophyll a holds importance to the photosynthesis

and transfer absorbed light from light harvesting complexes to reaction center (Yurkov and Csotonyi, 2009; Yurkov and Beatty, 1998).

In the "Shared light" project AAP bacteria are isolated from plant species and studied if the bacteria can aid the plant growth by sharing energy from bacteria photosynthesis. If this link between the plants and the AAP bacteria is found, AAP bacteria could be used to enhance plant photosynthesis and growth. This master's thesis is part of the research project, but it does not focus on finding the link between plants and AAP bacteria. Instead, it focuses on creating a workable method for pigment analysis, identification and finding photosynthetic pigments.

The aims of this study are centered around four areas which answers will help to understand pigments within *Sphingomonas glacialis* bacteria. The first aim is to develop a working method for pigment extraction and analysis from the bacteria culture. Two methods for the extraction were compared to perceive which is better for the pigment extraction. The prepared sample was analyzed with high performance liquid chromatography (HPLC). The working extraction method would be seen at the chromatogram as retention time signals. Once this was successful, the second aim was to test if the pigment composition changes under different growth conditions of bacteria, growth period length and light wavelength exposure changes. The pigment composition analysis was done from HPLC data of the samples. Retention time peaks from the chromatogram gave the number of the pigment peaks and the absorbance intensity to each pigment. Closer inspection was done to the relation between BChl a and carotenoids. The third and fourth aims were based on the photosynthesis part of the study and address the pigments photosynthetic capabilities. The third aim of the study is to identify the pigments based on the retention times and absorption spectra from HPLC data using literature sources. The properties of the identified pigments and possible link with photosynthesis were searched from literature. The fourth aim focuses on the singlet state energy transfer between carotenoids and bacteriochlorophyll a. The aim is to find out whether the carotenoids are capable of transferring excitation energy to

BChl a using fluorescence excitation. And if so, which carotenoids could be transferring.

Some preliminary hypotheses can be made from the aims of the study. The first hypothesis of the extraction method functionality was based on S. Taipale (2020) and Wiltshire et al. (2000) studies where one of the used extraction methods has worked on fatty acid extraction with algae. It is expected that the method can be applied to the bacteria at some level and the pigments can be extracted from the bacteria. The second hypothesis is that the alterations to the growth conditions affect the pigment composition. Takamiya et al. (1992) research has found blue light to be inhibitor on BChl and carotenoid production under aerobic photosynthesis. The most abundant BChl a production in the organism was enabled in darkness (Palokas, 2019). Bacteria culture grown under blue light is hypothesized to have more scarce pigment composition synthesis and the most abundant pigment synthesis in darkness. The pigments have protective properties (Dieser et al. 2010; Woodall et al., 1997; Britton 1995; Hairston Jr. 1976), and hypothesis is that the pigment compositions changes are based on protection against harmful growth environments. Third hypothesis regards the photosynthetic region of the study, and it is based on Ihalainen et al. (2001) study. The expectation is that photosynthesis linked pigments are found from the *Sphingomonas glacialis* strain S2U11 and they can transfer excitation energy into BChl a.

2 MATERIALS AND METHODS

2.1 Material

Carotenoid distribution determination methods were tested using *Spinghomonas glacialis* strain S2U11. Isolation was performed from *Diapensia lapponica* (pincushion plant) leaves endosphere found in Kilpisjärvi Finland (Nissinen et al. 2012). The strain S2U11 was cultured with varying conditions to determine whether the changes cultivate different ratios of pigments within the bacteria. The quantitative

pigment determination was performed using HPLC machinery (Manufacturer: Shimadzu, Program: Nexera Labsolutions) with YMS carotenoid column (Genetech YMC-Triath HPLC column C30 250x4.6mm, 5µm pore size) as a stationary phase and 25:75 combination of HPLC grade methanol (Supelco, Methanol for liquid chromatography) and MTBE (VWR chemicals, tert-Butyl methyl ether for HPLC) as a mobile phase. Qualitative determination of the pigment analysis took place from absorption spectra constructed from the HPLC data with MatLab program. Fluorescence excitation spectra of the bacteria strain was measured from cultured bacteria culture.

2.2 Methods

2.2.1 Bacteria isolation process

The bacteria strain was isolates as described in Nissinen et al., 2012, where the strain origin is also described. *Diapensia lapponica* leaves were sterilized using 3% chlorite and washed with sterilized water before crushed to form homogenized solution in KPi buffer (20mM, pH 6.5). A dilution series of 10^{-3}, 10^{-2}, 10^{-1} and 1 was prepared from the homogenized solution. 100 µl of each dilution was plated into ½ x R2A (pH 6.5) plates. The grown endosphere bacterial were separated with inoculation loop and grown into pure isolated cultures. The bacteria strain is stored at -80°C or -20 °C. The stored bacteria culture was thawed and 50µl were plated into new ½ x R2A (pH 6.5) plates.

2.2.2 Bacteria growth conditions

Two sets of growth condition alterations were applied to the growing bacteria cultures. In Table 1, are presented the first experiment alterations, the growth period alterations. The bacteria strain is first grown in ½ x R2A (pH 6.5) plates at RT (room temperature) under natural light. The plates were moved to +4°C temperature in darkness. The period under the darkness and +4°C altered from one week to four weeks. This experiment studied whether the pigments are produced in the beginning of the growth or does the production take place later in some pigments.

The second alteration changed the exposure light wavelength (Table 2). The strain S2U11 was cultured into the ½ x R2A (pH 6.5) plates in complete darkness and exposed to the different wavelengths for three days. After the exposure, the plates were moved into darkness at 4°C temperature for four days. The alterations were applied to document whether different light produces different pigment compositions within the bacteria. The light condition alteration experiment was performed based on the master's thesis by Arla Palokas (2019).

Table 1. The growth period alterations in the growth period experiment. Four sets of bacteria cultures were grown using the same starting conditions but altering the growth period length under +4 °C darkness.

Growth period	Light exposure; normal daylight at RT	+4 °C exposure in darkness
1 week	2 days	5 days
2 weeks	2 days	12 days
3 weeks	2 days	19 days
4 weeks	2 days	26 days

Table 2. The wavelength alterations in the light exposure experiment. Seven sets of bacteria cultures were grown under different visible light wavelengths for three days at RT and in darkness for four days at +4°C.

Sample type	Light exposure at RT
Dark	No light
White	Daylight
Blue	446 nm
Green	520 nm
Red	620 nm
Far red (FR)	760 nm
Near infrared (NIR)	870 nm

The light condition experiment samples consisted of three replicas in each light condition. Each of the replicas was weighed and analyzed with HPLC individually. The carotenoid abundance data was averaged from the three replicas.

2.2.3 Pigment isolation

Absorption and fluorescence excitation example spectra (Figure 14 and 15 in Appendix 1), and HPLC chromatogram (Figure 1) were measured from industrial made pigments to gain control spectra of β-carotene and BChl a. The absorption and fluorescence excitation measurement samples were prepared by dissolving the pigments into pure acetone. Used commercial BChl a pigment was from Frontier Specialty chemicals (Bacteriochlorophyll a under argon from *Rhodopseudomonas sphaeroides* purple bacteria, 17499-98-8) and the commercial β-carotenoid syntethised by Fluka Sigma Aldrich (β-carotene, 7235-40-7). The HPLC chromatogram measurement samples were prepared by dissolving the pigments into HPLC grade methanol. The control pigment measurements are the baseline on how the data of the pigments from bacteria should occur.

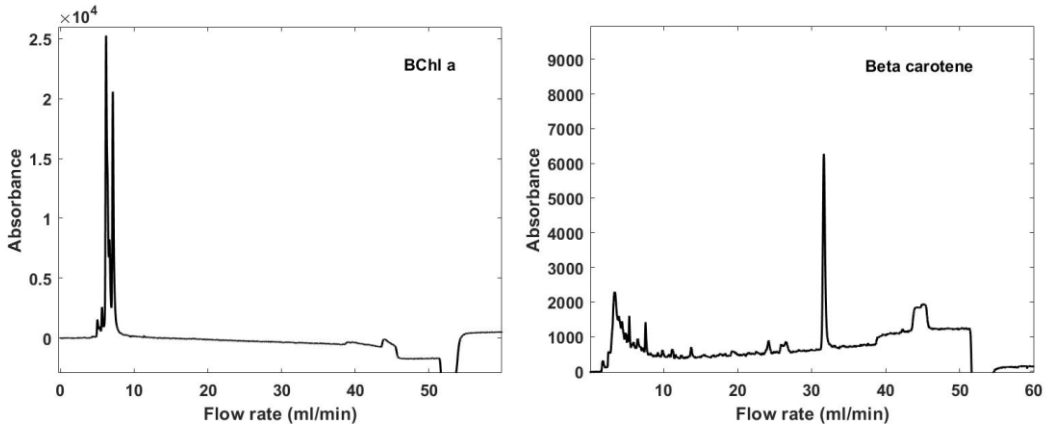


Figure 1. The HPLC chromatograms of example pigments BChl a (left) and β-carotene (right). The x axis displays the run time and y axis absorbance intensity. The peaks represent exited pigments form the HPLC column. The example measurements display how the BChl a and carotenoids work with the solvent and HPLC. The drop in β-carotene chromatogram after 50 minutes is a result from eluent concentration changes. The MTBE solvent percentage in the eluent dops to 0 %.

2.2.4 UV-VIS absorption and fluorescence emission and excitation spectroscopy

Absorption and fluorescence excitation spectra were measured with Tecan Spark Multimode microplate reader. Absorption spectrum demonstrates wavelengths that are absorbed by the pigments. The absorbed light is transferred as energy from the pigments if the singlet state energy transfer is possible. The transfer can be proven with fluorescence excitation spectroscopy. The natural state of the pigments in the bacteria was inspected by measuring unprocessed bacteria culture. Whether the strain S2U11 contains photosynthetic pigments can be proven with fluorescence excitation spectrum. The Tecan data was processed into spectra with Microsoft Excel (Microsoft corporation).

2.2.5 High Performance Liquid Chromatography

All measurements with HPLC equipment were performed with the same principle but two sample preparation methods were compared to examine which works best for giving out pigment signals. The first preparation method was to add the bacteria culture from plates into 1 ml of HPLC grade 100% methanol and incubated 2h at room temperature in darkness. The suspension was centrifuged for 4 minutes at 13 000 x g. The separated supernatant was utilized as the HPLC sample. Internal standard (ISTD) trans-β-apo-8-carotenal (Sigma Aldrich, CAS: 1107-26-2) was added into half of the sample replicates.

The second extraction method was chloroform:methanol (2:1) extraction. The pigments were separated from the bacteria culture before analysing with HPLC. The culture was first frozen overnight and cold dried until completely dry. The dried bacteria culture was weighed and diluted into solvent as described in Table 3. The suspension was treated with ultrasonication for 10 minutes to separate the pigments from the bacteria culture into the chloroform phase. The methanol phase contained impurities and was discarded. Nitrogen evaporator was used to remove the chloroform from the pigments. The dried pigments were diluted into HPLC grade methanol to prepare the final HPLC sample. Table 4 shows the parameters used in the HPLC measurement and Table 5 the eluent gradient during the HPLC

measurement. The light condition experiment samples were prepared using only the cold drying preparation method.

Table 3. The solvent for diluting the pigments from strain S2U11 culture in the chloroform:methanol extraction.

Dilution solvent for chloroform methanol exctraction	
Needed solvent	Volume
Chloroform:methanol (2:1) solution	3 ml
H_2O	750 µl
ISTD (20 mg/ml)	20 µl

Table 4. The measurement parameters for all HPLC measurements.

HPLC parameters	
Wavelength measurement area	250-800 nm
Injection volume	10 µl
Run time	60 min
Measurement interval (s)	640 ms
Flow rate	1 ml/min

Table 5. Eluent gradient in change in all HPLC measurements. The eluent is a mixtrure of HPLC grade methanol and MTBE. The change is reported as MTBE percentage changes at different flow rate timepoints.

Flow rate time	MTBE percentage in the eluent
1 min (start)	0 %
35 min	35 %
40 min	50 %
42 min	70 %

48 min	70%
50 min	0 %
60 min (stop)	0 %

2.2.6 Data-analysis

The data from the measurements were in chromatograms and spectra. Whether the strain S2U11 contains photosynthetic carotenoids can be determined by comparing the absorption spectrum and fluorescence excitation spectrum from the bacteria culture. Photosynthetic carotenoids move the excitation energy from LH complexes with singlet state energy transfer into the RC. The excitation energy transfer causes the fluorescence excitation spectrum to behave identically to the absorption spectrum of the BChl a molecule. The method does not necessarily identify which carotenoids are photosynthetic only if the bacteria strain contains photosynthetic carotenoids.

The identification of the individual carotenoids was based on HPLC chromatograms, absorption spectra of the carotenoids and retention timetable of the carotenoids scaled with ISTD. Data from the HPLC was analyzed with MatLab. Chromatograms of carotenoids and BChl a were prepared by plotting time and absorbance intensity data from 450 nm and 775 nm regions. Absorption spectra for each chromatogram signal were prepared by plotting wavelengths and absorption intensity from each retention time. The chromatograms show the exit times for each pigment molecule in the sample and the intensity of the signal. The absorption intensity is directly proportional to the pigment concentration in the sample. Carotenoids were tentatively identified with Table 10 in Appendix 2 and verified by comparing absorption spectrum of each pigment to absorption spectra from literature. Carotenoid absorption spectra are individual when compared and this is based on the structural differences.

3 RESULTS

3.1 Overview

Development of the pigment characterization method consists of various sectors of measurements and data processions. The growth condition comparison and light condition comparison interpret the possible parameters that influence the development of pigments. The composition and quantity of the pigments from the growth and light condition comparisons were received from reverse phase HPLC separation. The results are interpreted from the pigment molecule retention times, absorption intensity levels and absorption spectra of each pigment signal. Quantity of the different pigment signals reveals differences between the growth conditions. This is established by comparing the absorbance intensities within pigment signals along with number of pigment signals in different conditions. Pigment photosynthetic activity is linked with the capability to transfer excitation energy as singlet state energy transfer within the photosynthetic apparatus. Possible singlet state energy transfer between pigments is proven by comparing measured absorption and fluorescence excitation spectra.

3.2 HPLC analysis of the pigment composition of S2U11

The eluted pigments from both growth period comparison and light condition comparison bacteria culture were run through the reverse phase column in the HPLC instrument. The overall pigment separation is seen from a chromatogram, which shows the pigment signals as retention times in different wavelengths. Figure 2 shows the overall chromatogram from the instrument with all the measured wavelengths and Figure 3 MatLab constructed chromatogram at a chosen wavelength based on observed the pigment type. The wavelengths were chosen based on the areas where absorption of light takes place. From each pigment signal, an absorption spectrum was constructed to aid with recognition of carotenoids and BChl a.

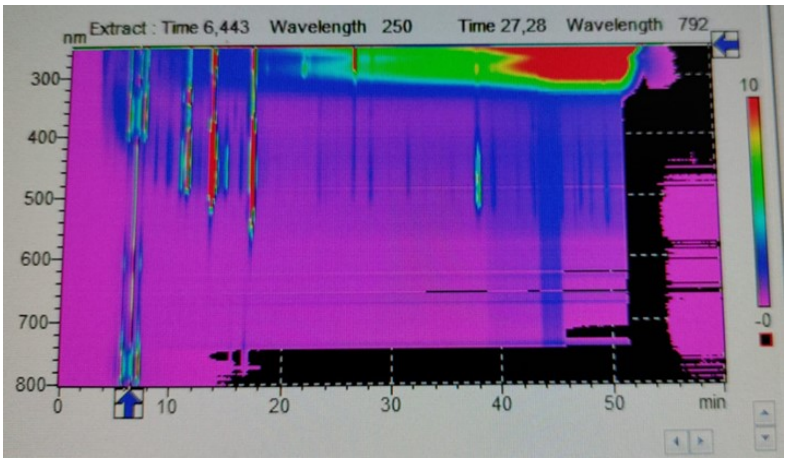


Figure 2. The chromatogram of all the pigment signals in second NIR sample taken from the HPLC instrument. The x axis holds the retention time range of the HPLC column run and the y axis the wavelength range where absorption of light energy takes place in the pigment signal. The peak colour represents the absorption intensity of the signal, blue being low intensity and red high. The signal area in the chromatogram is an estimate of the pigments based on the absorption wavelengths. BChl a absorbs in the 700 to 800 nm and 300 to 400 nm areas whereas carotenoids in the 400 to 550 nm area.

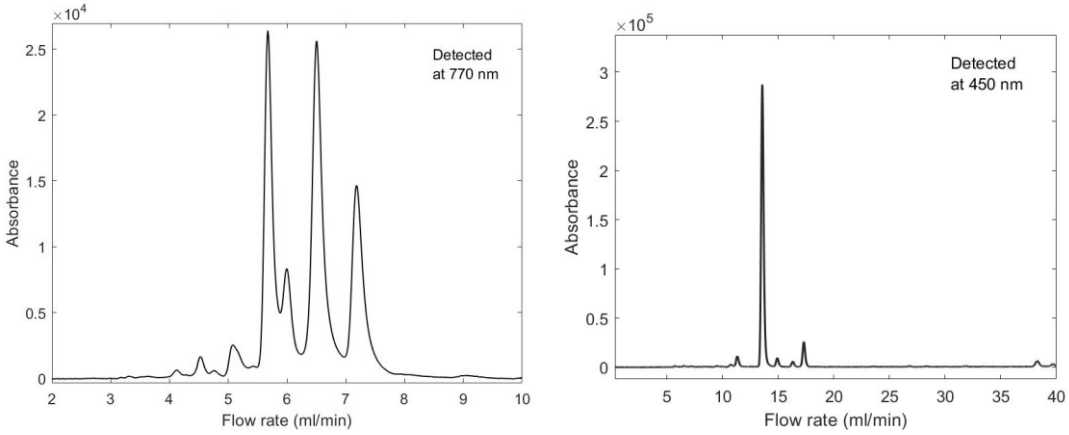


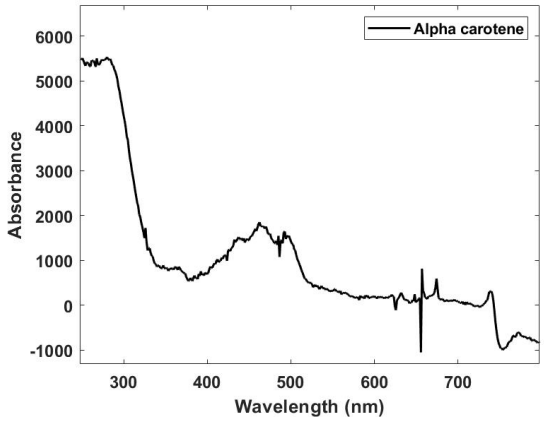
Figure 3. Matlab constructed chromatograms of the first NIR light exposure sample. The x axis holds the retention time range of the HPLC run and the y axis the absorption intensity of the pigment signal. The chromatograms were based on the wavelength where maximum absorption takes place on BChl a and carotenoids. BChl a (left figure) absorption maximum takes place around 770 nm and on carotenoids (right figure) 450 nm. The absorbance intensity scale shows directional abundances of the different pigments.

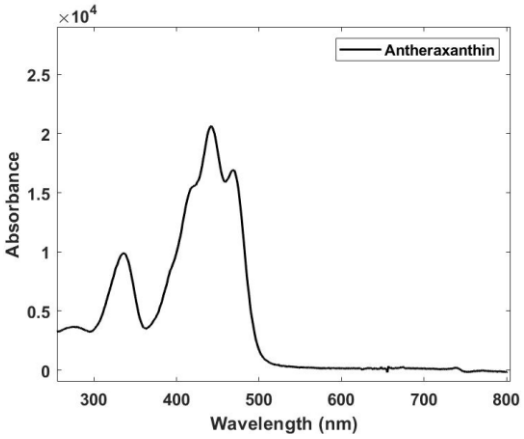
Retention times from both growth period comparison and light condition comparison sample chromatograms were cataloged into tables, which are listed in Appendix 3. The retention times indicate differences and similarities between the samples, but further examination is needed to gain proper results.

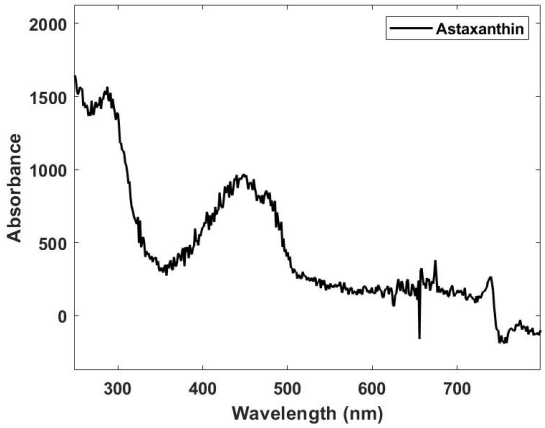
3.2.1 Pigment identification from the chromatograms.

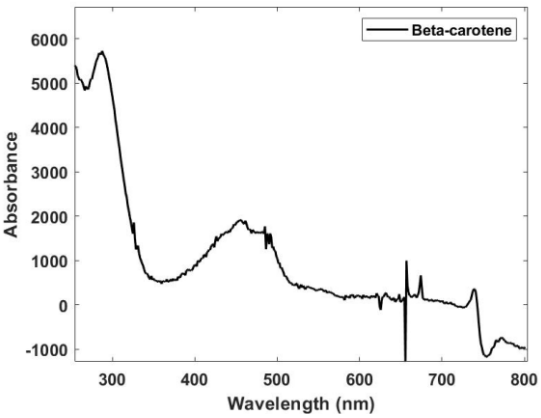
The pigment identification is performed using the retention times and absorbance spectra of the pigment signals. The different pigment molecules exit the reverse phase column at different rates based on the structure and polarity of the molecule (Harris and Lucy 2016). This exit rate is labelled as the retention time. Preliminary estimations of the pigment exit rate are done based on the chemical properties of the column and the pigments. Reverse phase C30 column binds stronger the non-polar and hydrophobic carotenes compared BChl a. The methanol:MTBE gradient release the pigments at different rate and the more non-polar carotenoids require higher gradient of the eluent. The oxygenated xanthophylls are more polar than non-oxygenated carotenes and therefore, their retention times exiting the column are lower.

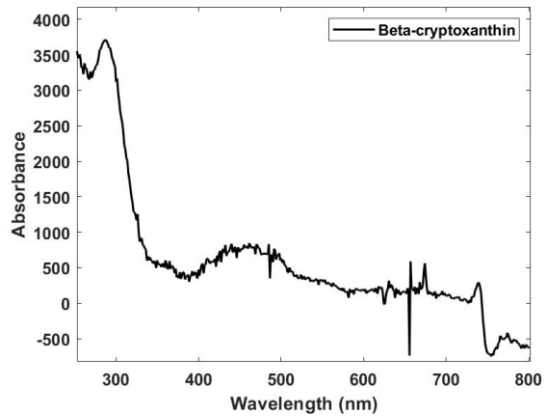
The carotenoids are abundant group and only some of the signals from the strain S2U11 can be preliminary identified with Table 10 in Appendix 2. The rest of the signals are marked as unknown (UN) and their retention times are compared to retention times found in literature. The verified carotenoids with the unknown are listed on Table 6. The ISTD retention time from the HPLC measurements is 0.3 digits off compared to the Table 10 ISTD. This indicates how much the carotenoid retention time can alter from the Table 10 carotenoid retention times. The verification of the retention time identified carotenoids was based on the absorption spectra of the carotenoids found in literature (Gupta and Sreelakshmi, 2015). Figure 4 contains the absorption spectra of identified carotenoids. The absorption spectra of the UN carotenoids are listed in Appendix 4 with all the carotenoid spectra peak maxima.

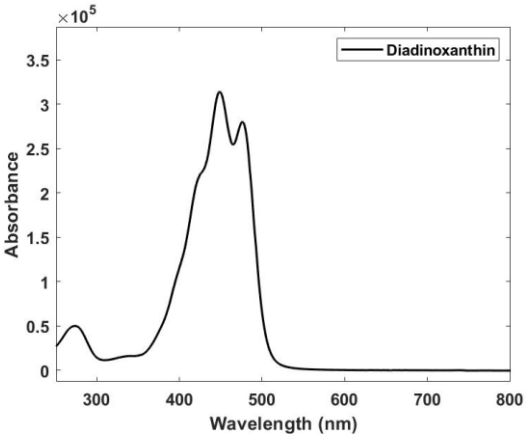

Alpha carotene
Absorbance
Wavelength (nm)


×10^4
Antheraxanthin
Absorbance
Wavelength (nm)


Astaxanthin
Absorbance
Wavelength (nm)


Beta-carotene
Absorbance
Wavelength (nm)


Beta-cryptoxanthin
Absorbance
Wavelength (nm)


×10^5
Diadinoxanthin
Absorbance
Wavelength (nm)

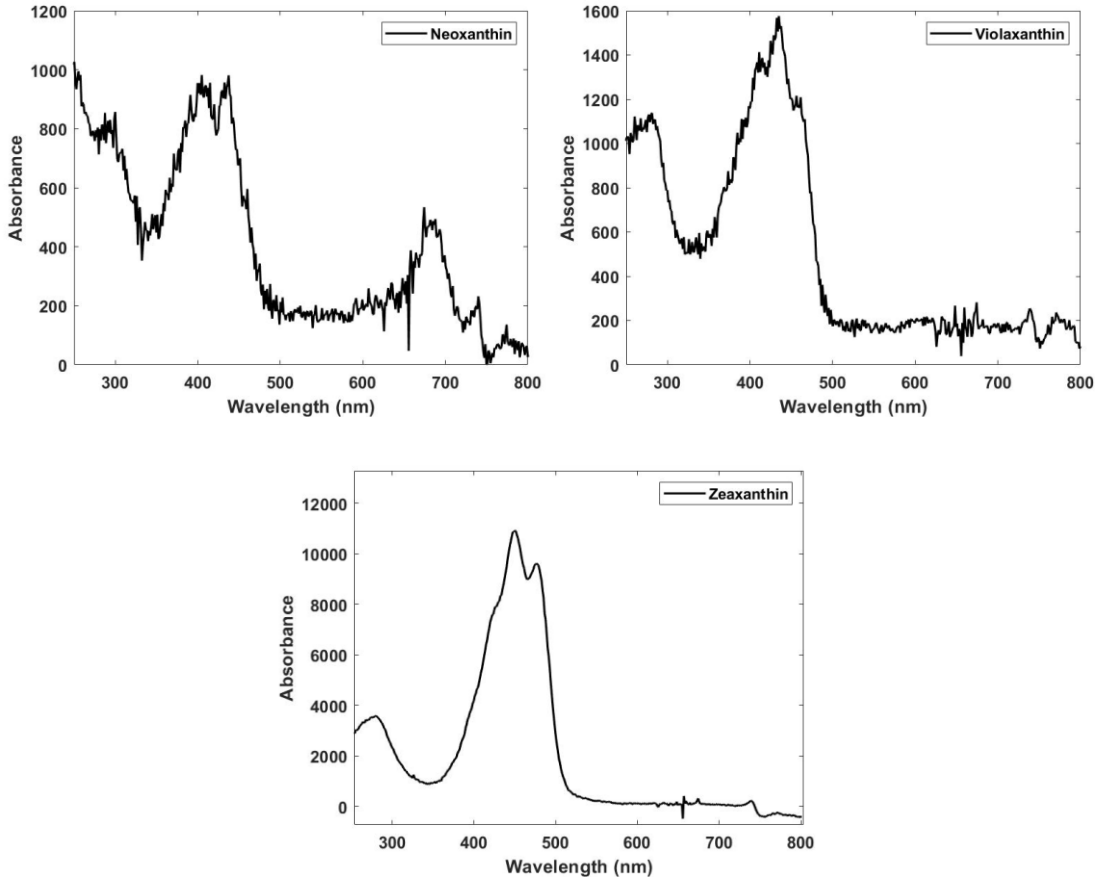


Figure 4. The absorption spectra of the HPLC retention time identified carotenoids. The x axis presents the measured wavelengths and the y axis the absorbance of the measurement.

Table 6. Pigments from the strain S2U11 cultures grown under different light exposures. The identification was performed using the retention times and absorption spectra of the HPLC measurement signals. The *-marked pigments are identified using the Table 9 in Appendix 2. The different light exposure during the growth period produces different pigment compositions in the bacteria. Blue light inhibits the pigment production whereas darkness, far red (FR) and NIR light are optimal conditions for the pigment growth.

Retention time (min)	Pigment	Light conditions						
		Dark	White	Blue (446 nm)	Green (520 nm)	Red (620 nm)	FR (760 nm)	NIR (870 nm)
4.1	BChl a						x	x
4.5	BChl a						x	x
5.1	BChl a				x	x	x	x

Continued the next page

5.7	BChl a	x	x	x	x	x	x	x
6.0	BChl a	x			x	x	x	x
6.5	BChl a	x	x	x	x	x	x	x
7.2	BChl a	x	x	x	x	x	x	x
7.9	UN1		x	x	x			
8.4	Violaxanthin*	x	x	x	x	x	x	x
8.8	Neoxanthin*	x					x	
9.4	UN2	x	x	x	x	x	x	x
9.6	UN3						x	
9.9	UN4		x	x			x	
10.7	UN5	x	x	x	x	x	x	x
11.3	Antheraxanthin*	x	x	x	x	x	x	x
12.4	UN6	x	x	x	x		x	x
12.9	Astaxanthin*		x			x	x	
13.6	Diadinoxanthin*	x	x	x	x	x	x	x
14.9	UN7	x	x	x	x	x	x	x
16.3	Zeaxanthin*	x	x	x	x		x	x
17.2	UN8	x	x		x	x	x	x
17.3	**ISTD (trans-β-apo-8-carotenal)**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
23.5	Beta-cryptoxanthin*						x	x
26.8	UN9	x	x		x	x	x	x
28.3	Alfa-carotene*					x	x	x
31.8	Beta-carotene*	x			x		x	x
38.2	UN10	x	x		x	x	x	x
39.7	UN11	x	x		x		x	x

3.2.2 Pigment composition related to the growth time of bacteria culture

The effect of the growth period length was inspected by comparing the pigment composition changes after one to four weeks of growth after two days of daylight. The inspected parameters were the composition of different pigments and the abundance of each pigment in each data set. Table 7 lists all the pigment signals as retention times from each growth period length datasets.

Table 7. Pigments from the strain S2U11 culture after different growth period lengths. The identification was performed using the retention times and absorption spectra of the HPLC measurement signals. The *-marked pigments are identified using the Table 10 in Appendix 2. The growth period length does not have an effect on the pigment composition within the bacteria culture.

		Growth period length			
Retention time	Pigment	1 week	2 weeks	3 weeks	4 weeks
5.1	BChl a	x	x	x	x
5.7	BChl a	x	x	x	x
6.0	BChl a	x	x	x	x
6.5	BChl a	x	x	x	x
7.1	BChl a	x	x	x	x
8.3	Violaxanthin*	x	x	x	x
9.4	UN2	x	x	x	x
10.6	UN5	x	x	x	x
11.2	Antheraxanthin*	x	x	x	x
12.3	UN6	x	x		x
13.4	Diadinoxanthin*	x	x	x	x
14.7	UN7	x	x	x	x
16.1	Zeaxanthin*	x	x	x	x
17.3	**ISTD (trans-β-apo-8-carotenal)**	**x**	**x**	**x**	**x**

Continued the next page

28.1	Alfa-carotene*	x	x	x	x
31.6	Beta-carotene*	x	x	x	x
38.1	UN10	x	x	x	x
39.5	UN11	x	x	x	x

Figure 5 shows the BChl a and carotenoid abundances as a sum of all pigment absorptions. In the three week sample the diadinoxanthin peak has significantly higher absorbances compared to the other sample peaks. It distorts the results, showing that in the three-week growth the carotenoid abundance would increase and then decrease after four weeks. When leaving the diadinoxanthin peak out from the sum the carotenoid abundance trend shows diminishing after longer growth period in a similar manner to BChl a.

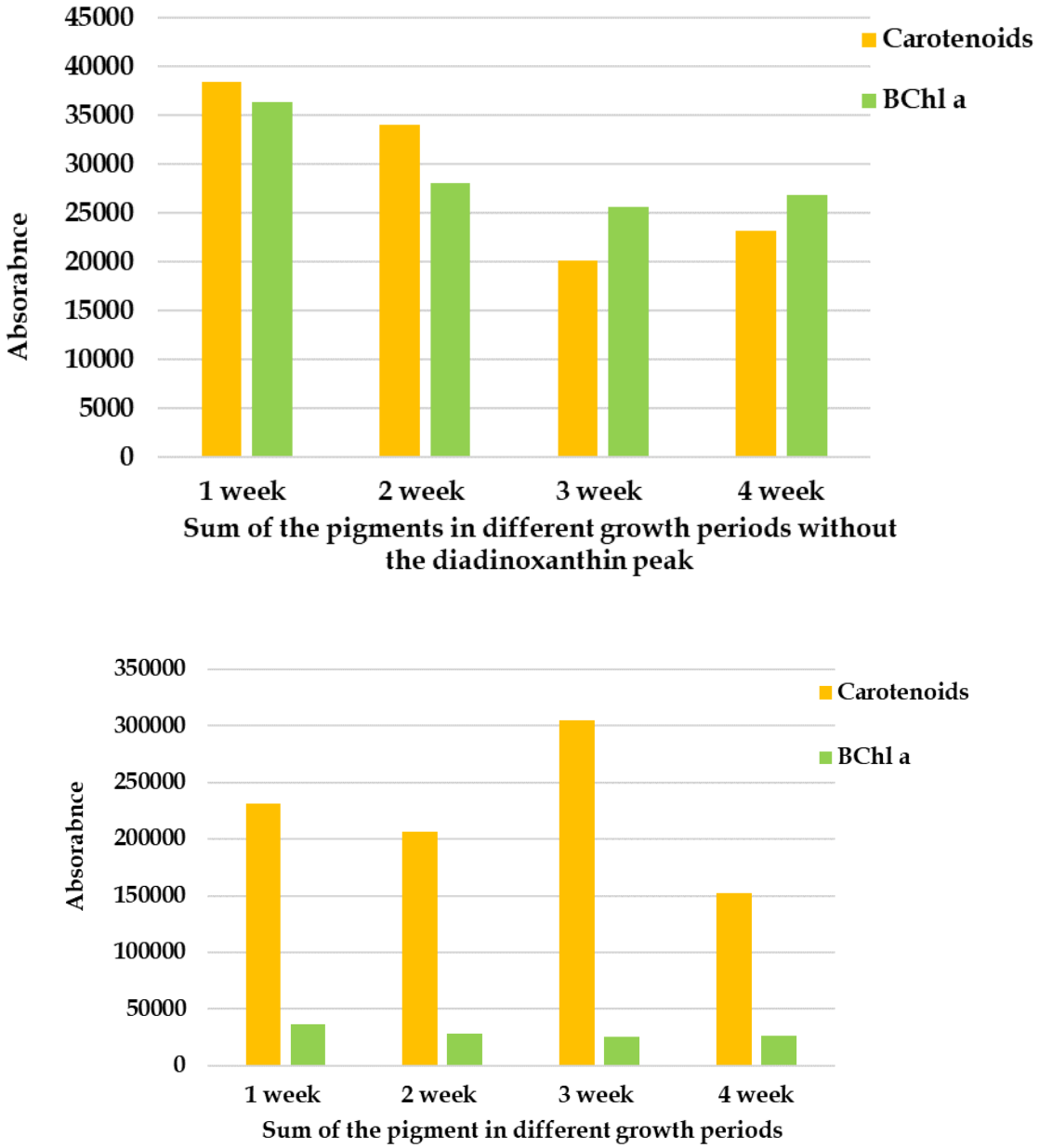


Figure 5. The abundance of the sum of carotenoid and BChl a signals from each growth period dataset. The data is normalized by taking into consideration the bacteria dry weight of the sample. On the third week data, the diadinoxanthin peak is relatively higher compared to the other week data and is taken out in the lower histogram. The upper histogram shows the sum of all pigments. The absorbance of BChl a is constant in both histograms. The pigments show a diminishing trend after longer growth period. Pigment development does not require long growth periods and the pigments are present after one week of growth.

There were no changes in the pigment compositions after elongation of the growth period (Table 8). Pigments are the same after one week of growth and four weeks of growth. The length of the growth period affects the abundance of the pigments within the bacteria culture. The abundance of the bacteria culutre also increases after longer growth period, which is confirmed by the dry weight of the bacteria culture taken from the growth plates shown in Table 19 at Appendix 5.

3.2.3 Pigment composition related to the light exposure growth conditions of bacterial culture

The light exposure had an effect on the pigment compositions seen at Table 6 where all carotenoid signals on each light condition are listed. Table 8 contains the identified carotenoids with the normalized absorbance values taken as a % from the sum of all carotenoid signal absorptions. The normalization was based on the bacteria culture dry weight on each sample. Table 6 and 8 show that antheraxanthin and diadinoxanthin are found in all data sets and diadinoxanthin is the most abundant carotenoid in each dataset. Three of the unknown carotenoids are synthesized only in darkness and beta-cryptoxanthin is synthesized only at FR and NIR. Under the blue light the pigment composition is scarcer compared to other conditions, which indicates that the blue light inhibits the production of pigments. This inhibition can be seen on the bacteria culture itself, which have different consistency in different light conditions. Under blue, white and green light the bacteria culture is more translucent and yellow in color and the viscosity is less compared to the dark, NIR and FR light condition culture. The color is also more vibrant and closer to orange in the dark, NIR and FR samples.

Table 8. The % values of the identified carotenoid signals in different light conditions. Each pigment signal is normalized by taking into consideration the dry weight of the bacteria culture and divided with the sum of all carotenoids to create % value of each pigment signal. The values inform the carotenoid distribution in each light exposure bacteria culture. Antheraxanthin and diadinoxanthin are found in all data sets. Diadinoxanthin is clearly the most abundant carotenoid in each dataset.

Retention time (min)	Pigment	Light conditions						
		Dark	White	Blue (446 nm)	Green (520 nm)	Red (620 nm)	FR (760 nm)	NIR (870 nm)
8.4	Violaxanthin	0.51	0.59	0.63	0.70	0.54	0.57	0.29
8.8	Neoxanthin	0.51					0.19	
11.3	Antheraxanthin	4.96	5.66	5.59	7.49	7.48	6.69	4.10
12.9	Astaxanthin					0.39	0.26	
13.6	Diadinoxanthin	81.54	79.97	72.91	82.42	86.72	81.21	83.74
16.3	Zeaxanthin	1.07	1.89	6.63	1.35		1.21	2.10
23.5	Beta-cryptoxanthin						0.09	0.28
28.3	Alfa-carotene					0.24	0.45	0.38
31.8	Beta-carotene	0.93			0.30		0.40	0.43

BChl a production is most abundant in darkness, far red illumination and NIR illumination as seen in Figure 5. The same conditions have also higher carotenoid abundance considering the pigment absorption intensities and the number of carotenoids produced. The blue light has the scarcest number of pigments but the abundance is lowest under green light. Blue light bacteria culture has the lowest BChl a ratio (Table 9) to the carotenoids, the BChl a production is heavily inhibited

by the blue light. The BChl a high abundance in dark, FR and NIR is seen in the Table 9 as a high relation value when compared to carotenoids.

Table 9. Relations between carotenoid and BChl a abundance in the strain S2U11. The sum of the BChl a absorbance intensities are divided with the sum of carotenoid absorbance intensities. From the results can be displayed that BChl a are less abundant than carotenoids, but this does not take into consideration whether all carotenoids are evenly more abundant or is there one highly abundant carotenoid which affects the relations. In the chromatogram data was interpreted that blue light has inhibiting affect in the pigments and the darkness far red and NIR areas produce more pigments, especially on BChl a. The same trend is seen on the relations where blue light has the lowest value on the BChl a relation to carotenoids and highest with darkness and NIR light. The β-carotene and zeaxanthin are photosynthetic linked carotenoids. Zeaxanthin was found from almost all light conditions, but β-carotene is present in the same conditions where BChl a is abundant.

Light condition	$\sum A_{775nm}^{BChl\ a} / \sum A_{450nm}^{Car}$	$\beta\text{-}car / \sum A_{450nm}^{Car}$	$zeaxanthin / \sum A_{450nm}^{Car}$
Dark	0.41	0,009	0,011
White	0.01	-	0,019
Blue (446 nm)	0.007	-	0,066
Green (520 nm)	0.03	0,003	0,014
Red (620 nm)	0.05	-	-
Far red (760 nm)	0.19	0,004	0,012
NIR (870 nm)	0.31	0,004	0,021

Based on literature, β-carotene and zeaxanthin are found to be photosynthetic (Frank and Cogdell, 1996; Polívika et al., 2009; Demmig-Adams et al., 1996; Yurkov and Beatty, 1998). Both carotenoids are present in the strain S2U11 in different light conditions. Zeaxanthin is found from all light conditions apart from red light. Under blue light zeaxanthin is most abundant and does not alter significantly between the rest of the light conditions. β-carotene is found in the same light conditions where BChl a production is higher, significantly in the dark growth conditions.

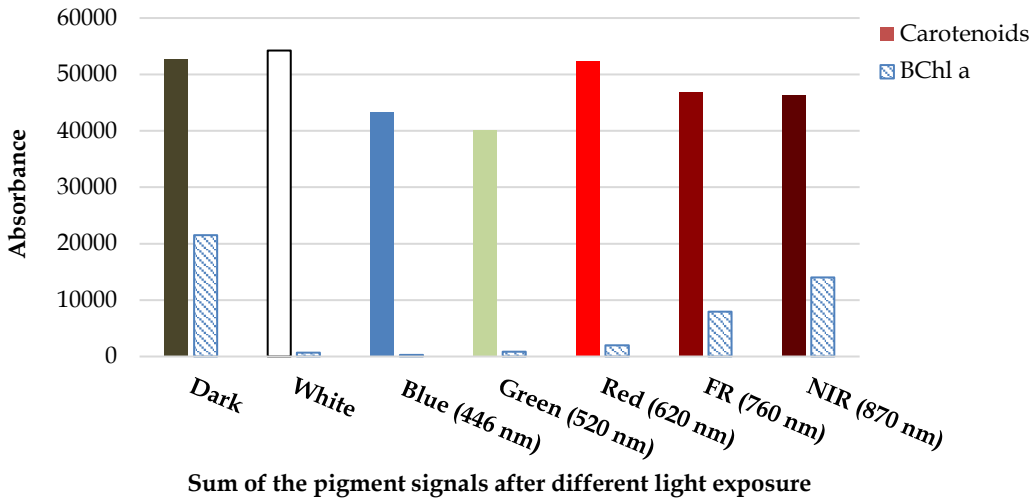


Figure 6. The abundance of the sum of the pigments in absorbance. Coloured colums hold the carotenoid abundance in the different light exposure. The striped columns hold the abundance of the BChl a. This figure verifies the table 8 relations between carotenoids and BChl a. The carotenoids are more abundant in the *Sphingomonas glacialis*. The light exposure influences the pigment production. Conditions where visible light was not accessible to the bacteria, the BChl a is produces more. The absence of light had no significant effect on the total amount of the carotenoids. Lower wavelengths, such as the blue and green light, lower the carotenoid and BChl a abundance.

The bacteria culture growth was documented with the dry weight of the bacteria culture in two plates. Table 19 in Appendix 5 shows the dry weight in the different light conditions with additional observations of the bacteria culture. FR replicate 3 only had one viable bacteria culture plate. The dry weight alters between the light exposures. Most growth takes place in the NIR and FR lights but interestingly, the dark sample has little bacteria culture growth although the pigments are abundant.

3.2.4 BChl a, pigment of multiple peaks in the HPLC chromatograms

HPLC chromatogram taken at 775 nm from NIR light exposure sample (Figure 7) shows multiple signals in five to seven retention time range area. When each of the signals are plotted as absorption spectra the signals appear to be all BChl a based on the identical shape when compared to the BChl a example spectrum in Appendix 1. The different signal spectra are identical to one another except for retention time signal 6.5 which has the first absorbance peak at 275 nm, whereas the other signals have the first peak at 260 nm.

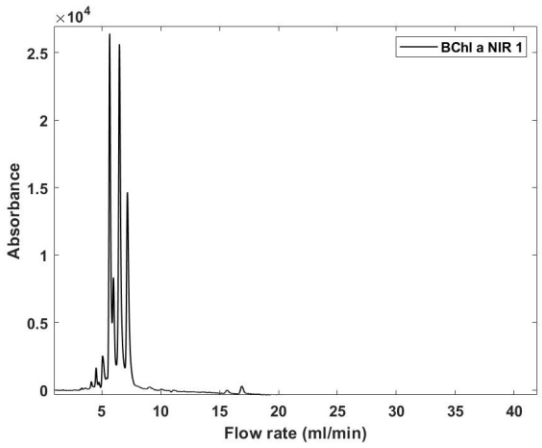


Figure 7. Chromatogram of the BChl a from NIR light exposed bacteria culture. The chromatogram shows seven different retention time signals 4.1, 4.5, 4.7, 5.1, 5.7, 6.0, 6.5 and 7.2 minutes. The retention times itself do not inform if all the retention times are BChl a. The signals need to be plotted as absorption spectra (Figure 8).

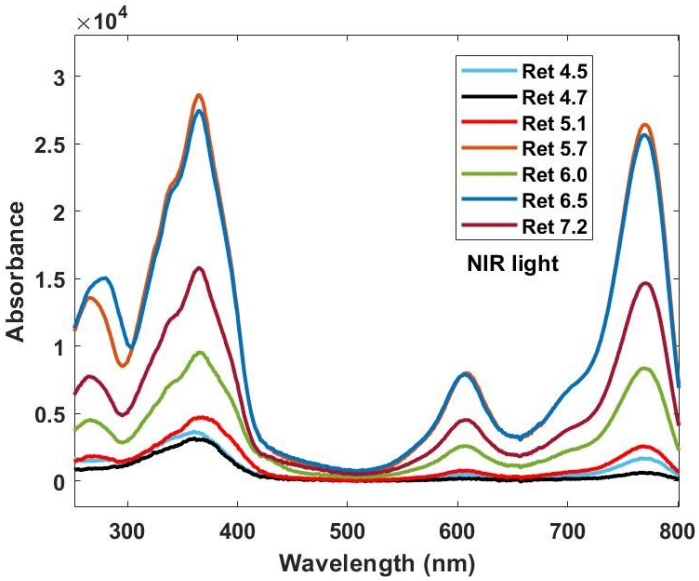


Figure 8. Absorbance spectra of the different BChl signals from the NIR light exposure bacteria culture. The HPLC chromatogram (Figure 7) shows multiple signals that can be verified as BChl a by the absorbance spectra shape. The spectra are identical except for retention time signal 6.5 that have the first absorbance maximum value at higher wavelengths. The multiple peaks of BChl a indicate that the pigment molecule have different conformations of properties, which affect the interaction between the BChl a molecule and the column leading to the multiple peaks.

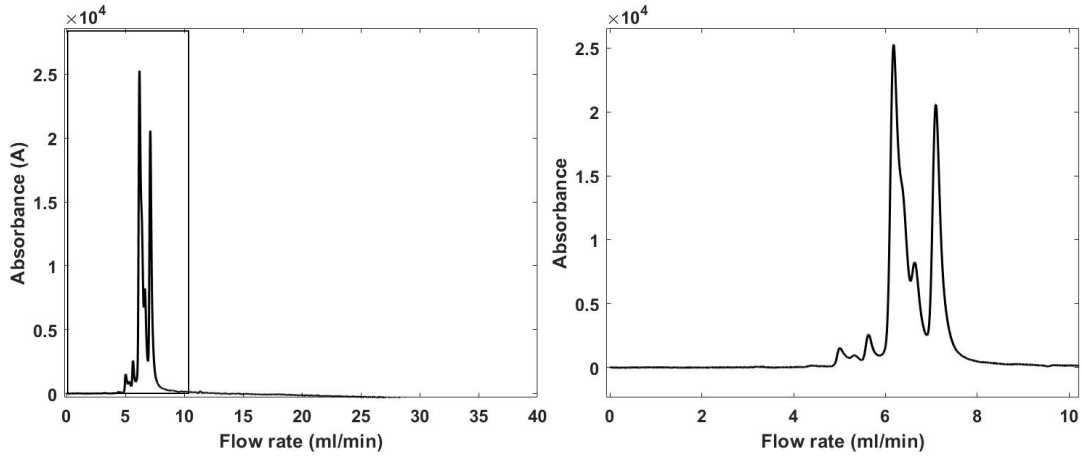


Figure 9. The HPLC chromatogram of commercial BChl a pigment diluted in methanol. The BChl a leaves the chromatogram column slighty different rates, which results in six different retention time peaks in the chromatogram. The segregated peaks are result of the changes in the BChl a molecule. The multiple peaks taking place in the sample chromatograms (Figure 7) and in example chromatogram indicates that the changes within the molecule are not due from changes within the bacteria growth, but rather from the pigment-solvent interaction.

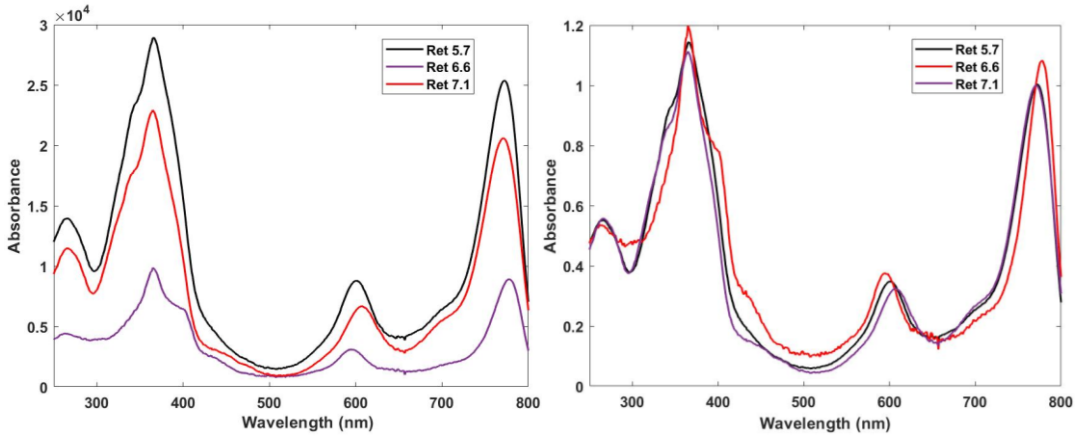


Figure 10. Three strongest BChl a absorbance spectra, retention times 5.7, 6.6 and 7.1 minutes, taken from the commercial BChl a pigment diluted in methanol. The left figure contains the absorption spectra of the retention times, and the right figure contains normalized spectra of the retention times. The small alterations are the indicator of the differences in the BChl a molecule.

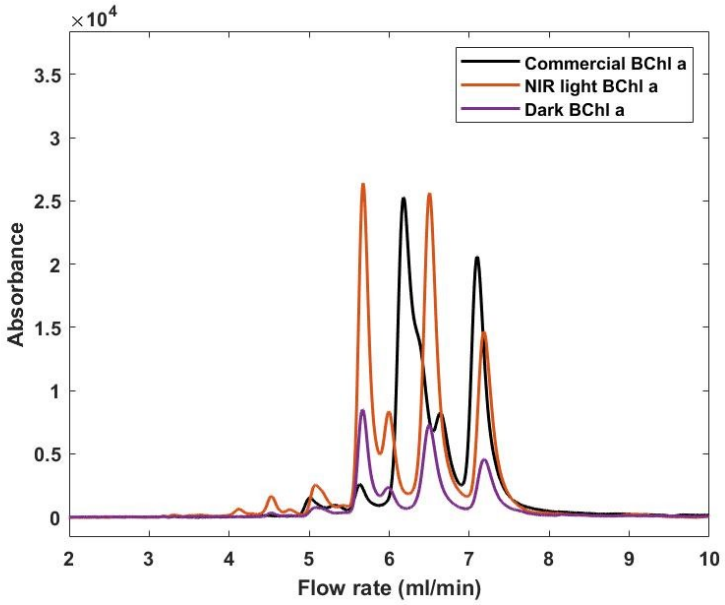


Figure 11. HPLC chromatograms of commercial BChl a and extracted BChl a from NIR and dark samples of *Sphingomonas glacialis* strain S2U11 growth. BChl a extracts exit the chromatogram at same rate, but the commercial BChl a exits slightly different rate compared to the NIR and dark sample produced BChl a.

Comparison of the NIR light exposure *Sphingomonas glacialis* BChl a extract chromatogram (Figure 7) with the commercial BChl a pigment chromatogram (Figure 9) confirms that the BChl a has multiple retention time peaks as a standard. The multiple retention times are not formed by the 870 nm light exposure but rather by the molecule's interaction with the methanol. In the Appendix 6 are the absorption spectra of the all BChl a retention times in each light exposure set, but in the Figure 12 are normalized retention times found in all light exposure bacteria cultures, retention times 5.7, 6.5 and 7.2 minutes. Only the blue light and white light exposed bacteria cultures had no other than the three retention times. The blue light and white light exposed bacteria had the most distorted chromatograms and absorbance spectra due to the low signal absorbance levels. The distortion affects the normalization, and the differences are more difficult to perceive in blue and white light BChl a spectra. The figure 11 shows the BChl a HPLC chromatograms of commercial BChl a, NIR exposure sample BChl a and under darkness produced BChl a. The chromatogram shows that the commercial BChl a exits the chromatogram at different rate compared to the strain S2U11 extracted BChl a.

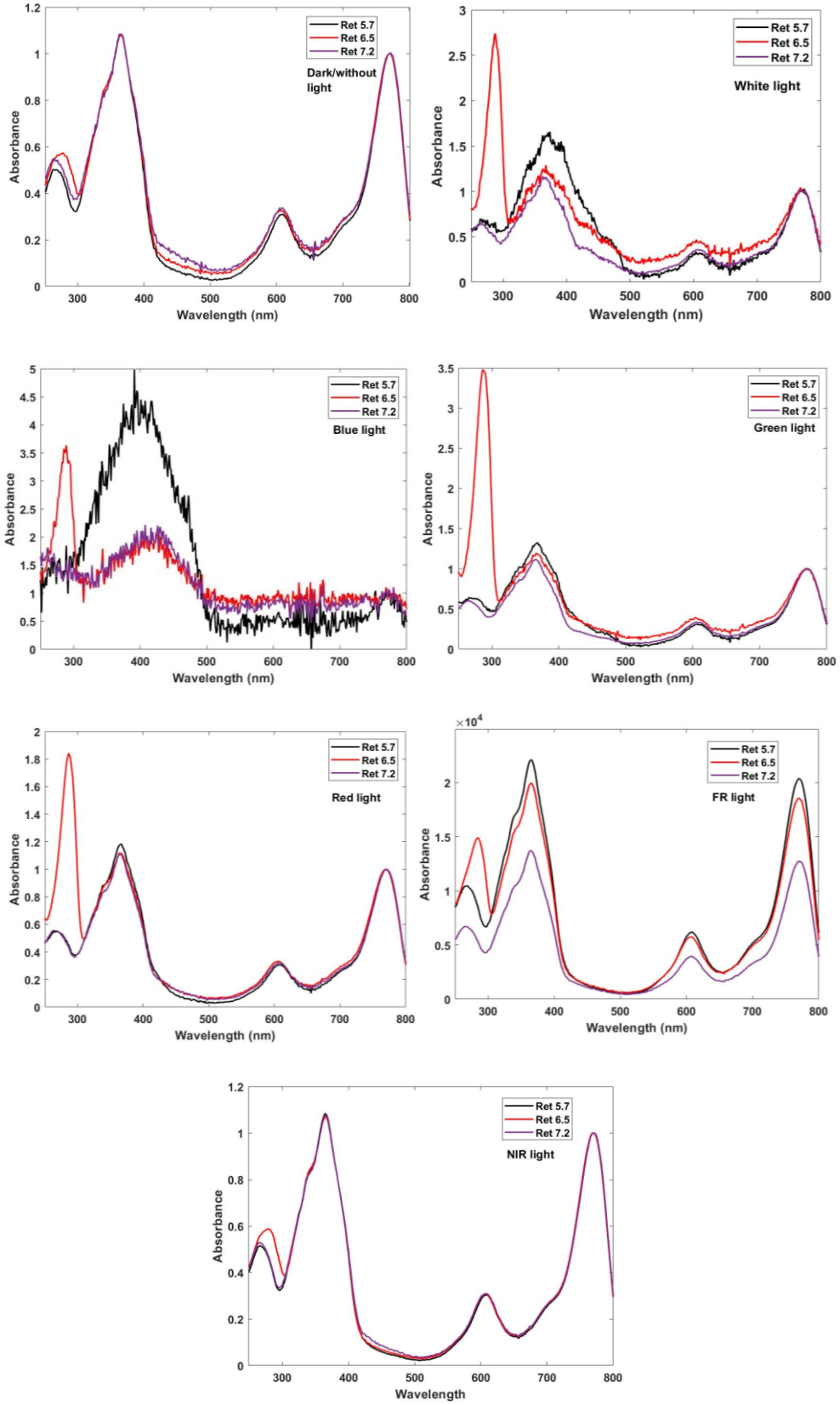


Figure 12. BChl a retention time absorption spectra from each light exposure sets. The spectra are normalised by multiplying the each wavelenght data point with $1/A_{770nm}$. Differences between each retention time absorbance spectrum are more visible after the normalization. The distortion of the spectra in the blue and white

light exposure affects the normalization. The retention times, 5.7, 6.5 and 7.2 minutes, are present in each light exposure samples and were the most abundant signals. Blue light and white light exposed bacteria culture produced only the three signals mentioned above. The absorbance intensity alters between the different light conditions.The slight alterations between the spectra indicate that the BChl a has small conformational changes in the molecule.

The difference of signal 6.5, which has the first absorbance peak at 275 nm whereas the other signals have the first peak at 260 nm, is visible in all light conditions. The signal 6.5 the normalized spectrum has higher 275 nm peak in the visible light sample spectra but is due from the increasing ratio when compared to other spectra. When the absorbance intensities in the other peaks spectrum lower and the peak 275 do not, the normalization creates a higher peak in the 275 nm. Nevertheless, in the specta at each light condition sample are visible small differences, due to changes in the BChl a molecule.

3.3 Fluorescence excitation and emission spectra of bacteria

Photosynthetic properties of the strain S2U11 pigments are investigated with absorption and fluorescence excitation spectra. Corresponding absorption and fluorescence excitation spectra in the 400 to 550 nm area are an indicator of the photosynthetic singlet state energy transfer between carotenoids and BChl a. The Figure 13 absorption and fluorescence excitation spectra do not correspond in the carotenoid absorption and excitation wavelength area. The strain S2U11 does not have carotenoids with photosynthetic properties and their importance to the bacteria are in photoprotection and structural stabilation.

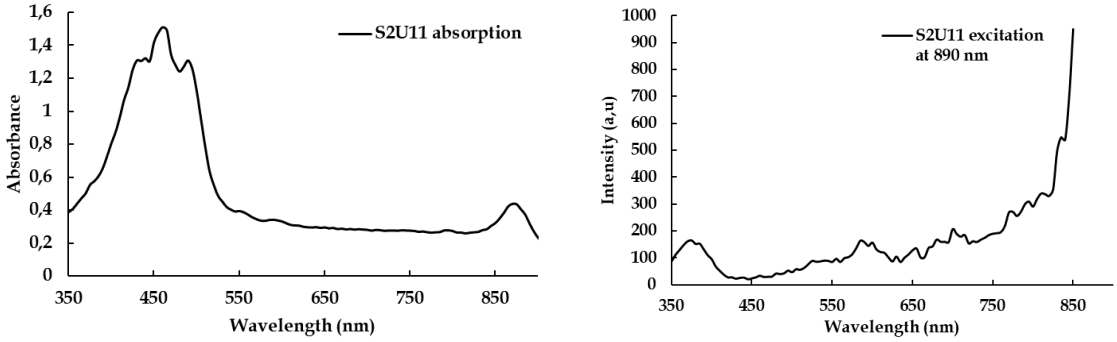


Figure 13. Absorption and fluorescence excitation spectra of *Sphingomonas glacialis* strain S2U11. Carotenoids photosynthetic properties are investigated by comparing whether the absorption and fluorescence excitation spectra correspond in the 400 to 550 nm area where carotenoid light absorption wavelengths are.

Literature of photosynthetic cartotenoids (Polívika et al., 2009 and Zia-Ul-Hag et al., 2021) lists β-carotene and zeaxhantin as photosynthetic which have been found from Sphingomonas glaicialis (see Table 6). This should conclude that fluorescence excitation should have excitation at the carotenoid area. The two carotenoids are not capable of transferring excitation energy to BChl a. The excitation and absorbance spectra show peak at the 850 nm area where the BChl a absorbance area are. BChl a is photosynthetic in Sphingomonas glaicialis.

4 DISCUSSION

The extraction of carotenoids and BChl a from the bacteria culture could be done with both methanol and the cholororm:methanol extraction. Both extraction methods had pros and cons. The methanol elution was faster and more straightforward but the pigment intensity in the HPLC chromatogram was lower. Exact dry weight of the bacteria culture could not be determined because the bacteria culture was added to the solvent straight from the plate. The chloroform:methanol extraction needed more than 24 hours to complete and had multiple steps, but it yielded more intense pigment signals. Dry weight of the bacteria could be weighed to exact if needed. Better control of the samples and higher peak intensity resulted that the chloroform:methanol pigment extraction was

used in the growth condition experiments. The decision was made prior the use of MatLab.

The HPLC chromatogram within the machine screen showed the pigment signals as seen in the Figure 1. This, however, was not clear enough to make conclusion of the pigments because the smaller peaks easily blurred into the background. The data was transferred into MatLab as chromatograms and absorption spectra. The MatLab chromatograms were easy to interpret because of clear signals which could be detected from the background. The column in the HPLC was the same in each measurement which kept the samples comparable to each other. Slight alterations in the retention times are normal and the change is seen when comparing retention times of the ISTDs. The internal standard peak comes out at a certain point from the chromatogram and the pigment peaks can be scaled with the internal standard change. This is needed when identifying the peaks. If the retention times of the peaks from the samples were within the change area between the ISTDs, the identification was used. Each pigment signal was turned into absorption spectra and confirmed to be carotenoid or BChl a. The identified carotenoids were verified with the absorption spectra due to the individuality of each pigment spectra. Not all found retention times were identified in the Table 10 (Appendix 2) and identification of these relied on literature sources. The problem lies in the retention times, because when the HPLC column changes, the run time of the measurement changes along with the retention times of the pigments. The literature list of the carotenoid retention times correlates with the full run time of the measurement in each experiment and the retention times gotten from this experiment are only partially usable. The literature retention times need to be scaled as close as possible to the verified experiment signals and use the scale faction in unidentified pigments. If the used HPLC column in the literature is based on different separation method, has different carbon chains or the column dimensions alter the signals cannot be scaled to correlate. The chemical and physical properties within the column alter the eluation order of the pigments. If the retention times could not be unified the verification would be based on the absorption spectra. This would need clear and

precise spectra because the differences between the carotenoids are small. Without the exact peak maxima, the carotenoid absorption spectra can be mixed easily.

Growth condition changes have alternating effect on the pigment compositions when the changing factor is light. The pigments are produced in the beginning of the bacteria culture growth based on the fact that the pigment composition does not change when comparing one week bacteria growth and four week bacteria growth. The pigments absorbance diminishes which indicates that there are less pigments in the longer growth period bacteria culture. Because the pigments are used as protection (Dieser et al., 2010; Stahl and Sies, 1993 and 2003) the diminishing of the pigments would be explained by destruction of the carotenoids due to photoprotection in free radical quenching or excess excitation energy quenching. Clear differences in the pigment composition and ratios are seen when the bacteria culture is exposed to the different wavelengths of light or darkness. BChl a abundance has greater change compared to the carotenoids. The production is minimal in visible light spectrum area and takes place in darkness or in the far red and NIR wavelengths. This phenomenon of better BChl a synthesis in darkness was demonstrated by Arla Palokas in her Master's thesis (2019). This could be explained with oxidative stress or excess excitation energy photoprotection. The absorption sum of all carotenoids has minor effect between the light exposures. The carotenoid production is smaller in the blue and green wavelength areas. The overall carotenoid absorption sum consists mostly of diadinoxanthin, which is over 90% of the whole absorption sum in all light conditions. The distribution changes if diadinoxanthin is left out from the data and the blue light exposure data becomes most abundant and the red, far red and NIR diminish. This can be seen in Figure 19 in Appendix 7. When inspecting the number of carotenoid retention time signals, the carotenoid composition changes significantly, and the blue light has fewer peaks along with white light. Interesting is that green light has more pigments compared to blue light, but the abundance is smaller. It indicates that the small number of pigments produced under blue light endure the blue light and become abundant. The green light, in turn, does not have the detrimental effect of the blue light but it is either unfavorable or slows the synthesis of the pigments. The highest number of

different carotenoids were synthetized under darkness, far red light and NIR light. These are conditions without visible light, which could indicate that the carotenoids, same as BChl a, synthetize better without visible light wavelengths. Taking into consideration that usually the bacteria growth takes place in the nature, the scarce pigment composition under white light could have been thought to be more abundant. Natural day cycle can be the cause of the results and carotenoids in nature grow better at dusk or at the night. Carotenoids have photoprotective properties within the organism (Dieser et al., 2010; Stahl and Sies, 1993 and 2003), which could explain the differences in the light condition. Similarly, as hypothesized with the BChl a, the carotenoid synthesis is disturbed by free radical quenching or excess light energy quenching when exposed to excess light. It is also possible that visible light inhibits the production of the carotenoids which are absent in their conditions. Astaxanthin was found in Spingomonas glacialis strain S2U11 from the white, red and FR light samples, and it has been found to have photoprotective properties (Hairston Jr., 1976). The difference between light exposures samples was clearly visible when preparing the samples. The bacteria culture grown under darkness, far red and NIR light had more vibrant orange colour and the blue and white light had dull yellow colour. Darkness, far red and NIR light exposed bacteria culture was thick and viscose whereas the blue and white light exposed bacteria culture was thinner and more watery.

For the future experiments, few changes could be made. Knowing that the blue, green and white light exposed bacteria culture grows less than the other conditions, in the given time, there should be more plates in these conditions. A higher amount of pigments in the elution can ensure that the infrequent pigments do not blend with the background. Dry weight should be the same in all different samples. This decreases the chance of error in the measurement. In this experiment, the dry weights in the light condition experiment were not the same due to the better bacteria culture growth in some conditions but were normalized by making the smallest sample dry weight a coefficient and multiplying rest of the data with it. This can increase the possibility of error and it can be seen in one FR sample replicate, which had half weight compared to other replicates. It increased the

absorbance of the most abundant carotenoid in the one replicate, which then affected the average of the sum of the all carotenoid absorbances. This was the reasoning for leaving the FR 3 replicate out from the Figure 5 data. The light condition effect experiment had only three replicates at each light condition due to the limited amount of time and resources. The light exposure was performed in a self-built machine found at Figure 19 in Appendix 8, which had room for six plates and each replicate needed two plates worth of bacteria culture. Each experiment required three days of light exposure, which was 21 days for all the seven light conditions. Prior to this experiment was done the one-to-four-week growth period experiment and the extraction method comparison experiment. More replicates in the future ensure more room to view errors and leave out clear outliers. However, this Master's thesis did not investigate the precise amount of the pigments found in the Sphingomonas glacialis, but research if the extraction methods extract carotenoids and BChl a from bacteria culture and does the alterations in the growth conditions affect the pigment composition. In the light conditions one aspect to experiment in the future is the natural day-to-night cycle. In this experiment, the bacteria culture was exposed to three days of non-stop light. It would be interesting to see if the period of darkness in between the light exposure has effect on the carotenoid growth.

HPLC chromatogram taken at the 770 nm area shows multiple BChl signals even if the measured pigment is commercial BChl a. When each of the signals are plotted as individual absorption spectra, they are recognized as BChl a but with small changes in the molecule. This can be seen in the Figure 11 where commercial BChl a is plotted with two light condition experiment sample BChl a. The commercial pigment exits slightly different rate compared to the sample BChl a. The commercial BChl a is produced from Rhodopseudomonas sphaeroides purple bacteria whereas the sample BChl a are from Sphingomonas glacialis bacteria and the different source of BChl a could be enough to have alterations in the molecule or behave differently. The HPLC column separates the molecules based on size, shape, and chemical properties. BChl a undergoes changes in the solvent, which are enough for the pigment to exit the column at different rates. Each exciting alteration of the pigment

would need to be collected as a fragment and examined further with mass spectrometry to see what changes cause the pigment to exit at different rates. Without mass spectrometry, it is impossible to discover these changes. The changes could be due to conformational or structural changes within the molecule, or that the BChl a molecule has bonded with another molecule or atom, and this affects the pigment interaction with the HPLC column.

Looking at the fluorescent excitation spectra of the native state Sphingomonas glacialis strain S2U11 culture it does not show the excitation at the carotenoid area. The carotenoids do not transfer excitation energy to BChl a. When looking at the found pigments and searching photosynthetic linked carotenoids the β-carotene and zeaxanthin has been found to have photosynthetic properties (Polívika et al., 2009 and Zia-Ul-Hag et al., 2021) and it is possible that the unknown carotenoids share the fate with the two carotenoids. Zeaxanthin abundance is highest under blue light and in the other light conditions stays in the same level. β-carotene grows under same conditions where BChl a production is abundant. The carotenoids need to locate close enough to the BChl a in the LH complex for the singlet state energy transfer to concur (Zia-Ul-Hag et al., 2021). Nevertheless, the photosynthetic properties consist of photoprotective functions such as excess light quenching and free radical quenching which are important. The carotenoids could gather the excess light energy in the LH complexes and prevent the creation of free radicals. This protects the LH complexes from damage. The abundant zeaxanthin production under blue light could be explained by protection against UV wavelength size, which is close is size with the blue light wavelength size. The β-carotene synthesis in the same conditions with BChl a could indicate a link between β-carotene and BChl a. The light harvesting complex is delicate process which the excess light energy can be detrimental, and the β-carotene could act as a photoprotector in these light conditions. Interestingly, the higher synthesis in β-carotene and BChl a is in the light conditions where is no visible light, which could indicate that the protection is against the excess excitation energy from the BChl a. This protects the BChl a from creating excess energy which could lead to the single oxygen creation. The fluorescence excitation was performed with Tecan Spark multimode microplate

reader which was loaned to university for a limited time and was returned before the light exposure experiment was completed. But in future experiments it would be interesting to study if the photosynthetic carotenoids appear functional under different light exposure.

5 CONCLUSIONS

The pigments are possible to analyze and identify with the used method. The first hypothesis considering the extraction method when applied to bacteria should be functional was proven right. Extraction and elution of the pigments can be done with two methods, which both give out distinct pigment signals. The analysis of the pigment signals can be done with a program where the HPLC data is plotted into chromatograms with particular detection wavelength or into absorption spectra are plotted with each retention time. BChl a and carotenoids have different absorption wavelengths and can be separated based on the absorption spectra alone. The difficult part is the identification of the different carotenoid signals without verified carotenoid retention time list based on the same chromatography column. The identification without them would require intensive literature research where the different column properties would be considered, which has more work than average Master's thesis should include. The second hypothesis of growth conditions altering the pigment composition was correct with the light conditions. It was hypothesized that the growth period length would also affect the composition, but the pigments are produced at the beginning of the bacteria culture growth and no new ones grow after a certain time point. No long growth periods are needed to analyze pigments. It was expected from the blue light to inhibit the pigment synthesis and the most abundant BChl synthesis take place in the darkness, which were proven right by the experiments. The third hypothesis was proven wrong. The photosynthetic carotenoids were found from the Sphongomonas glaciais but they cannot transfer excitation energy to BChl a. But their importance could be in the protection against excess light. The photosynthetic apparatus is delicate complex,

and the energy transfer occurs when the excitation states have similar energy levels. The energy transfer requires correct placement of the carotenoids, and the transfer cannot occur if the distance of carotenoids and BChl a grows excessively wide.

Each HPLC measurement contains considerably data and creation of each chromatogram and absorptions spectra form each sample replicate build up the workload. Program which automatically could plot the different chromatograms and spectra from the data with commands would speed up the process and allow more replicates or data to be processed. Creating the absorption spectra individually based on the chromatogram was time consuming and took more time than expected, which is a factor to consider in the future when planning the number of samples and replicates. Nevertheless, the whole method from the extraction and elution to the pigment analysis and photosynthetic property experiment is functional to analyze different pigments from bacteria. The next step is to experiment and verify the applicability with other bacteria than Sphingomonas glacialis strain S2U11 and compare the pigment composition between the different AAP bacteria.

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APPENDIX 1. EXAMPLE SPECTRA OF B-CAROTENE AND BACTERIOCHLOROPHYLL A

Measurement of the absorption and fluorescence excitation spectra of commercial pigments BChl a and β-carotene dissolved in acetone. The absorption maximum in each peak in the spectrum are the wavelengths where the light energy is absorbed into the pigment. These maxima correspond the different excitation states in the molecule. In BChl a these are around 350 nm, 560 nm, and 770 nm. In β-carotene the range is from 350 nm to 500 nm. The excitation range are the peak maxima in the fluorescence excitation spectrum. If the pigments can transfer the energy from the excitation states, the excitation spectrum corresponds to the absorption spectrum. This is true with BChl a but not with β-carotene.

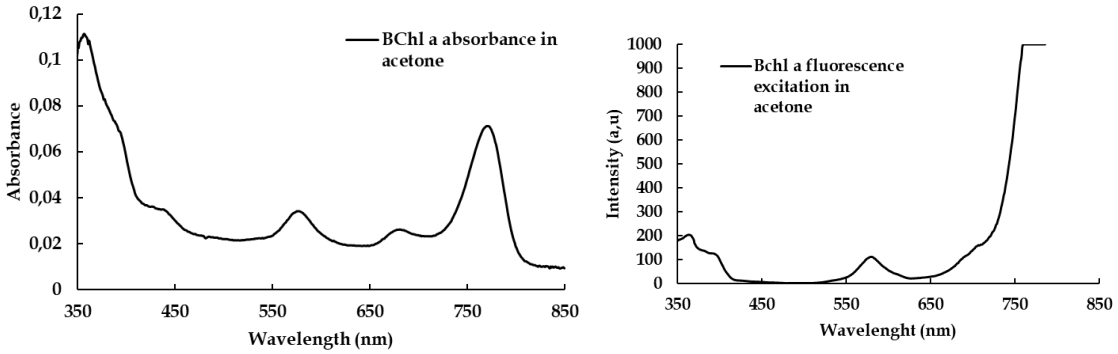


Figure 14. Absorption (left figure) and fluorescence (right figure) excitation spectra of commercial BChl a pigment diluted in acetone. The spectrum peak maxima correspond with the excitation states of the molecule. The BChl a is capable to transfer excitation energy from all excitation states when the fluorescence excitation spectrum corresponds with the absorption spectrum. The fluorescence excitation spectrum has too high signal after 760 nm due to scattering of the light, but the trend of the spectra shows excitation peak at the 770 nm area.

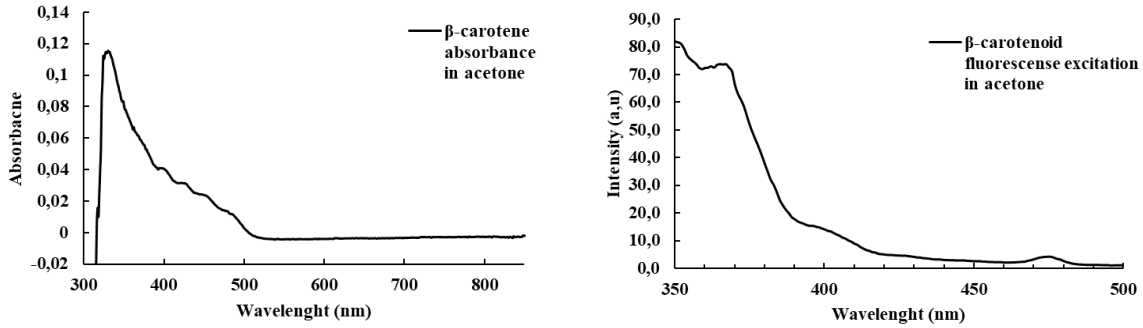


Figure 15. Absorption (left figure) and fluorescence (right figure) excitation spectra of commercial β-carotene pigment diluted in acetone. The spectrum peak maxima correspond with the excitation states of the molecule. The absorption spectrum does not correspond with the fluorescence excitation spectrum and the β-carotene does not transfer the excitation energy from the excitation states. The fluorescence excitation spectrum peak should be in the 400 to 500 nm range if the pigment is transfers energy.

APPENDIX 2. THE VERIFIED CAROTENOID TABLE

Table 10. Retention times for carotenoids, with the internal standard trans-β-apo-8-carotenal. The retention times of the carotenoids are proportioned to the internal standard and are dependent on the used column. The retention times were given by Hannu Pakkanen and Sami Taipale and are used as a course material for extraction of carotenoids from algae.

Carotenoid	Retention time (min)
Peridinin	5.6
Fucoxanthin	6.4
Violaxanthin	8.5
Neoxanthin	8.74
Dinoxanthin	8.9
Antheraxanthin	11.8
Astaxanthin	12.9
Diadinoxanthin	13.4

Lutein	13.8
Monadoxanthin	15.6
Zeaxanthin	16.1
Trans-β-Apo-8'-carotenal (ISTD)	17.0
Cantaxanthin	17.9
Diatoxanthin	18.1
Myoxoxanthophyll	18.6
Aphanizophyll	18.6
Aphanizophyll**	19.4
Alpha-Cryptoxanthin	20.1
Alloxanthin	20.4
Crocoxanthin*	21.9
Beta-Cryptoxanthin	23.1
Echinenone	26.0
Alfa-carotene	28.3
Beta-carotene	31.4

APPENDIX 3. PIGMENT RETENTION TIMES OF ALL LIGHT EXCPOSURE SAMPLE REPLICAS

Table 11. All pigment retention time peaks from darkness/no light exposure sample replicates.

Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
4.5	4.5	4.5
5.1	5.1	5.1
5.7	5.7	5.7
6.0	6.0	6.0
6.5	6.5	6.5
7.2	7.2	7.2
	8.3	8.4
	8.8	
9.5	9.5	9.5
10.7	10.7	10.7
11.3	11.3	11.3
12.4	12.4	
13.6	13.6	13.6
14.9	14.9	14.9
16.3	16.3	16.2
		17.2
26.8	26.8	26.8
31.8	31.8	31.8
38.3	38.3	38.3
39.7	39.7	39.7

Table 12. All pigment retention time peaks from white light exposure sample replicates.

Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
5.7	5.7	5.7
6.5	6.5	6.5
7.2	7.2	7.2
7.9	8.0	7.9
8.4	8.4	8.4

9.5	9.5	9.5
9.9	9.9	9.9
10.8	10.7	10.7
11.3	11.3	11.3
12.5	12.4	12.4
13.6	13.6	13.6
14.9	14.9	14.9
16.3	16.3	16.2
		17.2
26.8		
38.3		38.3
39.7		

Table 13. All pigment retention time peaks from blue light exposure sample replicates.

Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
5.7	5.7	5.7
6.5	6.5	6.5
7.2	7.2	7.2
7.9	7.9	7.9
	8.4	8.4
9.5	9.5	9.5
9.9	9.9	9.9
10.7	10.7	10.7
11.3	11.3	11.3
12.4	12.4	12.5
13.6	13.5	13.6
14.9	14.9	14.9
16.3	16.3	16.2
		17.2

Table 14. All pigment retention time peaks from green light exposure sample replicates.

Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
5.2	5.7	5.7
5.7		

	6.0	
6.5	6.5	6.5
7.2	7.2	7.2
7.9	7.9	7.9
8.3	8.4	8.4
9.5	9.5	9.5
10.7	10.7	10.7
11.3	11.3	11.3
12.5	12.4	
13.5	13.6	13.6
14.9	14.9	14.9
16.3	16.3	16.2
		17.2
26.8	26.8	
31.8	38.3	38.3
38.3		
39.7		

Table 15. All pigment retention time peaks from red light exposure sample replicates.

Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
5.7	5.1	5.1
6.5	5.7	5.7
	6.0	
	6.5	6.5
7.2	7.2	7.2
8.4	8.4	8.4
9.4	9.5	9.4
10.7	10.7	10.7
11.3	11.3	11.3
12.9	12.9	12.9
13.6	13.6	13.6
14.9	14.9	14.9
26.8	26.8	
28.3		
38.3	38.3	38.3
	39.7	

Table 16. All pigment retention time peaks from FR light exposure sample replicates.

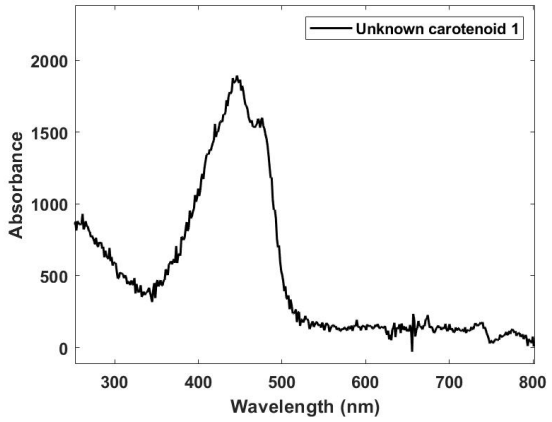
Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
		4.1
4.5	4.5	4.5
5.1	5.1	5.0
5.7	5.7	5.7
6.0	6.0	6.0
6.5	6.5	6.5
7.2	7.2	7.2
8.4	8.3	8.4
8.8		
9.6	9.5	9.5
	10.0	
10.7	10.7	10.7
11.3	11.3	11.3
12.4	12.4	
13.0		
13.6	13.6	13.6
14.9	14.9	14.9
16.3	16.3	16.2
		17.3
23.6	23.6	
26.8	26.8	26.8
		28.8
31.8	31.8	31.8
38.3	38.3	38.3
39.7	39.7	39.7

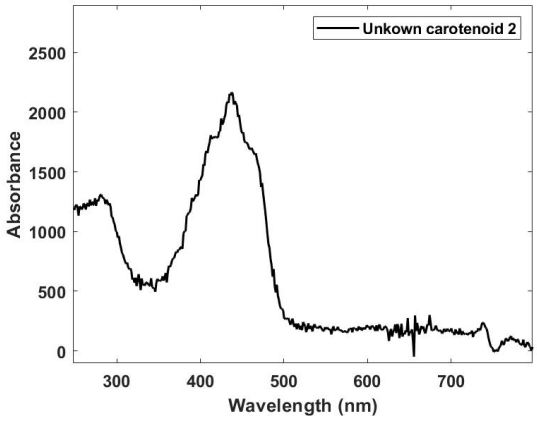
Table 17. All pigment retention time peaks from NIR light exposure sample replicates.

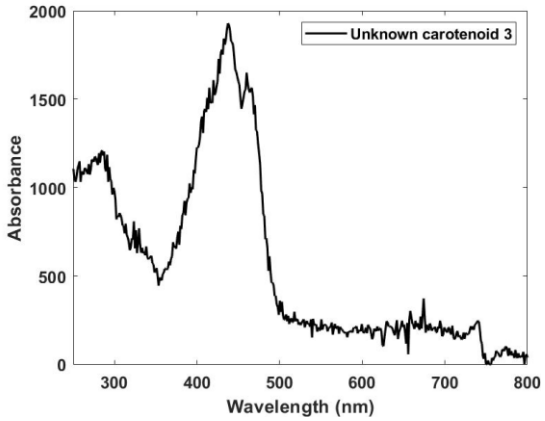
Replicate 1 retention times	Replicate 2 retention times	Replicate 3 retention times
4.1	4.1	4.1
4.5	4.5	4.5

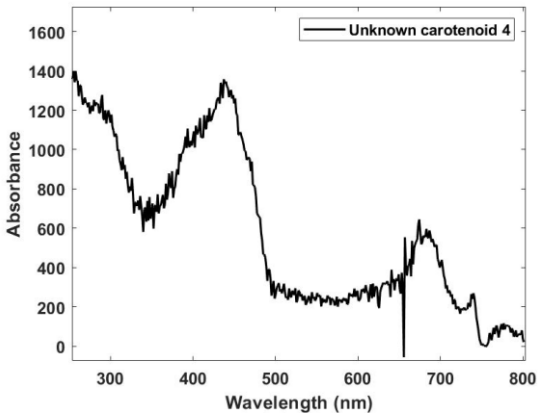
5.1	5.1	5.0
5.7	5.7	5.7
6.0	6.0	6.0
6.5	6.5	6.5
7.2	7.2	7.2
8.4	8.4	8.4
9.5	9.5	9.4
10.7	10.7	10.7
11.3	11.3	11.3
12.4	12.4	
13.6	13.6	13.6
14.9	14.9	14.9
16.3	16.3	16.3
		17.3
23.6		
26.8	26.8	26.8
		28.8
31.8	31.8	31.8
38.3	38.3	38.3
39.7	39.7	39.7

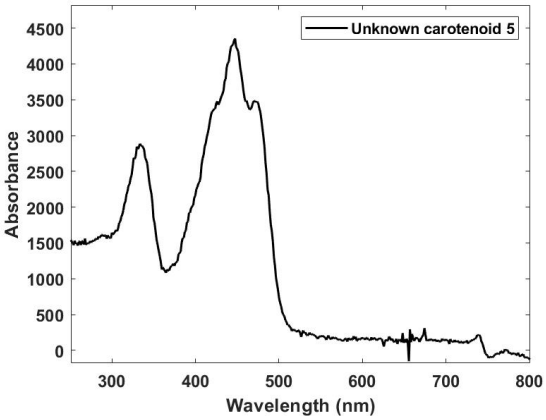
APPENDIX 4. ABSORPTION SPECTRA OF UNKNOWN CAROTENOIDS AND ABSORPTION PEAK MAXIMA FOR ALL CAROTENOIDS

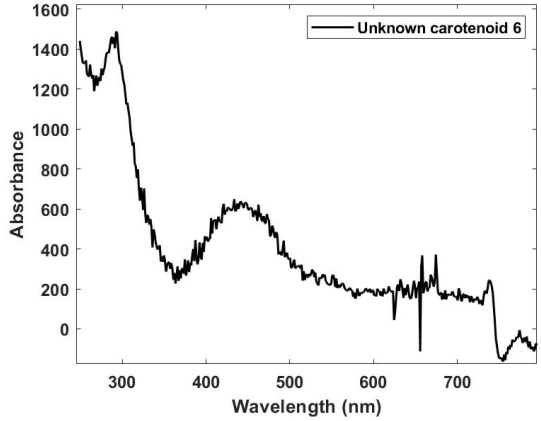


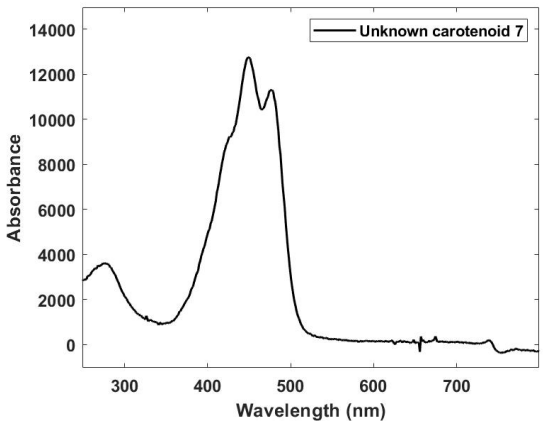


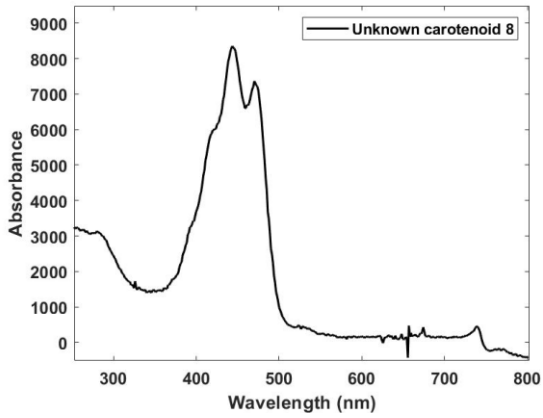

Unknown carotenoid 3
Absorbance
Wavelength (nm)

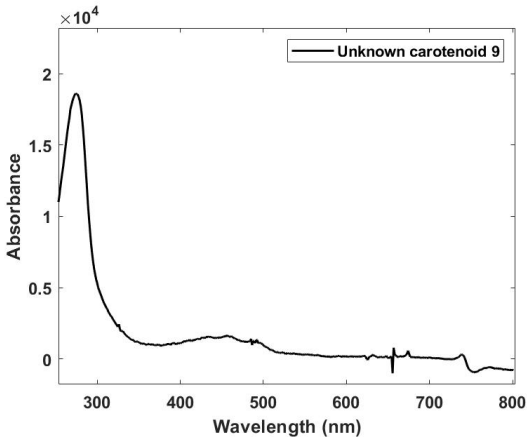

Unknown carotenoid 4
Absorbance
Wavelength (nm)

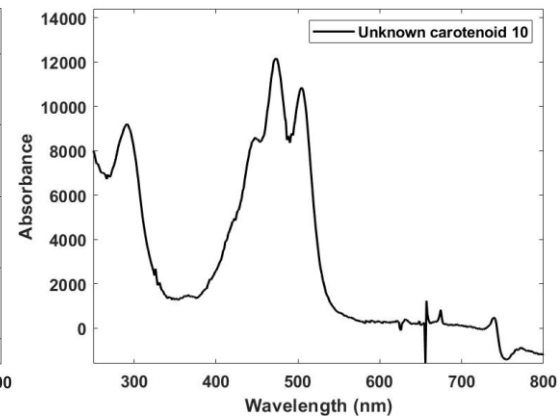

Unknown carotenoid 5
Absorbance
Wavelength (nm)


Unknown carotenoid 6
Absorbance
Wavelength (nm)


Unknown carotenoid 7
Absorbance
Wavelength (nm)


Unknown carotenoid 8
Absorbance
Wavelength (nm)


×10^4
Unknown carotenoid 9
Absorbance
Wavelength (nm)


Unknown carotenoid 10
Absorbance
Wavelength (nm)

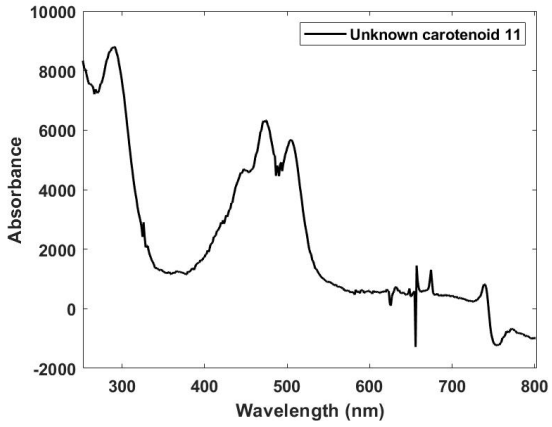


Figure 16. Absorption spectra of the unknown carotenoids. The x axis in each image holds the wavelenghts and y axis the absorbance intensity. The intensities change based on the abundance of each pigment. The less abundant pigment spectra has more noise.

Table 18. The absorption maximas for each of the found carotenoids.

Carotenoid	Spectra maxima (nm)
UN1	444, 473
Violaxanthin	280, 434, 456
Neoxanthin	299, 404, 437
UN2	280, 436
UN3	283, 437, 463
UN4	437
UN5	334, 447
Antheraxanthin	336, 442, 470
UN6	292, 443
Astaxanthin	287, 448, 475
Diadinoxanthin	273, 447, 475
UN7	278, 448, 477
Zeaxanthin	278, 451, 476
UN8	282, 444, 470
Beta-cryptoxanthin	287, 461
UN9	272, 358

Alfa-carotene	280, 437, 462, 492
Beta-carotene*	283, 453
UN10	291, 446, 473, 505
UN11	291, 446, 437, 504

APPENDIX 5. THE DRY WEIGHT GROWTH PERIOD SAMPLES AND THE LIGHT CONDITION REPLICATES

Table 19. The dry weight of the growth period samples. The three week data had larger weight compared to other. The used three week sample was from different sample preparation set due from destruction of the original sample. The original sample weight was 13,42 mg, which had similar weight to the two week and four week samples.

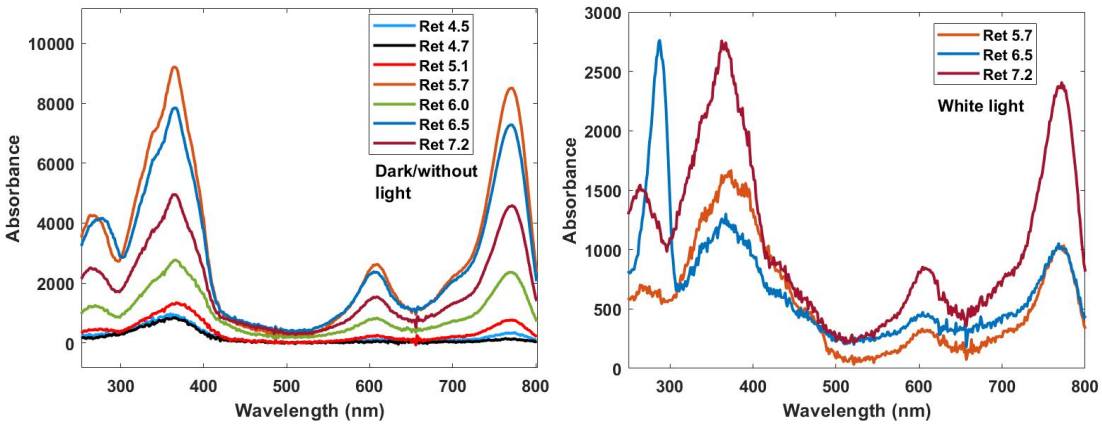
Sample	Dry weight (mg)
Week 1	9,4 mg
Week 2	17.02 mg
Week 3	31.41 mg*
Week 4	15.62 mg

Table 20. The dry weight of the light exposure samples. FR 3 sample had the bacteria culture of one plate due from destruction of the second plate.

Replicate	Dry weight (mg)	Number of bacteria culture plates
Dark 1	2,24 mg	2 plates
Dark 2	3,49 mg	2 plates
Dark 3	8,57 mg	2 plates
White 1	9,84 mg	2 plates
White 2	3,46 mg	2 plates

White 3	7,04 mg	2 plates
Blue 1	6,64 mg	2 plates
Blue 2	6,69 mg	2 plates
Blue 3	6,32 mg	2 plates
Green 1	18,37 mg	2 plates
Green 2	6,78 mg	2 plates
Green 3	12,82 mg	2 plates
Red 1	17,47 mg	2 plates
Red 2	14,75 mg	2 plates
Red 3	16,64 ng	2 plates
FR 1	17,04 mg	2 plates
Fr 2	16,16 mg	2 plates
FR 3	7,54 mg	1 plate, the second was destroyed
NIR 1	12,41 mg	2 plates
NIR 2	14,13 mg	2 plates
NIR 3	21,74 mg	2 plates

APPENDIX 6. ALL BCHL A SIGNAL ABSORBANCE SPECTRA FROM THE LIGHT EXPOSURE SAMPLES



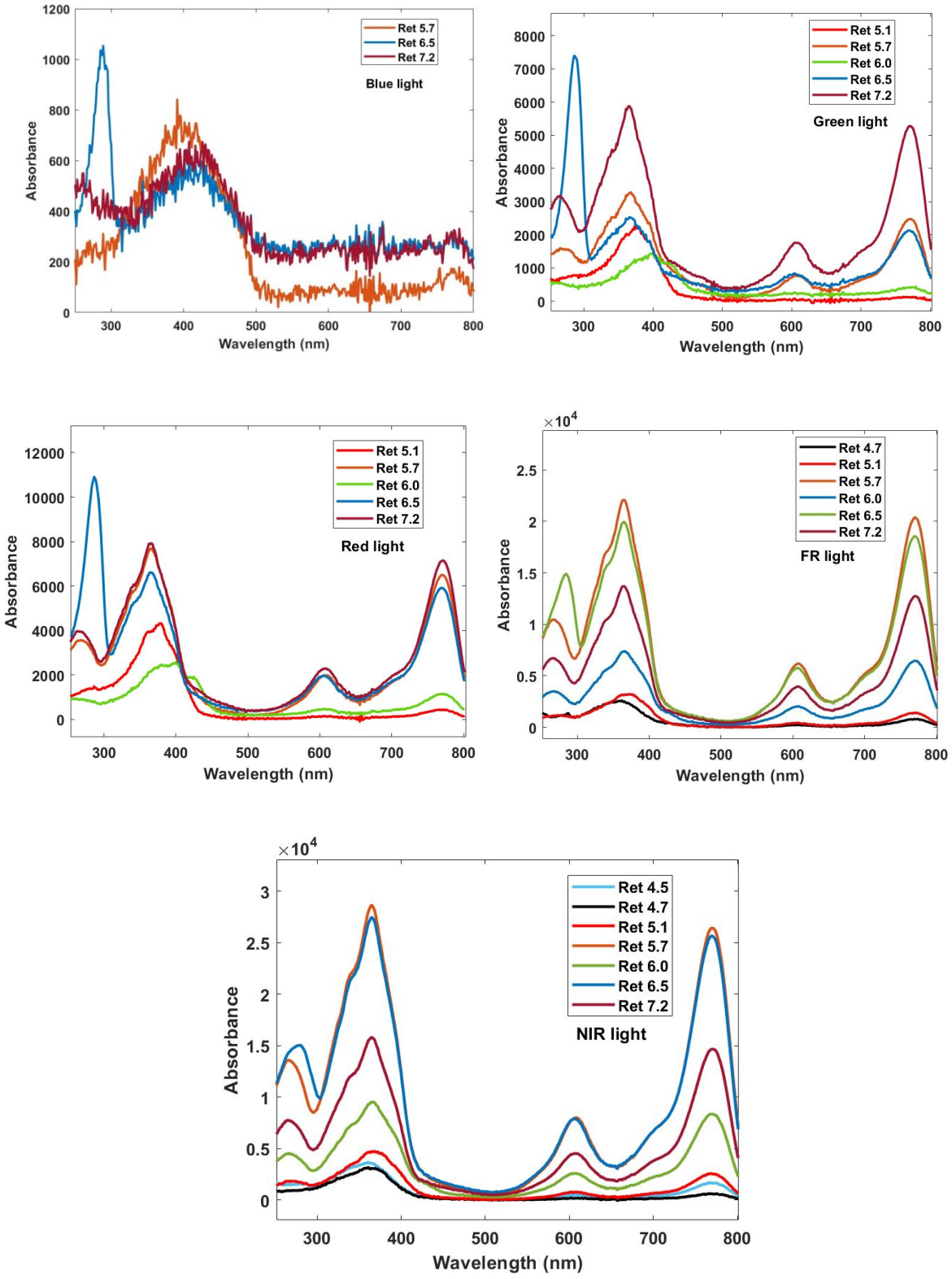


Figure 17. Absorption spectra of all BChl a retention time signals from different light exposure samples.

APPENDIX 7. CAROTENOID ABUNDANCE WITHOUT DIADINOXANTHIN PEAK

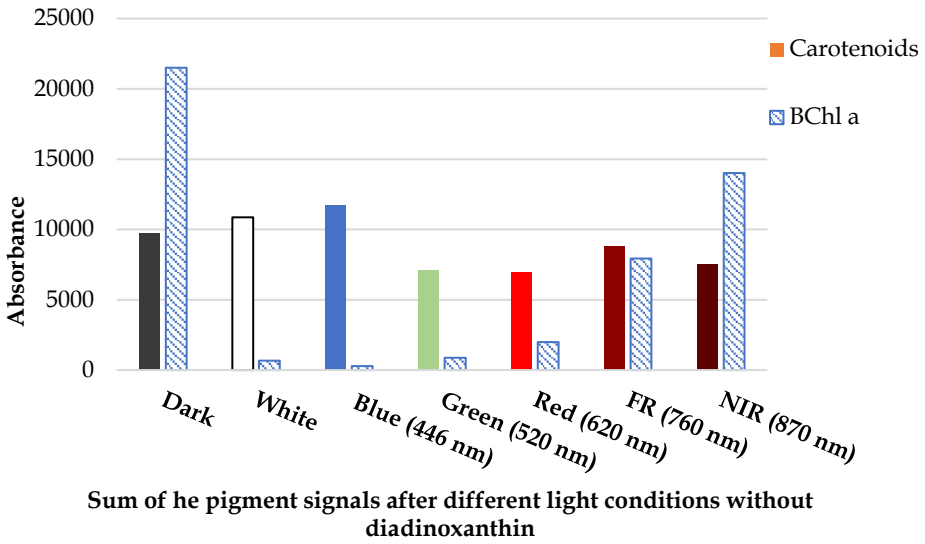


Figure 18. The abundance of the sum of the pigments in absorbance without the diadinoxanthin peak. Coloured columns hold the carotenoid abundance in the different light exposure. The striped columns hold the abundance of the BChl a. Without diadinoxanthin the carotenoids are less abundant than BChl a. The less abundant carotenoids have better growth in the blue and white light, which is opposite to the abundance with diadinoxanthin.

APPENDIX 8. THE LIGHT EXPOSURE MACHINE

Figure 19. Image of the light exposure experiment machine