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Photoacclimation and photoprotection strategies in siphonous green algae of the order Bryopsidales (*Codium tomentosum* and *Bryopsis plumosa*)

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Contents

To my mum Miriam.

When you dedicated your thesis to me I was just a little girl,

wearing a tiny blue dress with daisies.

Abstract

Several species of Bryopsidales (Chlorophyta) are known for displaying functional absence of the xanthophyll cycle, a common photoprotection mechanism responsible for qE component of NPQ. To cope with the extreme variability of their natural environment, these algae must be able to avoid photodamage. Previous works reported significant accumulation of *all-trans*-neoxanthin and violaxanthin under high light acclimation in different Bryopsidales, and speculated that these xanthophylls might control the amount of energy that reaches the photosystems, causing photoprotection. In this work, we investigated photoacclimation and photoprotection strategies in two species of Bryopsidales (*Codium tomentosum* and *Bryopsis plumosa*). We first characterised the acclimation state of algae exposed for 7 days to low light or high light (respectively 20 and 1000 μmol photons m^2 s^{-1}) in terms of pigment content (HPLC) and chlorophyll *a* variable fluorescence (PAM). We confirmed that high light triggers significant alteration of pigment content with accumulation of *trans*-Neoxanthin and Violaxanthin, and for the first time we characterised thoroughly how the pigment pool is altered during acclimation. We also confirmed that no evidence of any xanthophyll cycle is present in high light acclimated cultures. On a second note we tried to answer another major question: are *trans*-neoxanthin and violaxanthin photoprotective? Using a novel chlorophyll *a* variable fluorescence approach (pNPQ assessment) and performing quantification of PSII repair capacity (*via* lincomycin treatment) we were not able to give a clear answer to this question. Nonetheless, we concluded that despite *trans*-Neoxanthin and Violaxanthin might contribute to photoprotection, this process in Bryopsidales algae is likely given by the coordination between different mechanisms that deserve to be further investigated, including chloroplast movement, PSII repair/ modulation, state transitions, and PSI cyclic electron transport.

Key words

Bryopsidales, photoacclimation, photoprotection, *Codium tomentosum*, *Bryopsis plumosa*, stress physiology, photosynthetic pigments, xanthophylls, non-photochemical quenching (NPQ).

1. Introduction

The term “algae” does not always have a positive connotation in modern society, although these organisms are of key importance for our planet: algae are the main primary producers of aquatic ecosystems and are thought to be responsible of about half of the total oxygen production of the planet (*Chapman, 2013*).

The definition of algae is not straight-forward. They can be broadly described as a polyphyletic group of aquatic organisms that are mostly capable of doing oxygenic photosynthesis, but are not higher plants (*Raven & Giordano, 2014*). Others also define algae as thallophytes, plant-related organisms that have chlorophyll *a* as their primary photosynthetic pigment but lack roots, stems, leaves and a sterile covering of cells around the reproductive cells (*Lee, 2008*).

In this work I will present a study on photoacclimation and photoprotection strategies in two species of algae, *Codium tomentosum* and *Bryopsis plumosa*, belonging to the order Bryopsidales. These are high-interest species as are thought to lack a functional Xanthophyll Cycle, a widespread photoprotection mechanism, and display other peculiar physiological behaviours.

1.1 Bryopsidales

Bryopsidales (*Schaffner, 1922*) is a fascinating order of green algae, belonging to the Phylum Chlorophyta (*Reichenbach, 1834*). In general, the chlorophytes clade includes unicellular and multicellular algae that possess both chlorophyll *a* and *b* and form starch as storage product in the chloroplast (*Lee, 2008*). Up to 90% of the green algae is represented by freshwater species, while only about the 10% is marine (*Smith, 1955*). Some species can also be found in terrestrial environments, in symbiotic interactions with fungi, as lichens (*Raven & Giordano, 2014*).

The order Bryopsidales (also known as Caulerpales, Codiales or Siphonales) includes mostly macroscopic green algae, almost entirely marine (*Silva, 1982*; *Graham & Wilcox, 2000*), with the exception of the genus *Dichotomosiphon*, that occurs also in freshwater (*Škaloud et al. 2018*). Bryopsidales includes 589 species, divided in 12

families (**Fig. 1.1**) and is considered of high ecological relevance for coastal ecosystems, due to the presence of major primary producers and fast-growing invasive species (*Provan et al. 2005*; *Verbruggen et al. 2009*).

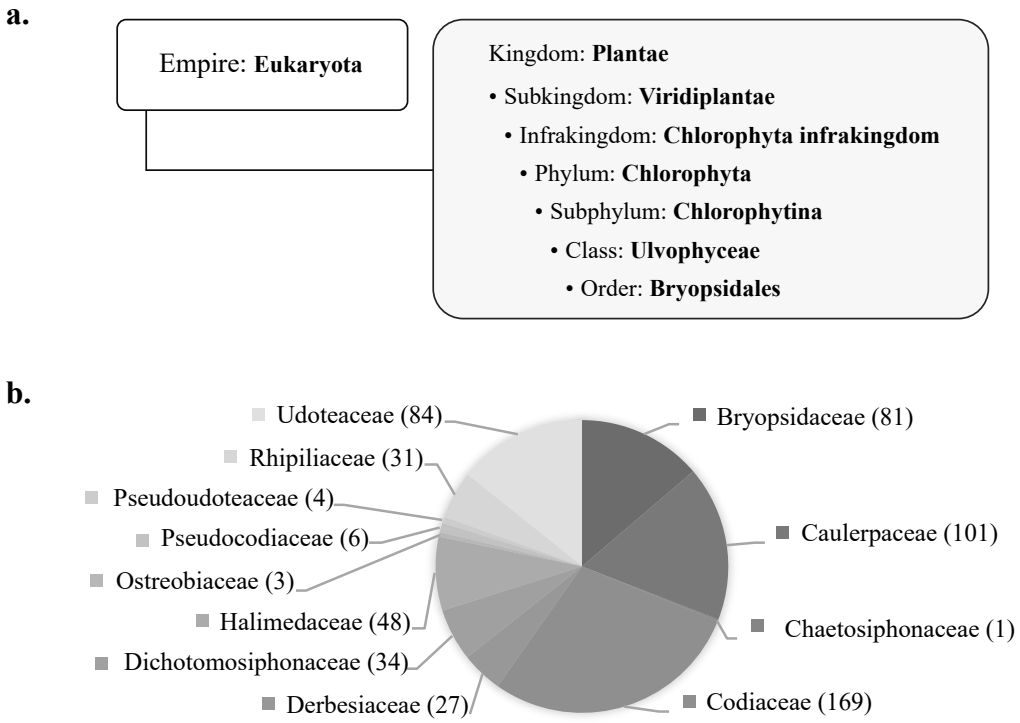


Fig. 1.1 Bryopsidales updated Taxonomy, as proposed by Algaebase (*Guiry & Guiry, 2020*). **a.** Higher taxonomy. **b.** Subordinate taxa (Families). Number of accepted species is indicated in brackets.

Bryopsidales is a widespread Order that inhabits tropical or subtropical waters, with normal salinity, and can be observed in association with biotic or abiotic substrates in nearshore environments, in both shallow and deeper waters. Like other related taxa, these algae display a peculiar cell morphology, called "siphonous" (from the Greek *siphon*, tube): the algae body is composed by a coenocytic multi-nucleate tallum either uniaxial (single branched siphon) or multiaxial (with multiple dichotomisations) (*Graham & Wilcox, 2000*). In other words, these algae consist of a unique, "giant" cellular space that contains several nuclei, chloroplasts and other organelles.

Despite this peculiar cellular organisation, Bryopsidales can reach surprising sizes (up to more than one meter), due to an extraordinary wound-healing ability that prevents loss of cytoplasm, by a rapid, actin-mediated contraction of the protoplast (*Graham &*

Wilcox, 2000). Moreover, these algae can display an incredible wide range of biological and morphological specialisations, including calcification (**Fig. 1.2**).

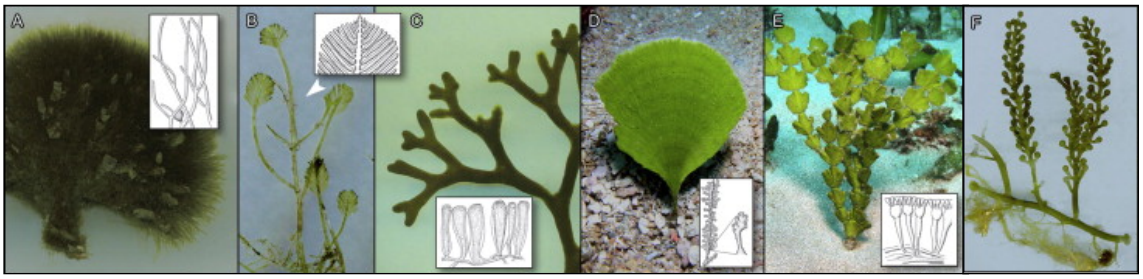


Fig. 1.2 Morphology and anatomy of siphonous green algae from the order Bryopsidales (adapted from *Verbruggen et al. 2009*). **A.** *Derbesia sp.* **B.** *Bryopsis sp.* **C.** *Codium sp.* **D.** *Udotea sp.* **E.** *Halimeda sp.* **F**. *Caulerpa sp.*

The siphonous morphology has several implications for the biology of these organisms: *Giovagnetti et al., 2018*, have reported that this peculiar body architecture, together with other physiological adaptations, ensures an efficient fitting to the extreme light changes of intertidal shores in *Bryopsis corticulans*. It is also known that some multiaxial siphonous algae can regularly change the position of their significantly abundant chloroplasts: plastids are normally kept around the surface, but they can be relocated into an internal colourless region (called "medulla") during the night, and repositioned into the surface at the beginning of the day (*Graham & Wilcox, 2000*). The possible implications of chloroplast movement for the present study will be discussed in **Chapter 6**.

Finally, Bryopsidales own a characteristic carotenoids pool, including siphonaxanthin and siphonaxanthin-dodecenoate, not normally present in other Chlorophyta (*Yokohama at al. 1977*; *Lee, 2008*), and at least some genera lack a functional Xanthophyll Cycle (*Franklin, 1996*; *Raniello et al. 2004*; *Christa et al. 2017*). The implications of such features will be discussed in **Section 1.3** of this Chapter.

Codium tomentosum

The *Codium* genus belongs to the family Codiaceae (see **Fig. 1.1**) and includes about 150 accepted species, following the most recent taxonomy data available on Algaebase (*Guiry & Guiry, 2020*).

Codium species commonly inhabit tropical and temperate coastal waters, reaching up to 70 m depth. Several are considered pests (e.g. of oyster beds) due to their ability of growing on hard substrates. The general morphology can vary between species. In the more common ramified forms, the multiaxial syphon separates in several branches that grow from a crustose portion at the base of the tallum, that secures the algae to the substrate (*Graham & Wilcox, 2000*; *Lee, 2018*). *Codium* species can exhibit many environmental adaptations, such as changes in number and size of the chloroplasts, pigment content or development of frond hairs (*Couteau & Coiffard, 2016*).

Codium tomentosum (*Stackhouse, 1797*), also known as "deadman fingers" or "sponge-weed", is considered the holotype of this genus (**Fig. 1.3**). It can be recognised for its dark-green, ramified, cylindric tallum, with spongy and elastic texture, often covered in epiphytes. It is normally found along Atlantic rocky shores where, in Autumn, can grow up to 50 cm of length (*Pereira & Correia, 2015*). This species is also found in the Mediterranean Sea and Indian Ocean (*WoRMS Editorial Board, 2020*), and can in general grow more than one meter (*van den Hoek, 1995*).

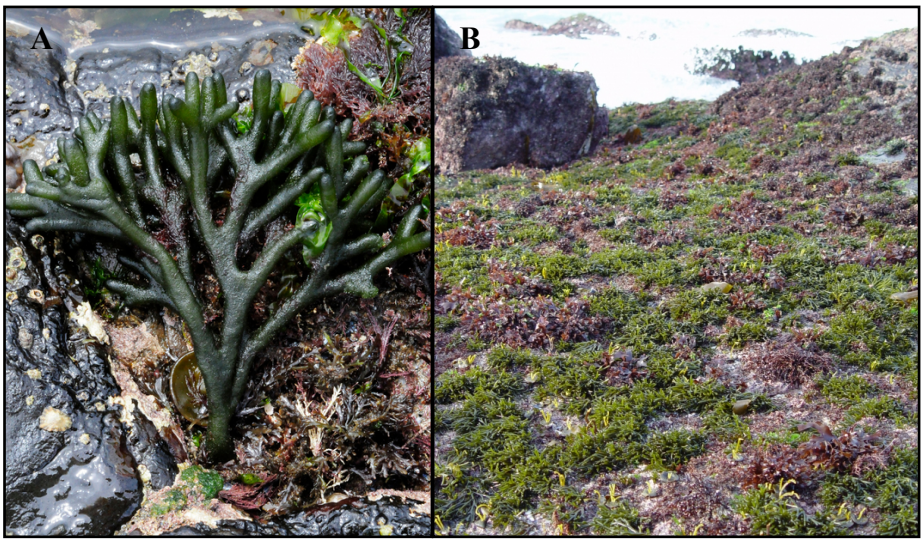


Fig. 1.3 *Codium tomentosum* (adapted from Algaebase, *Guiry & Guiry, 2020*). **A.** Single tallum documented at Spanish Point, Ireland, 2010 © Michael D. Guiry. **B.** Middle-intertidal community rich in *Codium tomentosum*, Redonda Island, Galicia, Spain, 2004 © Ignacio Bárbara.

The most well known characteristic of *Codium tomentosum* is the dichotomic growth of its tallum. As in other related species, the dichotomic branches have an internal colourless region (medulla) that generates inflated portions, called utricles, that form a

green, palisade-like layer around the external portion of the tallum. The utricles contain numerous discoidal chloroplasts in the periphery of the cytosol (*van den Hoek, 1995*; *Lee, 2018*). As mentioned above, in algae bearing this kind of morphology plastids can migrate from one region to the other under certain light conditions (*Graham & Wilcox, 2000*).

As other green seaweeds, *Codium* species are a known food source in several areas of the planet, particularly appreciated for their vitamin and other nutrients content (*Couteau & Coiffard, 2016*). Moreover, *Codium tomentosum* has recently been reported to be highly rich in polar lipids, including molecules known for having several nutritional and health benefits (e.g. PUFAs, Poly-Unsaturated Fatty Acids) (*da Costa et al. 2015*). This confirmed *C. tomentosum* as a species of high commercial value, whose possible applications for the production of feed, functional foods, nutraceuticals or new therapeutic agents have still to be explored.

Bryopsis plumosa

The *Bryopsis* genus belongs to the family Bryopsidaceae (see **Fig. 1.1**) and includes about 60 accepted species, following the most recent taxonomy data available on Algaebase (*Guiry & Guiry, 2020*).

Bryopsis species have been reported in both temperate and tropical seas, and are in general common in quiet waters, like tide pools or other sheltered locations (*Lee, 2008*). The genus includes all uniaxial species, meaning that they are composed of a single branched siphon. The base of the tallum grows horizontally over substrates and from it departs a system of erect main axes having many long, branched divergences (known as "laterals" or "pinnae"), that can grow up to about 10 cm (*van den Hoek, 1995*; *Graham & Wilcox, 2000*).

Bryopsis plumosa (Hudson, *Agardh, 1823*), commonly known as "green feather" (**Fig. 1.4**) is widely distributed worldwide: its presence is reported in the Mediterranean, North and Wadden Sea, Atlantic Ocean, Gulf of Mexico, Pacific and Indian Ocean (*WoRMS Editorial Board, 2020*). In this species, from the main axes of the tallum depart

two opposite rows of pinnae that resemble tiny feathers (*van den Hoek, 1995*), hence the name *plumosa*, from the latin word for "feather".

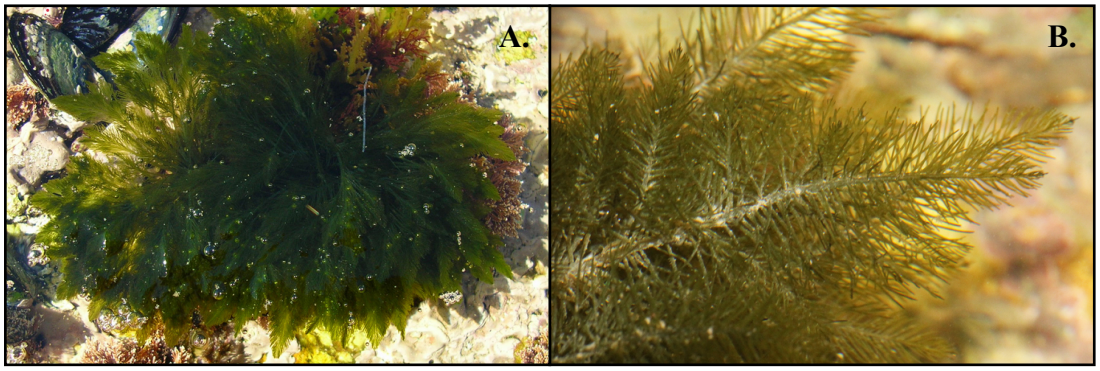


Fig. 1.4 *Bryopsis plumosa* (adapted from Algaebase, *Guiry & Guiry, 2020*). **A.** *Bryopsis plumosa* from an intertidal rocky pool, A Coruña, Galicia, Spain, 2005 © Ignacio Bárbara. **B.** Pinnae detail, from mid-tidal pool, Mount Edgcumbe Country Park, Cornwall, England, UK, 2011 © David Fenwick.

In *Bryopsis* species the protoplast forms a thin peripheral layer that contains nuclei towards the outside and discoidal chloroplasts towards the inside (*van den Hoek, 1995*). Due to the siphonous morphology is not to be excluded that, even in this species where a proper medulla is not present, chloroplast can migrate between different portions of the siphon in response to specific light stimuli.

Extensive literature described the reproduction and life history of this species, unlike other algae of the same order (*van den Hoek, 1995*; *Graham & Wilcox, 2000*). This, together with the fact that this algae is widespread and relatively easy to grow *in vitro*, makes *Bryopsis plumosa* an interesting potential model for investigating the physiology of Bryopsidales.

1.2 Photoacclimation, photoinhibition and photoprotection

Photosynthetic organisms are able to harvest Sun energy for oxygenic photosynthesis thanks to the transfer of light energy from the antenna complexes to the reaction centre complexes, which are able to create chemical energy in form of redox power (as summarised in **Fig 1.5**). In general, the reaction centres are located in the two photosystems: Photosystem I (PSI), that absorbs the far-red region, and Photosystem II (PSII), that absorbs in the red region (*Taiz, Zeiger et al. 2014*).

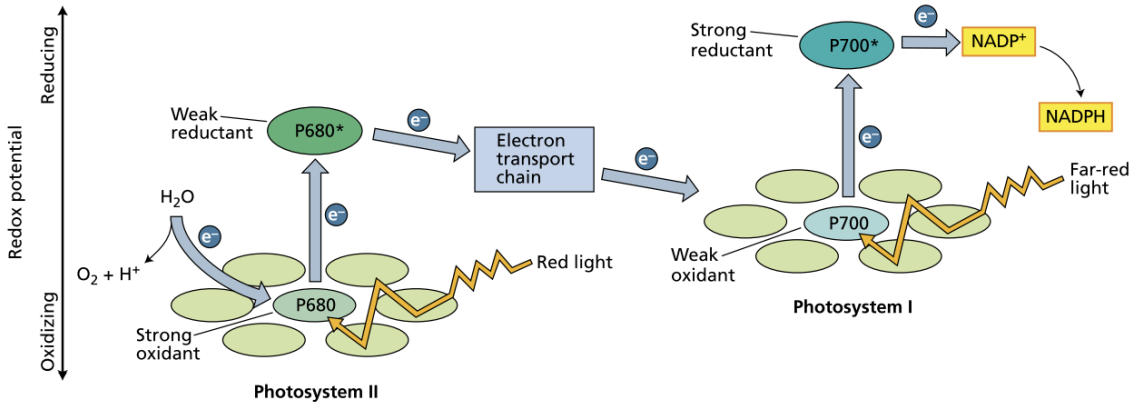


Fig. 1.5 Z scheme of photosynthesis (*Taiz, Zeiger et al. 2014*). P680 and P700 refer to the wavelengths of maximum absorption of the reaction center chlorophylls in PSII and PSI, respectively.

The antenna systems are composed by proteins associated with specific pigments, that are responsible of capturing and conveying the light energy to the reaction centres. While the reaction centres structures are well conserved through evolution, the antenna complexes can vary remarkably between taxa, due to adjustments in protein and pigment composition, including both chlorophylls and carotenoids (*Larkum et al. 2003; Taiz & Zeiger et al. 2014*). Antenna complexes make light harvesting efficient, physiologically competent and flexible at the same time, within a wide range of light environments on our planet. Moreover, the plasticity of antenna systems plays a central role in controlling the amount of energy delivered to the reaction centres, in response to fluctuations of the natural light environment (*Ruban, 2009*).

Photoacclimation or photoadaptation?

At this point, it's necessary to make some clarification. Following what proposed by *Falkowski & La Roche, 1991*, we define "photoadaptation" a set of genetic changes that allows a species to become fitted to its light environment. This process is therefore irreversible, and is the result of evolutionary forces acting upon several generations. In contrast, we define "photoacclimation" those reversible, phenotypic adjustments that take place in response to changing light conditions. Photoacclimation is omnipresent trough the evolution of photosynthetic organisms, and even if direct evidence of its effectiveness is hard to obtain, it's common opinion that this ability must be essential for survival (*Wilhelm et al. 2014*).

Indeed, the photosynthetic machinery is more plastic than we are accustomed to think. Plants and algae are known for being able of varying the composition of their photosynthetic apparatus in response to the variability of natural environments, mostly in terms of incident light. In general, when acclimating to high light the organisms are able to increase the maximum photosynthetic rate and decreases the total pigment content; the opposite happens in low light. These processes involve rearrangements in the relative levels of enzymes and membrane proteins, whose adaptive advantages remain still in part unclear (*Walters, 2005*).

The problem of photoinhibition

Despite light is vital for any photosynthetic organism, an excess can cause serious damage to the photosynthetic machinery. When not all the excitation energy is utilised, due to the fact that electron transport is much slower than energy transfer, the reaction centres become saturated. This excess of energy can damage the reaction centres, primarily of PSII, leading to a decline of photosynthetic efficiency, growth and productivity, called "photoinhibition". Coping with light-induced damage is therefore crucial for maintaining the organism well being and survival. (*Barber, 1995*; *Takahashi & Badger*; *2011*, *Ruban et al. 2012*).

In response to photodamage, photosynthetic organisms have developed quick and efficient mechanisms of repair of damaged PSII reaction centres (*Aro et al. 2005*). We can therefore say that net photoinhibition occurs when the rate of damage exceeds the rate of the repair. However, an excess of light does not only directly damage the PSII complexes (primary photodamage), but can also inhibit the PSII turnover (*Takahashi & Badger, 2011*). Thus, the active PSII repair is essential for the organism survival but is not enough for completely avoid photoinhibition.

For this reason, plants and algae have evolved a wide variety of strategies to alleviate net photoinhibition. This set of mechanisms, called "photoprotection", is associated with avoiding light absorption and successfully consuming or dissipating the light energy absorbed by light harvesting pigments. The main known photoprotective strategies include: light avoidance (due to the organism and/or chloroplasts movement), screening of photoradiation (e.g. UV), scavenging of Reactive Oxygen Species (ROS),

Non Photochemical Quenching (NPQ), PSI Cyclic Electron Flow (CEF) and the photorespiratory pathway (*Takahashi & Badger, 2011*; *Goss & Lepetit, 2015*).

Of these mechanisms, only Non Photochemical Quenching (NPQ), significant for the present study, will be further discussed.

D1 repair cycle and its importance for investigating photoprotection

As mentioned above, an excess of light can both cause primary photodamage to the PSII and inhibit its repair (*Takahashi & Badger, 2011*). Such inhibition is associated with the action of Reactive Oxygen Species (ROS), generated in the light harvesting complexes, able to inhibit protein *de novo* synthesis and/or reduce the efficiency of protein repair cycles under stress. The design of PSII gives protection to most of its protein components and the photodamage is addressed almost only to the D1 protein, belonging to the core of the photosystem (*Aro et al. 2005*; *Nishiyama & Murata, 2014*).

The repair cycle of PSII is a complicated process, that partly takes place contextually with its assembly. Notably, only the D1 protein (and occasionally D2, CP43 and PsbH subunits) are replaced, while other components are recycled (*Järvi et al. 2015*). Since the chloroplast originated from endosymbiosis of ancient cyanobacteria, all the mechanisms necessary for its biogenesis and homeostasis are governed by the protoplast itself, including PSII subunits assembly and maintenance (*Kato & Sakamoto, 2009*). The reaction centre turnover (summarised in **Fig. 1.6**.) is rapid, specific and light-dependent and allows the organism to maintain high functionality of PSII over a wide range of environmental conditions (*Zhang & Aro, 2002*).

D1 turnover cycle can be blocked with specific inhibitors (e.g. Lincomycin, Di-Thio-Threitol (DTT) and Chloramphenicol). The use of these substances is well known in plant biology (*Olaizola et al. 1994*; *Bachmann et al. 2004*; *Takahashi & Badger 2011*) because they allow to study the photodamage of PSII: if D1 turnover is inhibited, it's possible to quantify the extent of damage that hits the photosystem (in terms of percentage protein drop), therefore obtaining indirect information on the effectiveness of the global photoprotective mechanisms in action (*Pokorska et al. 2009*).

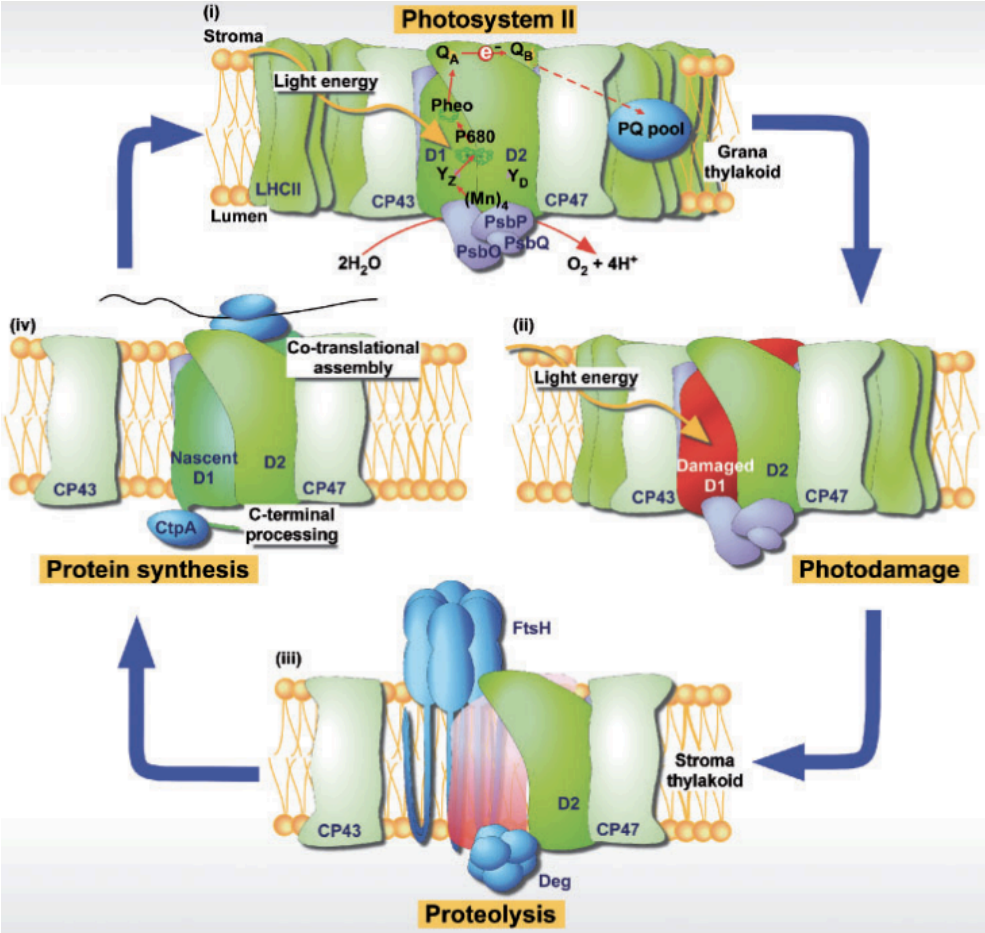


Fig. 1.6 Schematic drawing of the PSII repair cycle (*Kato & Sakamoto, 2009*). (i) Functional PSII complex. (ii) Light-induced D1 damage. (iii) D1 proteolysis, by FtsH and Deg proteases. (iv) Synthesis of D1 nascent chain and co-translational insertion in the thylakoid membrane.

Non Photochemical Quenching and the Xanthophyll cycle

As mentioned above, Non Photochemical Quenching (NPQ) is one of the main photoprotective strategies in photosynthetic organisms, and allows a safe dissipation of the excess excitation energy as heat. Therefore, this energy does not reach PSII and is not used for photochemistry (*Derks et al. 2015*). The photoprotective role of this mechanism it's still debated. On one side, NPQ is known as a rapid process, perfectly suited for coping with sudden light fluctuations and avoiding photodamage (*Goss, & Lepetit, 2015*). However, some experimental evidence has shown that little or no role seems to be played by NPQ in protecting the D1 protein from light induced degradation in Spinach (*Santabarbara et al. 2001*). Still, due to its widespread presence and to the huge amount of related molecular data, NPQ is still considered one of the main

photoprotective mechanisms in photosynthetic organisms (*Goss, & Lepetit, 2015*; *Ruban, 2016*).

The term "Non Photochemical Quenching" derives from the fact that the thermal dissipation becomes visible as a quenching of the measurable Chl *a* fluorescence (*Krause & Jahns, 2004*). NPQ (also referred to as qN) consists of three principal components that contribute to the overall energy dissipation: high-energy-state quenching (qE), photoinhibitory quenching (qI) and state transitions quenching (qT). qE is known as the fastest and most important NPQ mechanism (*Goss, & Lepetit, 2015*; *Ruban, 2016*).

As reviewed by *Ruban et al. 2012*, qE is triggered by the proton gradient formed as a consequence of the light reactions that happen on the thylakoid membrane. This generates a structural reorganisation of the antenna proteins, from light harvesting to light energy dissipative mode, and allows the thermal dissipation of energy thanks to quencher molecules. Several proteins and pigments have been proposed to serve as quenchers, but the major role is thought to be played by xanthophylls.

Briefly, xanthophylls are able to regulate the excitation state of chlorophylls thanks to a cycle of related reactions, called xanthophyll cycle. In the classic cycle described for higher plants and several algae taxa (**Fig. 1.7**), violaxanthin is converted into zeaxanthin, that acts as a trap for the excess excitation energy accumulated on the chlorophyll *a* pigments within the reaction centres. After the stress, zeaxanthin is converted again into violaxanthin, thus re-establishing the cycle (*Frank et al. 1994*).

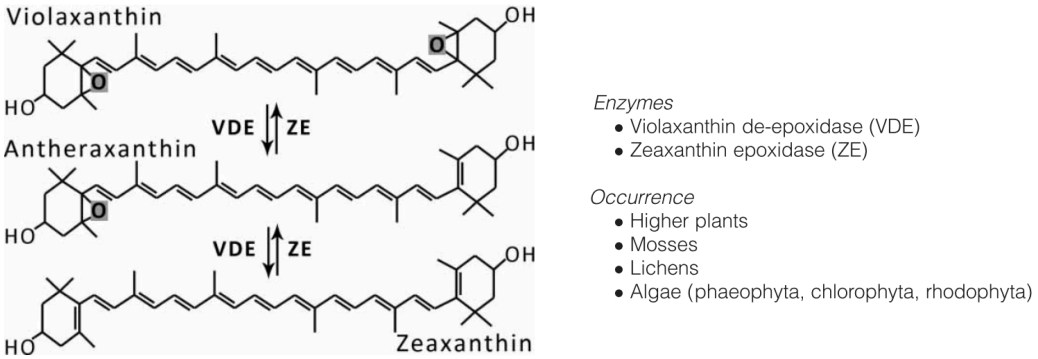


Fig. 1.6 Violaxanthin-Antheraxanthin-Zeaxanthin (VAZ) cycle (*Latowski et al. 2011*).

Six different xanthophyll cycles have been described in taxa of photosynthetic organisms. All of them protect the photosynthetic apparatus from photodamage caused by light-induced oxidative stress, either directly quenching the overexcitation energy (qE) or participating indirectly in the process of photoprotection (*Latowski et al. 2011*). Therefore, in plants and algae xanthophylls are essential for protecting the photosynthetic machinery from the damage caused by an excess of light.

1.3 Bryopsidales: peculiar pigment composition and physiological implications

Siphonaxanthins and the light-harvesting

As mentioned above, Bryopsidales own two characteristic carotenoids, siphonaxanthin and siphonaxanthin-dodecenoate (also known as siphonein), not normally present in other Chlorophyta (*Yokohama at al. 1977*; *Lee, 2008*). Since these pigments are found in ancient species, it has been speculated that they might belong to ancient photosynthetic complexes evolved in deep water green algae (*Anderson, 1983*). siphonaxanthin is supposed to be an ancestor of lutein, a carotenoid often present in the antennae systems of green algae and higher plants (*Yoshii, 2006*; *Wang et al 2013*).

In both algae and higher plants, the light harvesting complexes of the two photosystems are associated mainly with chlorophyll *a*, allowing the organisms to harvest the light in the far red (PSI, maximum absorption of 700 nm) and red (PSII, maximum absorption of 680 nm) regions (*Taiz, Zeiger et al. 2014*). In addition to this very well conserved core structure, Bryopsidales light harvesting proteins are able to bind siphonaxanthin, siphonaxanthin-dodecenoate and relatively high levels of chlorophyll *b*. This particular Siphonaxanthin-Chlorophyll *a*/*b*-Protein complexes (SCP) are thought to be responsible of enhancing the absorption in the green region (518 and 538 nm), therefore allowing the algae to harvest those light wavelengths that penetrate deeper in the water column, where these organisms live. Similar strategies are adopted also by other green algae and higher plants, but in these cases the carotenoid associated with the antennae is normally lutein (*Anderson, 1983*; *Wang et al 2013*). At the end, energy is always transferred to chlorophyll *a* (*Kageyama et al. 1977*; *Nakayama et al. 1994*).

A recent study by *Qin et al. 2015*, also showed that in a *Bryopsis* species the PSI antenna is associated with a pool of 7 accessory carotenoid molecules, and has less red Chlorophylls compared to higher plants, resulting in a high carotenoid/chlorophyll ratio. Moreover, the presence of unique α- and ε-carotene complexes suggested a structural flexibility of the PSI core. The authors concluded that these features might help regulating the energy transfer network, allowing the organisms to cope with the high variability of the light conditions in their natural intertidal habitat.

Lack of a functional xanthophyll cycle

The carotenoid metabolism in Bryopsidales algae is indeed peculiar. In addition to the above mentioned features, some studies have suggested the lack of a functional xanthophyll cycle in this monophyletic branch of algae.

In particular, *Franklin et al. 1996*, showed that in *Chlorodesmis fastigiata* (Bryopsidales, Udoteaceae) NPQ was limited and during exposure to High Light no conversion to zeaxanthin or significant accumulation of antheraxanthin was detected. Moreover, *Raniello et al. 2004* suggested that light intensity was not sufficient to induce the complete activation of the xanthophyll cycle in patches of *Caulerpa racemosa* (Bryopsidales, Caulerpaceae), even if the samples exposed to higher light had a significant accumulation of antheraxanthin. Also *Cruz et al. 2015* observed lack of a functional xanthophyll cycle and qE component of NPQ in *Codium tomentosum* (Bryopsidales, Codiaceae).

Starting from previous observations, *Christa et al. 2017* were able to demonstrate that although Bryopsidales accumulate zeaxanthin under high-light conditions, NPQ formation is independent of a xanthophyll Cycle and photoprotection is not related to qE. The experiment was performed on several Bryopsidales species, showing that this feature seems to be widespread in the order, and only in *Caulerpa taxifolia* the presence of a xanthophyll cycle (even if not contributing to NPQ) was observed under high light.

Interestingly, *Raniello et al. 2006* suggested the existence of a peculiar xanthophyll-based energy quenching in *Caulerpa racemosa*, given by the interconversion between lutein and siphonaxanthin, and between violaxanthin and antheraxanthin. However, it

has to be noted that *Caulerpa sp.* was found able to perform the xanthophyll cycle under high light, despite an absence of measurable qE (*Christa et al. 2017*). Therefore, the physiological behaviour of this algae might be completely different from the one exhibited by other Bryopsidales species that do not display the xanthophyll cycle.

Finally, in a recent work on *Bryopsis corticulans* (Bryopsidales, Bryopsidaceae), *Giovagnetti et al. 2018* observed that even if sustained NPQ, triggered by the formation of trans-thylakoid proton gradient, was formed in filaments more exposed to light, the process was independent of a xanthophyll Cycle. Moreover, important light harvesting proteins (PsbS and LHCSR) did not seem to be active in NPQ mechanisms activated by this alga. The authors concluded that the sustained protective NPQ displayed by *B. corticulans* is not related to photodamage, and is probably due to either the occurrence of transient PSII photoinactivation or a fast rate of PSII repair cycle.

The putative photoprotective role of trans-neoxanthin and violaxanthin

Neoxanthin is a common carotenoid, present in photosynthetic organism in two isomeric forms: *9′-cis*- and *all-trans*-neoxanthin. Not all photosynthetic organisms have this pigment, and its appearance seems to be associated with that of chlorophyll *b* (*Takaichi & Mimuro, 1998*). *9′-cis*-neoxanthin is considered a major component of photosynthetic xanthophylls in both green algae and higher plants (*Roy et al. 2011*) and has a specific binding site in the light harvesting complexes, therefore is present on the membrane in association with the proteins, but is never the most abundant carotenoid (*Anderson, 1983*; *Wang et al. 2013, Qin et al. 2015*).

An important insight on the function of these pigments in Bryopsidales algae was given by a work from *Uragami et al. 2014* that reported an accumulation of *all-trans*-neoxanthin, in addition to the normal *9′-cis*-, in *Codium intricatum* cultured under High Light. Together with this pigment, also a significant accumulation of violaxanthin and α-carotene was observed. The authors suggested that, since the interactions between these pigments and the proteins are weak, *trans*-neoxanthin and violaxanthin might exist in the surface of the Siphonaxanthin-Chlorophyll *a*/*b*-Protein complexes (SCP) to promote their oligomerization. According to the authors, this process might be able to control the amount of energy transferred from the SCP to PSII by adjusting the distance

between the energy donor and the energy accepter in order to quench the excess amount of excitation energy.

The accumulation of these pigments in Bryopsidales algae exposed to high light was later confirmed in a study from *Cartaxana et al. 2018*, that reported a significant accumulation of *trans*-neoxanthin and violaxanthin in high light acclimated *Codium tomentosum*.

1.4 Gaps and needs

In general, the available information on Bryopsidales algae is not enough for outlining a comprehensive overview on the physiology of this Order.

First of all, despite the xanthophyll cycle is currently thought to be functionally absent in many genera of this order (*Franklin, 1996*; *Raniello et al. 2004*; *Christa et al. 2017*), some data suggested the existence of other functional xanthophyll-based quenching strategies in some species (*Raniello et al. 2006*). Moreover, it has to be noted that the available studies are still not sufficient to completely exclude the existence of a specific trigger for xanthophyll quenching reactions in Bryopsidales species. Therefore, further experimental studies are needed for throwing more light on this topic, confirming the widespread absence of a functional xanthophyll cycle in this order and understanding which carotenoids are actively involved in photoprotection in these algae and how they work.

Also, a question still open concerns the putative photoprotective role of the *trans*-neoxanthin. This pigment, which accumulates together with violaxanthin and α-carotene under excessive light conditions, might function as control of the excitation energy that reaches the photosynthetic machinery; however, this theory is still speculative (*Uragami et al. 2014; Cartaxana et al. 2018*). Thus, comprehensive studies that test this hypothesis are essential to move forward in this field.

Finally, we lack complete information on the physiological role of this characteristic pigment pool in the thylakoid environment of Bryopsidales algae. The photoprotective carotenoids might just work as antioxidant, dissolved in the lipid matrix, and/or work as

quenchers only when coupled with specific membrane proteins. Both these behaviours are known for xanthophyll-cycle pigments in diatoms (*Lepetit et al. 2010*). Studies on the isolation and identification of pigment-antenna protein complexes in Bryopsidales are available in literature (*Anderson, 1983*, *1985*; *Chu & Anderson, 1985*; *Wang et al. 2013*; *Qin et al. 2015*) but the functioning of these structures *in vivo* needs to be further investigated.

In the end, we still do not know which photoprotective mechanisms are activated in these algae. We cannot exclude that due to the particular environment where they live, Bryopsidales might have partially (or completely) lost the ability of quenching excess of excitation energy using xanthophylls, and might activate other photoprotective strategies, like transiently inactivating PSII or enhancing its repair (*Giovagnetti et al. 2018*), activating proteins responsible for the ROS scavenging or simply avoiding an excess of light through chloroplast movement and reciprocal shading. Therefore, all the previous hypotheses still need to be tested.

1.5 The projects CtLight and HULK and their goals

The present work was developed in collaboration with the University of Aveiro and CESAM (Center for Environmental and Marine Studies), Portugal, in the framework of the projects CtLight (PTDC/BIAFBT/30979/2017) and HULK (PCIG11-GA-2012-322349, POCI-01-0145-FEDER-016754), funded by FCT and FEDER, in the framework of Portugal 2020 and COMPETE 2020.

CtLight - Effects of light on the photobiology and growth of the commercially valuable macroalgae Codium tomentosum

The project CtLight focuses on the culture optimisation of the green macroalga *Codium tomentosum*, produced by the company ALGA+® in a sustainable Integrated Multi-Trophic Aquaculture system and sold in the food, nutraceutical and cosmeceutical industry.

As mentioned above, *Codium tomentosum* is an algae of high commercial interest, due to its possible use as food and its known high nutritional value (*da Costa et al. 2015*,

Couteau & Coiffard, 2016). The main aim of the CtLight project is to understand the effect of light intensity and quality on the physiology of this algae, in terms of growth, photosynthetic performance and metabolism, to determine the best conditions for implementing the production in open culturing tanks.

Due to the fact that the target species belongs to a monophyletic group of algae that lack a functional Xanthophyll Cycle (*Cruz et al. 2015*; *Christa et al. 2017*), the study of its physiology in terms of photoprotection and photoacclimation ability is essential for achieving the goal of the project.

HULK - Functional Chloroplasts inside Animal cells: cracking the puzzle

The project HULK focuses on the ability of some Sacoglossa sea slugs of sequestering chloroplasts from the algae they feed on, and maintaining them photosynthetically active inside specialised digestive gland cells for very long periods. The physiological role of these "stolen" plastids ("kleptoplasts") in this peculiar slug-algae interaction is still debated (*Christa et al. 2013*).

The aim of the HULK project is to understand the role of kleptoplasts in the host metabolism and to determine what are the mechanisms responsible for their survival inside the animal cell. For answering this question, one section of the project focuses on the analysis of the photoprotection mechanisms in the algae that act as food source.

Sacoglossa sea slugs of the genus *Elysia* are known to feed on algae of the order Bryopsidales, including several *Codium* and *Bryopsis* species (*Lee, 2008*, *Cruz et al. 2013*, *Baumgartner & Toth 2014*, *Rauch et al. 2018*, *Middlebrooks et al. 2019*). Therefore, the study of the physiological and biochemical processes that drive photoprotection in this particular group of algae is of primary importance for understanding how and why the plastids are maintained by the animal, and whether these processes can contribute to kleptoplast longevity.

2. Aim of the present study and goals

This work aims to fill some of the gaps that concern our knowledge on the photoacclimation and photoprotection strategies in Bryopsidales algae (see **Section 1.4**). The main objective is to further investigate the role of xanthophyll pigments in Bryopsidales metabolism, with two distinctive approaches.

The first part of the present study focuses on understanding the pattern of accumulation of xanthophyll pigments in Bryopsidales algae acclimated to high light, trying to answer two main questions: which is the trigger for accumulation of *trans*-neoxanthin and violaxanthin, and how is their accumulation carried on over prolonged high light exposition? Also, do these algae eventually display alternative xanthophyll cycle-like mechanisms that might be responsible for photoprotection? This first set of analyses will consolidate the knowledge on the existing high light acclimation patterns in these algae and analyse all the potential target pigments that might contribute to photoprotection.

Another goal of this work is to set the basis for answering another major question: are *trans*-neoxanthin and violaxanthin really photoprotective? This hypothesis, based on theories previously postulated (*Uragami et al. 2014*), is in principle really hard to prove with a single set of experiments. Therefore, the main aim concerning this topic is to start investigating the existence of this putative photoprotection mechanisms in Bryopsidales algae, analysing the global photoprotective capacity and the extent of damage to the PSII machinery in individuals acclimated to High Light and containing higher levels of *trans*-neoxanthin and violaxanthin. We believe that a first experimental work focused on this hypothesis might solve a part of the pending question and give interesting insights to be elaborated in future studies.

Finally, the putative role of other mechanisms possibly responsible for photoprotection in Bryopsidales (transient inactivation of PSII or repair enhancement, activation of ROS scavenging, light avoidance through chloroplast movement and/or reciprocal shading) will be discussed.

3. Preliminary experiments

Basing on information available in literature, including previous work from our research group (*Uragami et al. 2014*, *Cartaxana et al. 2018*), I first tried to establish the best experimental conditions for achieving high light acclimation state in *Codium tomentosum*, characterised by significant accumulation of pigments of interest (*trans*-neoxanthin and violaxanthin) and fluorescence analysis. The methodology and results of such preliminary experiments are briefly discussed in this chapter.

3.1 Experiment 1: Two weeks exposure to moderate High Light

Materials and Methods

Sample collection and maintenance

Codium tomentosum samples were collected in March from the rocky shores of Praia de Aguda, Portugal (Lat 41.046622°; Lon −8.653272°) during morning low tide, causing algae to be completely or partially exposed to air.

In laboratory, grazers and macro-epiphytes were removed manually and the samples were randomly transferred into 3 lab-acclimation plastic tanks, with 2L volume (**Fig. 3.1**). For 4 days the algae were pre-cultured in growth chamber under a long day light period (16:8 h) with Low Light conditions (LLac, 40 µmol photons m^{-2} s^{-1}).

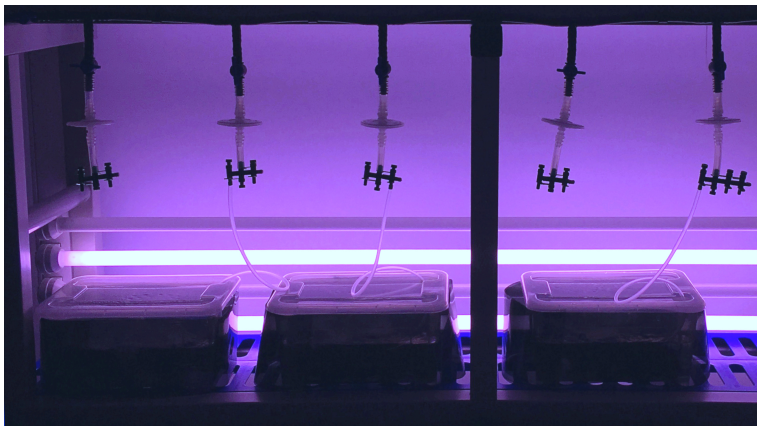


Fig. 3.1 Pre-culture setup. Samples were split into rectangular plastic boxes and the desired light intensity was obtained adding two layers of neutral filters.

Cultures were maintained in a thermostatically controlled growth chamber at 17°C, in Artificial Sea Water, 33‰ salinity (ASW 33‰), prepared with osmose water and RED SEA SALT (Red Sea), supplemented with f/2 medium components (*Guillard & Ryther 1962*; *Guillard, 1975*; *Andersen,* 2005; see **Supplementary material: Appendix A**).

Bubbling aeration was provided with aquarium plastic tubes and aquarium air stones. Light was supplemented with FLUORA T8 lamps (L58 W/77, OSRAM) and different light intensities were obtained with neutral filters (209 0.3ND, LEE Filters). Light intensity was measured using Universal Light Meter (ULM-500, Heinz Walz GmbH) with Submersible Spherical Micro Quantum Sensor (US-SQS/L, Heinz Walz GmbH) in the middle of the culture tank filled with ASW 33‰.

Experimental setup and design

From the initial batch, unhealthy individuals were discarded and 2 batches of 5 samples each were randomly selected, within a weight range of 20 ± 5 g. Crude cultures for future experiments were established from remaining healthy samples.

Codium tomentosum was exposed for two weeks to different irradiance: 200 µmol photons m^{-2} s^{-1} (moderate-High Light acclimated, HLac) and 40 µmol photons m^{-2} s^{-1} (Low Light acclimated, LLac) with a long day light period (16:8 h). Cultures were maintained in growth chamber with the conditions described above (f/2 in ASW 33‰, 17°C, FLUORA T8 lamps), in cylindrical plastic tanks with a volume of 1.2 L (**Fig. 3.2**). After 7 days medium was replaced and supplemented with 0.25 mg/L GeO_2.

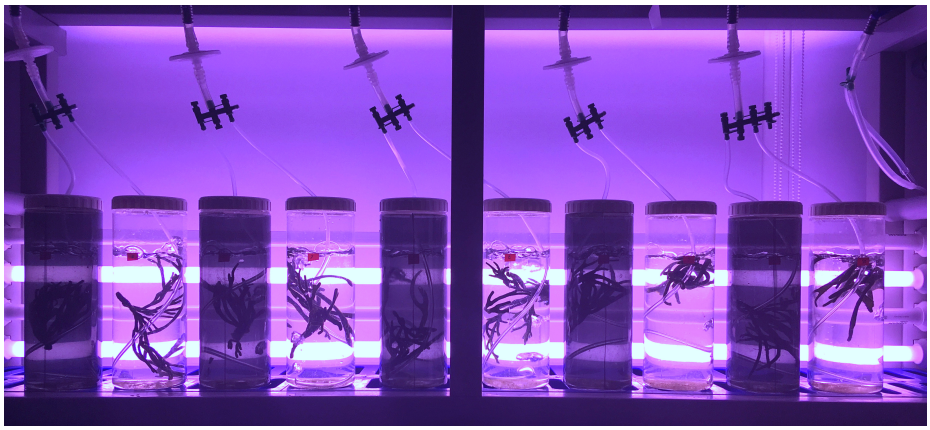


Fig. 3.2 Experiment 1 setup. Cultures were maintained into cylindrical plastic tanks, without filters (HLac) or with two layers of neutral filter (LLac).

Cultures were sampled at three time points: at the beginning of the experiment and after each week. First, biological samples were taken for HPLC analysis; approx. 1.5 cm of frond tip was cut, washed with ASW, frozen with liquid nitrogen and stored at −80°C. Samples were later freeze-dried in a lyophiliser at −50°C and stored at −20°C. Second, chlorophyll *a* fluorescence data was collected *in vivo* using a Pulse Amplitude Modulated fluorometer (PAM).

Pigment analysis

High-Performance Liquid Chromatography (HPLC) was performed as described by *Mendes et al. 2007*, following the C18 method (see **Chapter 4: Section 4.3** for complete method description), using a Shimadzu HPLC (Shimadzu, Japan) equipped with photodiode array detector (SPD-M10ADVP).

Fluorescence analysis

Fluorescence measurements were carried out using a Junior PAM Fluorometer (Heinz Walz GmbH). Due to the "thick" morphology of *Codium tomentosum*, the optic fibre was kept adjacent to the algae surface. Steady State Light Curves (SSLC) were constructed with 6 incremental steps of actinic irradiance (PAR: 0, 25, 65, 90, 190, 420 µmol photons m^{-2} s^{-1}), each lasting 10 min. For each step, the effective quantum yield of photosystem II (YII) was monitored after 8 min and rETR was calculated. The light response was characterised by fitting light curves to the model by *Platt et al. 1980*; α (initial slope of the light curve), rETRmax (maximum rETR) and Ek (light saturation coefficient) were estimated. The curves were fitted iteratively using MS Excel Solver. For parameters definitions and formulas see **Supplementary material: Appendix B**.

Statistical analysis

Differences in pigment content and SSLC parameters (α, rETRmax and Ek) between HLac and LLac cultures were tested using repeated measures partly nested analysis of variance (ANOVA, **Fig. 3.3**). Data exploration was performed using box-plots, interaction-plots and Pearson's residuals analysis to verify normality and homogeneity of variance, absence of block-by-within-block interactions and identify evident outliers (*Logan, 2010*). Sphericity assumption was verified using Mauchly's Test. Pairwise post-

hoc comparisons were performed using Welch's two-samples t-test (for individual comparisons) and pairwise t-test with Bonferroni correction (for multiple comparisons). Statistical analyses were carried out using R (*R Foundation for Statistical Computing, Vienna, Austria*).

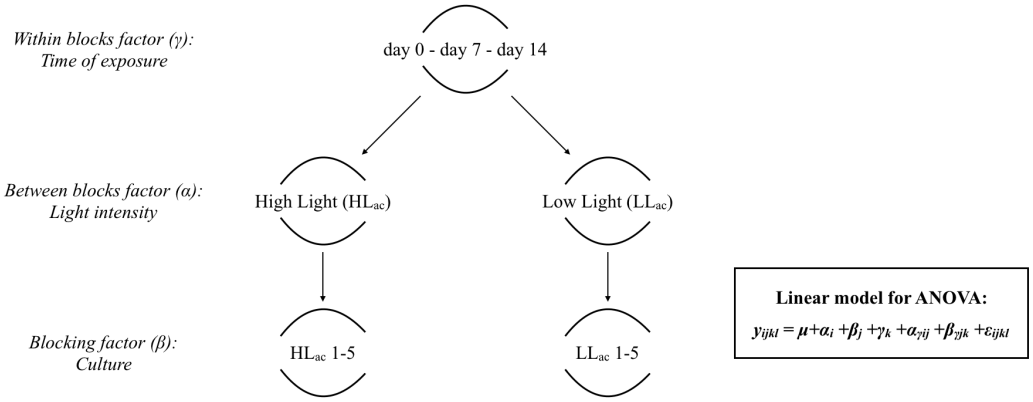


Fig. 3.3 Proposed experimental design and relative linear model for ANOVA analysis. Effect of light acclimation was tested using a repeated measure design, with one factor Between blocks (Light intensity, fixed with 2 levels), one factor Within blocks (Time of exposure, fixed with 3 levels) and one Blocking factor (Culture, random with 5 levels).

Results and discussion

Almost no significant variation in pigment content between HLac and LLac was observed (**Fig. 3.4**). Detected pigments with abbreviations and related parameters are reported in **Tab. 3.1**.

Pigment	*Abbreviation*	*Retention time (min)*	λ_{max} *(nm)*
Siphonaxanthin	Siph	12.02 (± 0.05)	448
all-trans-Neoxanthin	t-Neo	13.16 (± 0.05)	417; 441; 471
9'-cis-Neoxanthin	c-Neo	13.57 (± 0.05)	413; 437; 466
Violaxanthin	Viola	14.85 (± 0.04)	416; 441; 471
Siphonaxanthin dodecenoate	Siph-do	20.28 (± 0.03)	455
Chlorophyll *b*	Chl *b*	23.45 (± 0.03)	458; 597; 645
Chlorophyll *a*	Chl a	25.00 (± 0.03)	430; 617; 662
ε-Carotene	ε-Car	29.00 (± 0.07)	414; 441; 471
α-Carotene	α-Car	29.16 (± 0.04)	447; 476

Tab. 3.1 Pigment content of *Codium tomentosum* detected with HPLC. Pigment names, mean retention time (with standard deviation) and representative absorption maxima (λ_{max}) are reported.

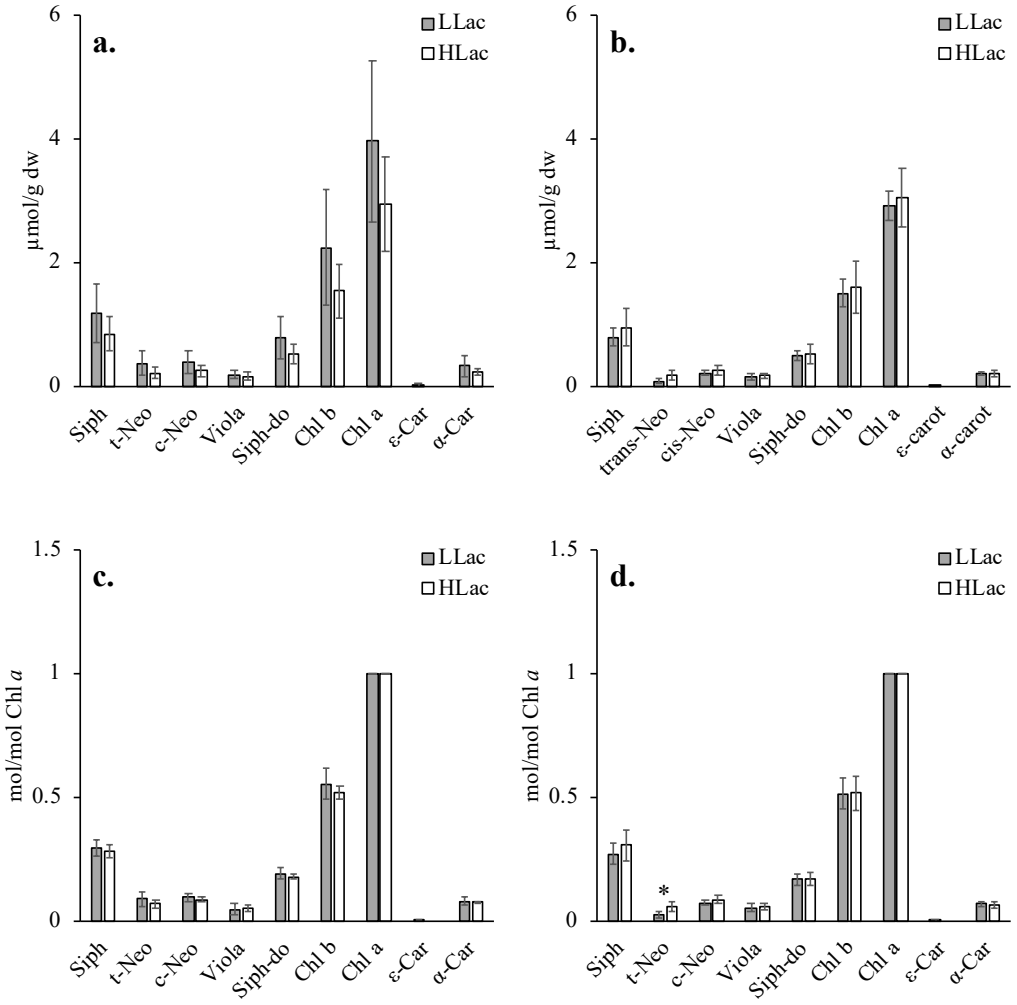


Fig. 3.4 Pigment concentrations of LLac (40 µmol photons m^{-2} s^{-1}) HLac (200 µmol photons m^{-2} s^{-1}) *Codium tomentosum*. Average points with standard deviation are reported (n=5). Results are expressed both as pigment concentration per dried weight (**a.** at day 0; **b.** after two weeks of acclimation) and as pigment:Chl *a* ratio (**c.** at day 0; **d.** after two weeks of acclimation). Asterisks indicate significant differences between LLac and HLac (t-tests): * $p < 0.05$.

ANOVA analysis was performed on pigment:Chl *a* ratios. In all cases, no relevant abnormalities were found during preliminary data exploration. When sphericity assumption was not met (Viola, $p = 0.01$; ε-Car, $p = 0.05$) Greenhouse-Geisser ε correction was applied. No significant effects ($p > 0.05$) correlated to a difference between LLac and HLac conditions were detected, with the exception of t-Neo (**Fig. 3.4-5**), for which significant effect of factor Time ($p = 0.0005$) and of the interaction Time × Light Intensity (0.01) were observed.

It has to be noted that for cis-Neo:Chl *a*, Siph-do:Chl *a* and α-Car:Chl *a* ratios a significant effect of factor Time was observed ($p = 0.023$; $p = 0.047$; $p = 0.015$ respectively), but with independent t-test no significant difference was observed between HLac and LLac conditions at any time point. Pairwise t-test with Bonferroni correction revealed a significant difference between day 0 and day 7 values for both cis-Neo:Chl *a* and Siph-do:Chl *a* ratio ($p = 0.015$ and $p = 0.027$ respectively), while α-Car:Chl *a* ratio day 0 resulted significantly different from both day 7 ($p = 0.025$) and day 14 ($p = 0.036$). Therefore, the significant effect of Time on these pigment:Chl *a* ratios was attributed to a variation completely independent from treatment.

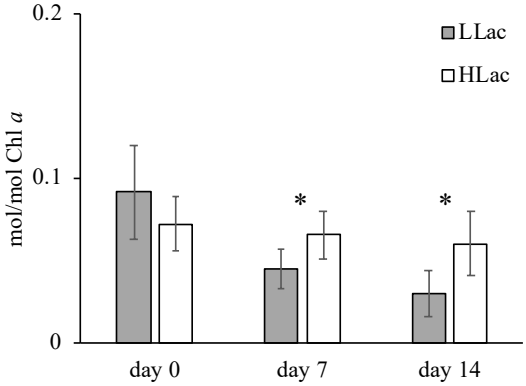


Fig. 3.5 t-Neo concentration in LLac (40 µmol photons m^{-2} s^{-1}) and HLac (200 µmol photons m^{-2} s^{-1}) acclimated *Codium tomentosum*. Results are expressed as t-Neo:Chl *a* ratio at day 0, after one week and two weeks of treatment. Average points with standard deviation are reported (n=5). Asterisks indicate significant differences in pigment concentrations between LLac and HLac (t-tests): * $p < 0.05$.

Overall, the significant difference reported for t-Neo concentration has to be attributed to a decrement under LLac, rather than a production in HLac conditions: the average content of t-Neo in LLac cultures drops from around 0.9 at day 0 to 0.3 at day 14, with a significant t-test result ($p = 0.017$), while t-Neo content under HLac conditions remained quite constant. This result might be explained by lack of sufficient time for establishing a stable laboratory culture. Indeed, it has to be noted that samples were maintained in growth chamber for only few days prior experimentation (**Fig. 3.1**). Therefore, they might still have been influenced by pre-existing acclimation conditions of their natural environment. This would also explain the relatively high standard deviation observed for pigment concentration per dried weight, particularly at day 0 and

the significant effect of factor Time, independent from treatment, observed for cis-Neo:Chl *a*, Siph-do:Chl *a* and α-Car:Chl *a* ratios (**Fig. 3.4.**). Nonetheless, we can not exclude that other physiological factors might have contributed to the above reported results. In any case, it is evident that the imposed experimental conditions were not sufficient to induce a clear High Light photoacclimation state in samples exposed to 200 µmol photons m^{-2} s^{-1}. This observation was confirmed by fluorescence data, according to which no evident difference in the photoacclimation state was observed between HLac and LLac cultures (**Fig. 3.6**).

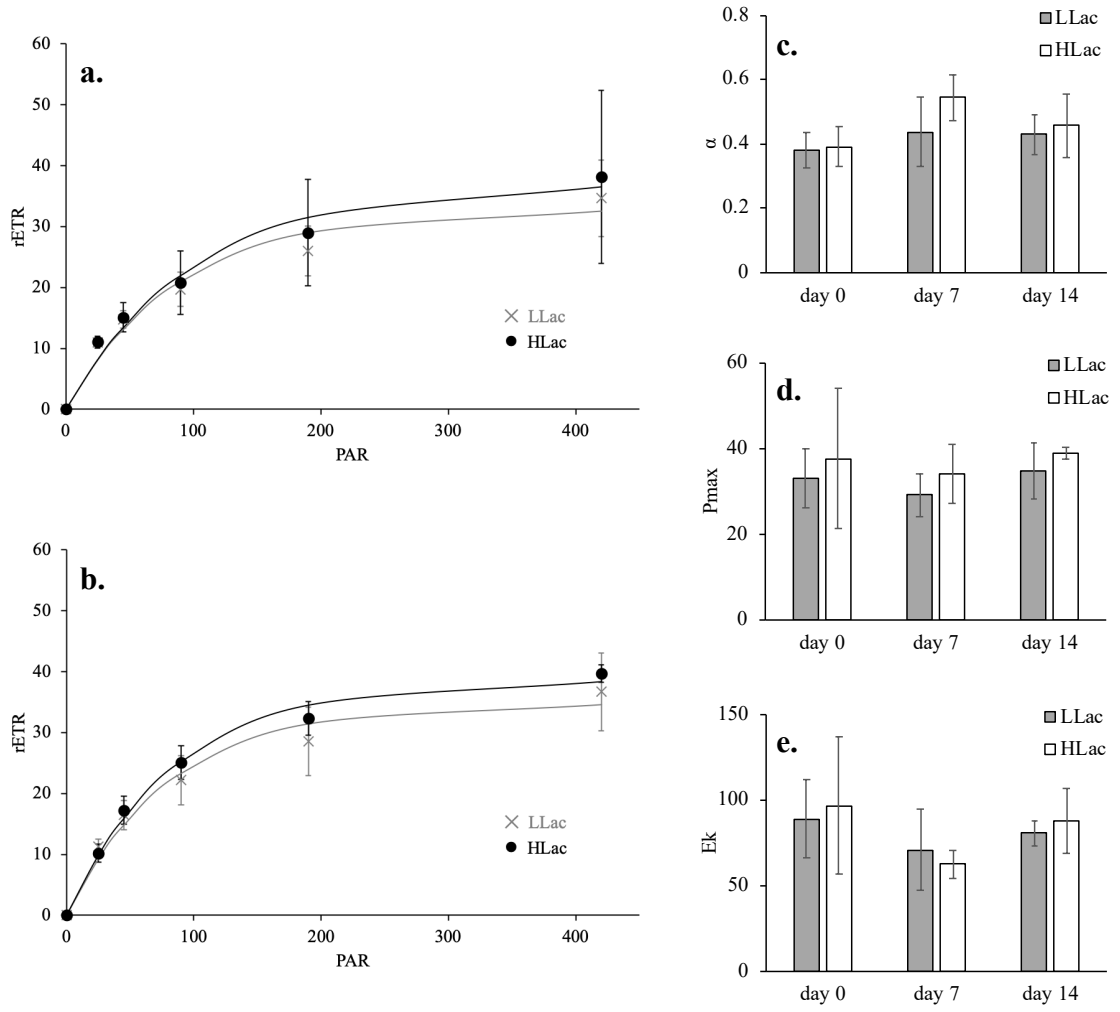


Fig. 3.6 Steady State Light Curves (SSLC) and related parameters from LLac (40 µmol photons m^{-2} s^{-1}) and HLac (200 µmol photons m^{-2} s^{-1}) *Codium tomentosum*. SSLC were fit using the model by *Platt et al. 1980*; residuals of fit were always good (r > 0.96). **a-b.** Fitted SSLC (rETR versus PAR) at time 0 (**a.**) and after two weeks of treatment (**b.**); average points with standard deviation are reported (n=5). SSLC after one week is not shown. **c-e.** Parameters α, rETRmax and Ek at three time points (day 0, day 7 and day 14) with standard deviation.

First of all, the SSLC after two weeks for LLac or HLac were almost identical, showing no evidence of High Light acclimation; the same result was obtained from ANOVA analysis performed on α, rETRmax and Ek. No relevant abnormalities emerged during preliminary data exploration; α and Ek respected the sphericity assumption ($p > 0.05$), while for rETRmax ($p = 0.023$) Greenhouse-Geisser ε correction was applied. No significant differences ($p > 0.05$) were detected for rETRmax and Ek, whilst a significant effect of Time ($p = 0.009$) was observed for α. This result is similar to the one obtained for cis-Neo:Chl *a* and Siph-do:Chl *a* ratios: no significant difference was observed between HLac and LLac conditions at any time point and pairwise t-test with Bonferroni correction revealed a significant difference between day 0 and day 7 ($p = 0.023$). Also in this case, the observed significant effect of Time was therefore attributed to a variation completely independent from treatment.

The absence of difference between LLac and HLac cultures was at first surprising, considering that a photoacclimation response, with highly significant accumulation of *trans*-neoxanthin and violaxanthin, was observed in *Codium tomentosum* cultures exposed to the same High Light intensity comparable to the present experiment (*Cartaxana et al. 2018*). Without excluding the possibility that the time of acclimation to artificial growth conditions preceding the experiment was not sufficient, this discrepancy might be explained by a difference of light quality provided. Actually, we know that Bryopsidales algae display peculiar antenna complexes, that enhance light harvesting in the blue-green region (*Anderson, 1983*; *Wang et al 2013*). Therefore, the given High Light intensity (200 µmol photons m^{-2} s^{-1}) provided with lamps with a predominantly red spectrum, might not have been sufficient for stimulating a HL photoacclimation. This hypothesis cannot be verified with the present data and will need to be further explored.

In conclusion, the two irradiance levels tested (low light: 40 µmol photons m^{-2} s^{-1}; high light: 200 µmol photons m^{-2} s^{-1}) did not induce a clear difference in the photoacclimation state of *Codium tomentosum*, considering both pigment content and fluorescence data.

3.2 Experiment 2: Ten hours exposure to extremely High Light

Materials and Methods

Culture maintenance

Codium tomentosum crude cultures were established from samples previously collected from Praia de Aguda, Portugal (as mentioned above) and maintained for 1.5 month in thermostatically controlled chamber at 17°C. Long day light period (16:8 h) was imposed, with an intensity of 130 µmol photons m^{-2} s^{-1}, supplemented with FLUORA T8 lamps (L58 W/77, OSRAM). Light intensity was measured using Universal Light Meter (ULM-500, Heinz Walz GmbH) with Submersible Spherical Micro Quantum Sensor (US-SQS/L, Heinz Walz GmbH) in the middle of the culture flask. Bubbling aeration was provided with sterilised glass tubes.

Cultures were kept clean via regular, manual removal of macro-epiphytes and medium was replaced approximatively every two weeks. Medium f/2 (*Guillard & Ryther 1962*; *Guillard, 1975*; *Andersen,* 2005; see **Supplementary material: Appendix A**) was prepared from Artificial Sea Water 30‰ salinity (ASW 30‰), obtained with distilled water and RED SEA SALT (Red Sea), and supplemented with 1 mg/L GeO_2.

Experimental setup and design

Algae from crude cultures were accurately cleaned from macro-epiphytes (with manual removal); fragments of 3 ± 1 g were cut and washed 2 times in a 50 ml tube with ASW 30‰, agitating vigorously for removing loosely adherent micro-epiphytes.

Eighteen *Codium tomentosum* fragments were randomly selected and cultured under Low Light condition (LLac, 20 µmol photons m^{-2} s^{-1}) for 3 days with long day light period (16:8 h). Subsequently, they were randomly split in 2 batches (of 9 cultures each) and exposed for 10h to different treatments: 1000 µmol photons m^{-2} s^{-1} (High Light acclimated, HLac) and 20 µmol photons m^{-2} s^{-1} (LLac), with a same light period.

A custom setup was built for this experiment (**Fig. 3.7**); two cooling baths (one for each light condition) were kept in a thermostated room (17°C). Temperature was constantly monitored with aquarium thermometers; in case of temperature rise cool water was

added. Bubbling aeration was provided with plastic tubes and aquarium air stones. Culture maintenance medium was used (f/2, ASW 30‰ + 1 mg/L GeO_2) and replaced after the preliminary 3 days of Low Light acclimation. Lamps were mounted above the cooling baths, using metallic supports. For LLac condition, light was provided with four REEF-SPEC blue-white T5 lamps, 24 W (Red Sea) set on top of the bath (approx. 50 cm far), while for HLac four REEF-SPEC blue-white T5 lamps, 54 W (Red Sea) were mounded immediately above the bath. Semi-conic mirrors were used for adjust light to the desired light intensity, measured using Universal Light Meter (ULM-500, Heinz Walz GmbH) with Submersible Spherical Micro Quantum Sensor (US-SQS/L, Heinz Walz GmbH) in the middle of the culture flasks, taking care that all the samples would receive the same irradiance.

Fig. 3.7 Experiment 2 setup. Cultures were maintained in 250 mL conic flasks, sealed with Parafilm. **a.** Low Light acclimation (LLac, 20 µmol photons m^{-2} s^{-1}). **b.** High Light acclimation (HLac, 1000 µmol photons m^{-2} s^{-1}).

Cultures were sampled at two time points: at the beginning of the experiment and after 10 h of exposure. Only biological samples for HPLC analysis were taken; approx. 0.5 cm of frond tip was cut and washed 2 times in a 50 ml tube (first with ASW 30‰, then with distilled water), agitating vigorously. Subsequently, samples were dried with paper towel and frozen with liquid nitrogen inside a 2 mL tube, and stored at −80°C. Samples were later freeze-dried in a lyophiliser at −50°C and stored at −20°C.

Pigment analysis

High-Performance Liquid Chromatography (HPLC) was performed as described by *Mendes et al. 2007*, following the C18 method (see **Chapter 4: Section 4.3**), with a Shimadzu equipment (LC-2030C 3D Plus, Shimadzu, Japan).

Statistical analysis

Significant differences in pigment content between HLac and LLac cultures were tested using using independent samples t-tests. Statistical analysis was carried out using R (*R Foundation for Statistical Computing, Vienna, Austria*; package: "stats").

Results and discussion

The two irradiance levels under which *Codium tomentosum* was maintained induced differences in pigment content (**Fig. 3.8**): in HLac cultures a significant decrement of c-Neo, ε-Car and α-Car content was observed, while Viola, Ant and Zea increased. This result is particularly interesting, considering that violaxanthin, antheraxanthin and zeaxanthin are known for being responsible of the conventional xanthophyll cycle described for higher plants and several algae taxa, also known as VAZ-cycle (*Frank et al. 1994*; *Latowski et al. 2011*). Detected pigments with abbreviation and related parameters are reported in **Tab. 3.2**.

Pigment	*Abbreviation*	*Retention time (min)*	λ_{max} *(nm)*
Siphonaxanthin	Siph	10.32 (± 0.04)	448
all-trans-Neoxanthin	t-Neo	11.27 (± 0.10)	417; 441; 471
9'-cis-Neoxanthin	c-Neo	11.95 (± 0.09)	413; 437; 466
Violaxanthin	Viola	12.86 (± 0.11)	416; 441; 471
Antheraxanthin	Anth	14.77 (± 0.18)	447; 474
Lutein	Lut	16.33 (± 0.10)	425; 447; 476
Zeaxanthin	Zea	16.45 (± 0.14)	451; 480
Siphonaxanthin dodecenoate	Siph-do	18.41 (± 0.12)	455
Chlorophyll *b*	Chl *b*	22.70 (± 0.13)	458; 597; 645
Chlorophyll *a*	Chl a	24.29 (± 0.12)	430; 617; 662
ε-Carotene	ε-Car	28.20 (± 0.12)	414; 441; 471
α-Carotene	α-Car	28.39 (± 0.13)	447; 476
β-Carotene	β-Car	28.60 (± 0.13)	450; 476

Tab. 3.2 Pigment content of *Codium tomentosum* detected with HPLC. Pigments names, mean retention time (with standard deviation) and mean absorption maxima (λ_{max}) are reported; standard deviation for λ_{max} was always low (between 0 and 3).

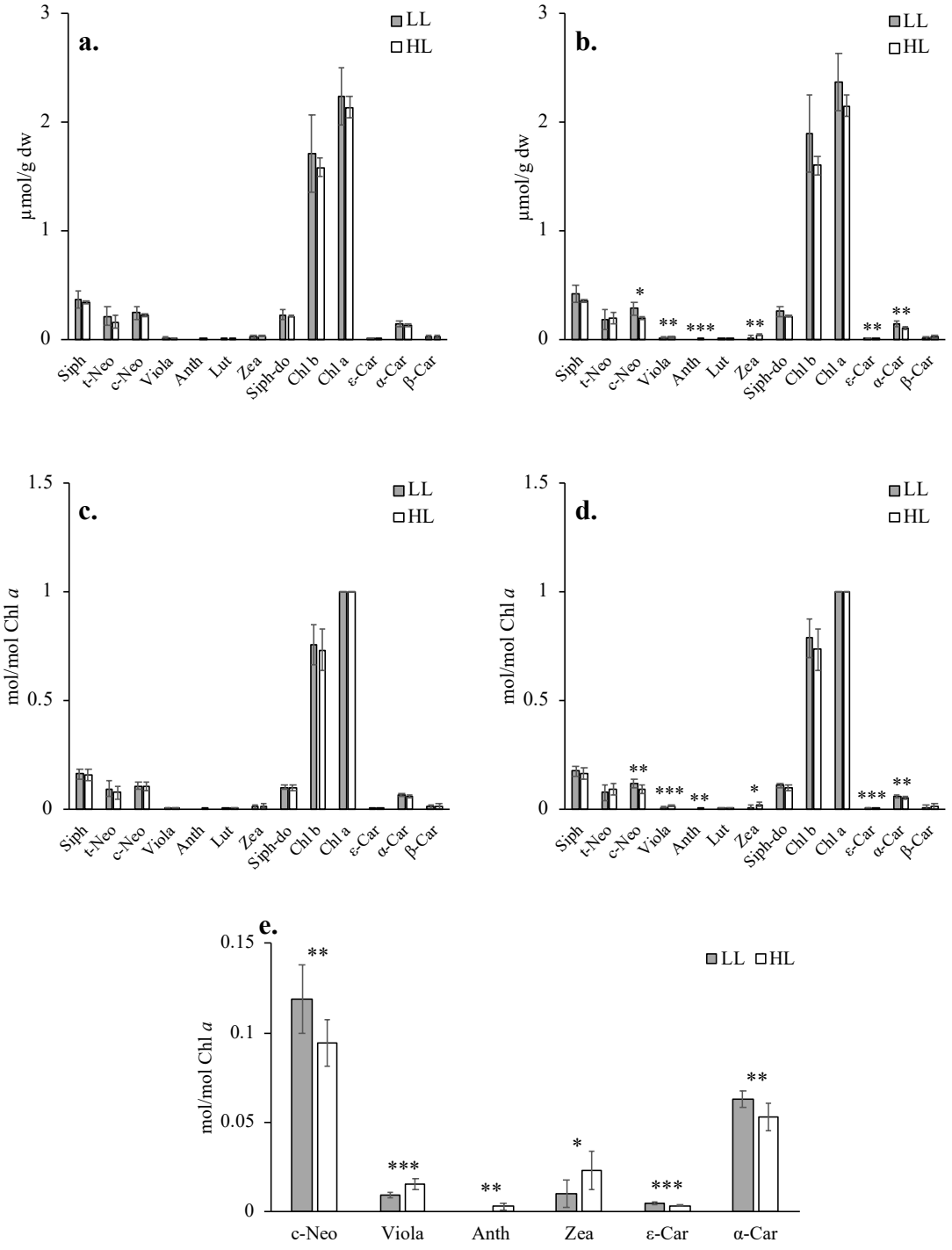


Fig. 3.8 Pigment concentrations of LLac (20 µmol photons m^{-2} s^{-1}) and HLac (1000 µmol photons m^{-2} s^{-1}) acclimated *Codium tomentosum*, with standard deviation (n=9). Results are expressed both as pigment concentration per dried weight (**a.** at time 0; **b.** after 10 h of acclimation) and as pigment:Chl *a* ratio (**c.** at time 0; **d.** after 10 h of acclimation). **e.** Close look to pigment:Chl *a* ratios with statistically significant differences between LLac and HLac after 10 h of exposure. Asterisks indicate significant differences in pigment concentrations between LLac and HLac (t-tests): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

It has to be noted that xanthophyll cycle pigments were reported to be present in other Bryopsidales algae, and in one case the presence of VAZ-cycle was also reported, despite the lack of ability of generating qE (*Christa et al. 2017*). Therefore, the accumulation of VAZ pigments in *Codium tomentosum* was not surprising, but took us to question the eventual presence of a vestigial xanthophyll cycle in this species.

The absence of significant accumulation of t-Neo and the significant decrement of α-Car in HLac cultures contradicts what observed by *Uragami et al. 2014*, that reported significant accumulation of both t-Neo and α-Car under High Light acclimation (500 µmol photons m^{-2} s^{-1}) in *Codium intricatum*. However, *Cartaxana et al. 2018* also reported a decrement of α-Car content in HLac *Codium tomentosum* (200 µmol photons m^{-2} s^{-1}), together with a significant accumulation of t-Neo. Therefore, alterations in α-carotene concentration seem to depend on additional not predicted factors (possibly determined also by specie-specific responses), while *trans*-neoxanthin accumulation seems to be more strictly correlated to high light. The lack of significant t-Neo accumulation in our experiment might be explained by the short time of exposure imposed (10h). If this hypothesis were true, it would mean that *trans*-neoxanthin accumulation is correlated with a slower High Light acclimation response, under long period of exposure.

In conclusion, despite the presence of some significant differences in pigment content between HLac and LLac cultures, a longer time of exposure to intense light might be necessary for achieving a decisive High Light acclimation state, with significant accumulation of both *trans*-neoxanthin and violaxanthin, in *Codium tomentosum*.

3.3 Conclusive remarks

While in the first experiment, two weeks exposure to high irradiance (200 µmol photons m^{-2} s^{-1}) was not sufficient to induce a clear High Light photoacclimation state in *Codium tomentosum*, the secondly imposed HLac condition (1000 µmol photons m^{-2} s^{-1}) triggered significant xanthophyll production, but the time of exposure (10h) was probably too short for achieving a significant accumulation of *trans*-neoxanthin. It has to be noted that direct comparisons between the results of the two experiences cannot be

applied, since completely different experimental conditions were imposed (in terms of setup, light intensity and spectrum, sample dimension and culture maintenance). Therefore, comprehensive conclusions must be taken with caution. Nonetheless, the development of these preliminary experiences allowed us to obtain useful information for conducing further physiological experiments on *Codium tomentosum*.

On a practical note, a lab-acclimation of at least 2 weeks, with regular medium changes and manual macro-epiphyte removals, is in my opinion necessary when harvesting *Codium tomentosum* samples from the wild, to obtain stable crude cultures (acclimated to laboratory conditions and with negligible contamination). The supplement of GeO_2 has also to be considered a good practice for preventing diatom overgrow (*Andersen, 2005*). Due to its morphology and relatively slow growth, the isolation of a pure culture of this species is hard to achieve and maintain, therefore the use of a clean and healthy crude culture is in my opinion the easiest and most practical way of obtaining *C. tomentosum* samples for this kind of physiological experiments.

As discussed above, the first experiment suggested the possibility that light quality (in terms of available light spectrum) might influence photoacclimation in Bryopsidales algae. Hence, future experiments on this topic will be needed for investigating this hypothesis.

Moreover, the second experiment opened some questions regarding the possibility that extreme high light might be able to trigger a vestigial xanthophyll cycle in these algae. This possibility will be further taken into account in the present study.

Finally, these preliminary experiments led to the conclusion that prolonged exposure to sensibly High Light might be necessary for achieving an evident High Light-acclimation state in *Codium tomentosum*, with significant accumulation of *trans*-neoxanthin and violaxanthin. For this reason, the setup developed for the second experiment (**Fig 3.7**) was maintained and a forthcoming experience, that tested prolonged exposure to extremely High Light in both *Codium tomentosum* and *Bryopsis plumosa*, was designed.

4. Materials and Methods

4.1 Sample collection and Culture maintenance

Codium tomentosum

Codium tomentosum was collected during Summer from the rocky shores of Praia de Aguda, Portugal (Lat 41.046622°; Lon −8.653272°) during morning low tide, causing algae to be completely or partially exposed to air.

In laboratory, grazers and macro-epiphytes were removed manually and crude cultures were established. Crude cultures were maintained for around 1 month prior experimentation in thermostatically controlled growth chamber (see below) and were kept clean via regular, manual removal of macro-epiphytes.

Bryopsis plumosa

Bryopsis plumosa was acquired in 2018 from the Kobe University Macro-Algal Culture Collection (KU-MACC, Japan). Strain details are summarised in **Tab. 4.1**.

Strain number		KU-0990
Taxonomy	*Scientific name*	*Bryopsis plumosa*
	Higher taxonomy	Chlorophyta; Ulvophyceae; Bryopsidales; Bryopsidaceae
	Author	(Hudson) C. Agardh
Collection information	*Locality*	Koshien-hama, Nishinomiya, Hyogo, Japan
	Collector name	S. Uwai
	Date of collection	2002.11.19
Culture conditions	*Medium*	PES/10L-15L
	Temperature	10°C
	Light period	16:8 (light:dark)
Genetic information	*Gene*	rbcL

Tab. 4.1 *Bryopsis plumosa*, strain KU-0990 details (KU-MACC, Japan).

Maintenance in growth chamber

Cultures of both species were maintained in a thermostatically controlled growth chamber at 17° C. Long day light period (16:8 h) was imposed, with an intensity of 130 µmol photons m^{-2} s^{-1}, supplemented with FLUORA T8 lamps (L58 W/77, OSRAM). Light intensity was measured using Universal Light Meter (ULM-500, Heinz Walz GmbH) with Submersible Spherical Micro Quantum Sensor (US-SQS/L, Heinz Walz GmbH) in the middle of the culture flask. Bubbling aeration was provided with sterile glass tubes.

Medium f/2 (*Guillard & Ryther 1962*; *Guillard, 1975*; *Andersen,* 2005; see **Supplementary material: Appendix A**) was prepared from Artificial Sea Water, 30‰ salinity (ASW 30‰), obtained with distilled water and RED SEA SALT (Red Sea), and supplemented with 1 mg/L GeO_2. Medium was replaced around every two weeks.

4.2 Experimental setup

For one week, algae fragments from stock culture (12 ± 3 g for *Codium tomentosum*; 20 ± 3 g for *Bryopsis plumosa*) were pre-cultured under Low Light condition (LLac, 20 µmol $_{photons}$ m^{-2} s^{-1}, **Fig. 4.1**) with long day light period (16:8 h). Subsequently, they were randomly split into two batches (of 5 samples each) and exposed for 7 days to different light irradiance: 1000 µmol photons m^{-2} s^{-1} (High Light acclimated, HLac) and 20 µmol photons m^{-2} s^{-1} (LLac), with same photoperiod (16:8).

A custom setup, built for preliminary experiments was used (**Fig. 4.1**). Two cooling baths (one for each light condition) were kept in a thermostated room (17°C); to ensure constant temperature even through prolonged High Light exposure, a chiller with water pump was added to the HLac bath. Temperature was constantly monitored with aquarium thermometers. Bubbling aeration was provided through air pumps with plastic tubes and aquarium air stones. Culture maintenance medium was used (f/2, ASW 30‰ + 1 mg/L GeO_2) and replaced after the first week (pre-culture). Lamps were mounted above the cooling baths, using metallic supports. For LLac condition, light was provided with four REEF-SPEC blue-white T5 lamps, 24 W (Red Sea) set on top of the

bath (approx. 50 cm far), while for HLac four REEF-SPEC blue-white T5 lamps, 54 W (Red Sea) were mounded immediately above the bath. Semi-conic mirrors were used for adjust light to the desired light intensity, measured using Universal Light Meter (ULM-500, Heinz Walz GmbH) with Submersible Spherical Micro Quantum Sensor (US-SQS/L, Heinz Walz GmbH) in the middle of the culture flasks, taking car that all the samples would receive the same irradiance.

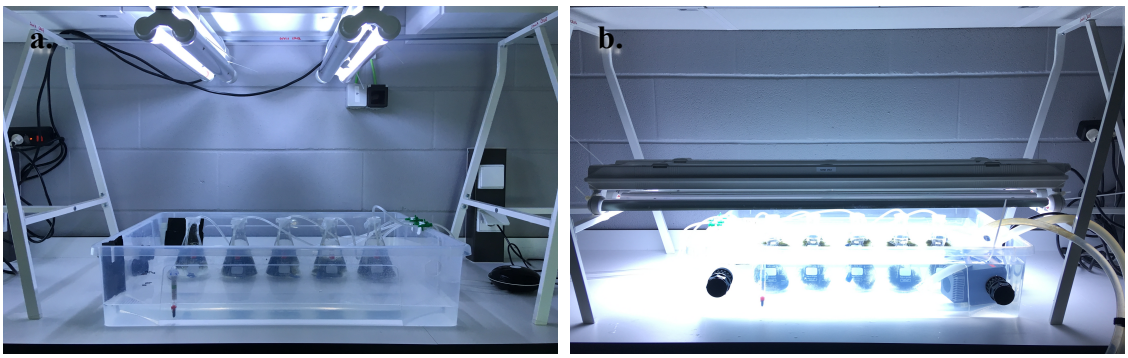


Fig. 4.1 Experimental setup. Cultures were maintained in conic flasks, sealed with Parafilm. **a.** Low Light acclimation (LLac, 20 µmol photons m^{-2} s^{-1}). **b.** High Light acclimation (HLac, 1000 µmol photons m^{-2} s^{-1}). Note that for *Codium tomentosum* 250 mL conic flasks were used, while for *Bryopsis plumosa* a higher volume was chosen (500 mL), due to different species-specific requirements.

For pigment analysis (HPLC), cultures were sampled at 6 time points: at the beginning of the experiment (day 0), each day for the first three days and then on alternate days, until the end of the week (day 7). Around 0.5 cm of frond tip was cut and washed 2 times in a 50 ml tube (first with ASW 30‰, then with distilled water), agitating vigorously[1]. Subsequently, samples were dried with paper towel and flash-frozen with liquid nitrogen inside a 2 mL tube, and stored at −80°C. Samples were later freeze-dried in a lyophiliser at −50°C and stored at −20°C.

For variable fluorescence analysis, cultures were sampled at two time points: at the beginning (day 0) and at the end of the experiment (day 7). Chlorophyll *a* variable fluorescence data was collected *in vivo* using a Pulse Amplitude Modulated fluorometer (PAM).

[1] Note that since *B. plumosa* samples came from a pure stock culture, and the excessive agitation could damage the filaments, the washing procedure was performed more gently on this species, while was more incisive on *C. tomentosum* due to the possible presence of epiphytes in the crude culture.

4.3 Pigment analysis

Freeze-dried algae samples were pestled (until powder-like) and weighted on an analytical balance (0.003 ± 0.001 g). Pigments were extracted in 1 mL of 4°C cool extraction buffer (95% Methanol + 2% Ammonium Acetate) and sonicated for 1 min; if necessary, an additional step of 30 s sonication was added. Subsequently, samples were transferred to −20°C for 20 min in the dark, to perform complete extraction. Extracts were filtered through 0.2 mm Fluoropore membrane filters (Merk-Millipore) and immediately injected into the HPLC system (LC-2030C 3D Plus, Shimadzu, Japan).

Chromatographic separation was performed with a SUPELCOSIL C18 column (Sigma-Aldrich, USA) for reverse phase chromatography and a 35 min elution programme, with injection volume of 50 µl and flow rate of 0.6 mL min^{-1} (*Kraay et al. 1992*; *Mendes et al. 2007*). Gradient profiles and mobile phase compositions are summarised in **Tab. 4.2**. Pigments were identified from retention times and absorption spectra (see **Supplementary material: Appendix C**). Concentrations were calculated from the signal (peak area) in the photodiode array (PDA) detector and calibration curves were constructed with pure crystalline standards (DHI, Denmark).

Time (min)	*% Solvent A Methanol:water (85:15 v/v)*	*% Solvent B Acetonitrile:water (90:10 v/v)*	*% Solvent C Ethyl acetate (100%)*
0	60	40	0
2	0	100	0
7	0	80	20
17	0	50	50
21	0	30	70
28.5	0	30	70
29.5	0	100	0
30.5	60	40	0
35	60	40	0

Tab. 4.2 Gradient profiles and mobile phase compositions of the chosen HPLC method.

4.4 Fluorescence analysis

Fluorescence measurements were carried out using a Junior PAM Fluorometer (Heinz Walz GmbH). Due to the "thick" morphology of *Codium tomentosum*, the optic fibre was kept adjacent to the algae surface, while for *Bryopsis plumosa* the filamentous branches of the siphon were aligned and the fibre was kept at about 0.1 mm distance. Measurements were taken at about 1-0.5 cm of distance from the frond tip.

Photoprotective effectiveness of NPQ was assessed with a method proposed by *Ruban & Murchie, 2012* and reviewed by *Ruban, 2017*, here called "pNPQ protocol". Schematic structure of the adapted pNPQ protocol is shown in **Fig. 4.2**. After dark acclimation (30 min for LLac; 1 h for HLac cultures) a saturation pulse was given in the dark for obtaining Fo (minimum fluorescence yield in the dark adapted state) and Fm (maximum fluorescence yield in the dark adapted state). After a short pause (1 m), samples were illuminated with 5 phases of progressively increasing Actinic Light (AL) intensities (PAR: 25, 65, 90, 190, 420 μmol photons m^{-2} s^{-1}) each lasting 10 min, with a total illumination time of 50 min. Under each AL phase, a saturation pulse was given for obtaining F (minimum fluorescence yield during steady state illumination) and Fm′ (maximum fluorescence yield during steady state illumination). After each phase, AL was switched off and 3 consecutive saturation pulses were given under Far Red (FR) light (3 s after switching off AL, with 7 s of distance between each other). With the pulses under FR, Fo′ (minimum fluorescence yield in the dark immediately after steady state illumination) and Fm′d (maximum fluorescence yield in the dark immediately after steady state illumination) were measured. Immediately after FR phase, another AL phase was initiated; all the phases were repeated for the 5 PAR intensities. The full measurement lasted about 55 min per sample. Note that all the pulses and light phases were imposed manually.

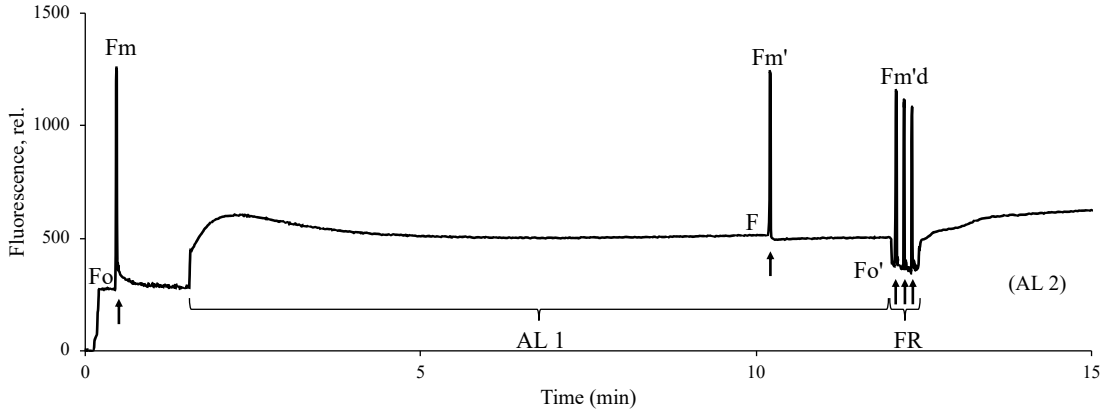


Fig. 4.2 Schematic representation of pNPQ protocol from a *Codium tomentosum* LLac sample. All measured parameters (Fo, Fm, F, Fm′, Fo′, Fm′d), Actinic Light (AL) and Far Red (FR) phases are reported. Arrows (↑) indicate saturation pulses. With the measured parameters, NPQ, YII, YIIth and qPd parameters were calculated (see **Supplementary material: Appendix B.**).

Steady State Light Curves (SSLC) were constructed with saturation pulses given during the 10 min incremental steps of actinic irradiance of the pNPQ protocol (PAR: 0, 25, 65, 90, 190, 420 µmol photons m^{-2} s^{-1}). Sat-pulses were given sufficiently far from FR phase: after 8 min for *Codium tomentosum* and after 7 min for *Bryopsis plumosa*, due species-specific responses. The light response was characterised by fitting the light curves to the model of *Platt et al. 1980* and by estimating the parameters α, rETRmax and Ek (**Fig. 4.3**). The model was fitted iteratively using MS Excel Solver.

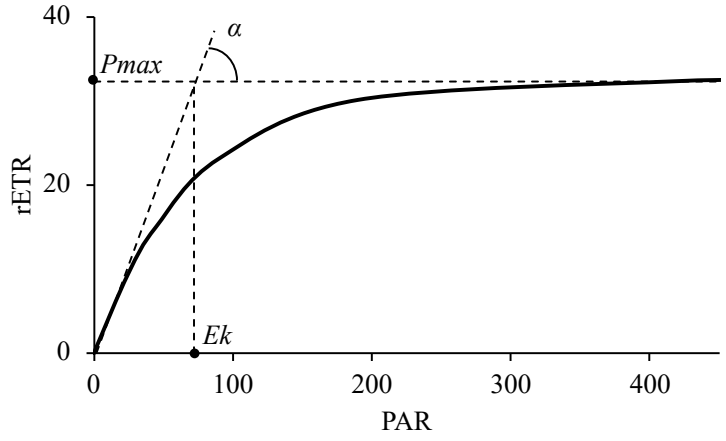


Fig. 4.3 Parameters α, rETRmax and Ek (*Platt et al. 1980*) from a representative Steady State Light Curves (SSLC) of *Codium tomentosum* (LLac). α: Initial slope of the light curve; rETRmax: Maximum rETR (µmol e− m^{-2} s^{-1}); Ek: Light saturation coefficient (PAR).

From the same protocol, complementary quantum yields parameters (Y(NPQ) and Y(NO); *Klughammer &Schreiber, 2008*) were calculated. For all parameters definitions and formulas see **Supplementary material: Appendix B**.

4.5 Second round

The experiment described in **Section 4.2** was repeated for both *Codium tomentosum* and *Bryopsis plumosa*; 2 batches of 5 algae fragments (7 ± 2 g) were randomly taken from the stock culture, and cultured for 7 days under either Low Light (LLac, 20 μmol photons m^{-2} s^{-1}) or High Light (HLac, 1000 μmol photons m^{-2} s^{-1}). Pigment and fluorescence measurements, at day 0 and after 7 days, were taken for validation and analysed with methods previously described (see: **Section 4.3-4.4**). Pigment samples at day 7 were taken at 3 time points: immediately after the night (6:00), at late morning (10:00, normal sampling time) and at the end of the light period (21:00), for assessing the presence/absence of a xanthophyll cycle. The newly acclimated cultures were used for performing the experiment of D1 protein repair quantification (see **Section 4.6**).

4.6 D1 protein repair quantification

After the 7 days of acclimation, 9 algae fragments (0.5 ± 0.1 g) were randomly cut from both LLac and HLac cultures and split into 3 batches (of 3 replicates each): "Before" (frozen with liquid nitrogen and stored at −80°C), "Treatment" (incubated for 30 min with 1 g/L of Lincomycin-HCl in ASW 30‰) and "Control" (incubated for 30 min with ASW 30%). After the 30 min of incubation, Treatment and Control samples were exposed to 1 h of light stress (1000 μmol photons m^{-2} s^{-1}). After the light stress, samples were rapidly frozen with liquid nitrogen and stored in the dark at −80°C.

It has to be noted that the experiment was performed after the last night of acclimation, and the algae were therefore kept in the dark for 8 h before the experiment started. The experiment was performed in duplicate, for obtaining backup samples. Light was provided with the same REEF-SPEC blue-white T5 lamps, 54 W used for High Light

Acclimation (see **Fig. 4.1.b**). Incubation and treatment were performed into transparent polycarbonate tubes with (dm: 25 mm; h: 89 mm; no cap).

Thylakoid proteins extraction was performed with a protocol adapted from *Chen et al. 2016*, in a cool room (17°C) under dim light. Equipment and buffers were pre-chilled (4°C) and samples were kept in ice. Samples stored at −80°C were grind with a tissue-homogeniser in 5 mL of Grinding Buffer (GB: 50 mM Hepes-KOH, pH 7.8; 330 mM sorbitol; 10 mM EDTA; 5 mM NaCl; 5 mM $MgCl_2$; 2,5 mM sodium ascorbate; 0.2% BSA). Homogenates were filtered into 50 ml centrifuge tubes through two layers of Miracloth (Merk-Millipore), with 20 µm pore size; 10 ml of GB were added and the suspension centrifuged. Supernatant was discarded and pellet was carefully resuspended in 2ml tubes with excess of GB. After another centrifuge, pellets were resuspended in excess of Shock Buffer (ShB: 50mM Hepes-KOH, pH 7.8; 5mM $MgCl_2$), using a 200 mL tip. Resuspension with ShB and following centrifuge step were performed two times. Subsequently, pellets were resuspended in excess of Storage Buffer (StB: 50mM Hepes-KOH, pH 7.8; 100mM sorbitol; 5mM $MgCl_2$; 10mM NaCl), using a 200 mL tip and a last centrifuge step was performed. Finally, supernatant was discarded and pellets containing thylakoid membrane proteins were resuspended in 200 µL of StB. Extracted samples were divided in aliquots of 50µL, flash-frozen with liquid nitrogen and stored at −80°C. Between each resuspension step samples were centrifuged at 1000 g, 4°C, for 5 min, repeating the cycle if necessary.

Sample preparation for ELISA was adapted from *Mujer et al. 1996* and *Hall et al. 2015*. *S*amples were de-frost in a cold room (17°C) with dim light and immediately centrifuged at 1000 g, 4°C, for 5 min. Supernatant was discarded and thylakoid protein solubilisation was performed with StB + 0.5% Triton-X100 in a heated shaker (37°C) for 30 min, under dim light; samples were gently resuspended with 1 mL tip after the first 15 min. Subsequently, extracts were centrifuged at high speed (approx. 12000 RPM) at room temperature for 10 min. Pellets of non-protein material were discarded and supernatants, containing extracted proteins were transferred to fresh tubes and directly loaded into the ELISA plate.

ELISA (Enzyme-Linked Immunosorbent Assay) was performed essentially as described in *Gimpel et al. 2016*. Samples were diluted 1:200 in buffer solution (TBS + 25% Isopropanol); appropriate buffer and dilution were chosen after several preliminary tests. Calibration curves were constructed using PsbA standard (D1 positive control/ quantitation standard, AS01 016S, Agrisera, Sweden). Diluted samples and standard were loaded in a 96-well microplate with high-binding coating (Greiner Bio One International GmbH): 50 µL per well, in triplicates from each sample. Protein binding was done overnight at 4°C, covering the plate with aluminium foil. Subsequently, 200 µL per well of blocking solution (1% BSA in TBS) were added and blocking was performed for 1.5 h at 37 °C, with shaking. Primary antibody (Rabbit anti-PsbA C-terminal AS05 084, Agrisera, Sweden) was added with a concentration of 1 µg/mL (diluted with 1% BSA in TBS; 50 µL per well). Incubation was performed at 37 °C, with shaking, for 1.5 h. Secondary antibody (Goat anti-Rabbit IgG, Alkaline Phosphatase (AP) conjugated, AS09 607, Agrisera, Sweden) was added with higher concentration (1.5 µg/mL, diluted with 1% BSA in TBS50; µL per well). AP substrate was prepared in the dark, mixing pNPP tablets and diethanolamine buffer (AP Substrate Kit #1721063, Bio-Rad Laboratories, Inc.); incubation, with 100 µL of substrate per well, was performed at 37 °C, in the dark, for about 20 min; results were read with a microplate reader at 405 nm. Between each incubation step the plate was washed 3 times with TBS + 0.05% Tween-20.

4.7 Statistical analysis

Differences in pigment ratios between HLac and LLac cultures were tested using repeated measures partly nested analysis of variance (ANOVA, **Fig. 4.4**). Data exploration was performed using box-plots, interaction-plots and Pearson's residuals analysis to verify normality and homogeneity of variance, absence of block-by-within-block interactions and identify evident outliers (*Logan, 2010*). Sphericity assumption was verified using Mauchly's Test. Pairwise post-hoc comparisons were performed using Welch's two-samples t-test (for individual comparisons) and pairwise t-test with Bonferroni correction (for multiple comparisons). t-tests were also performed for testing

differences in SSLC parameters (5 replicates per condition in the first experiment, 3 for the second).

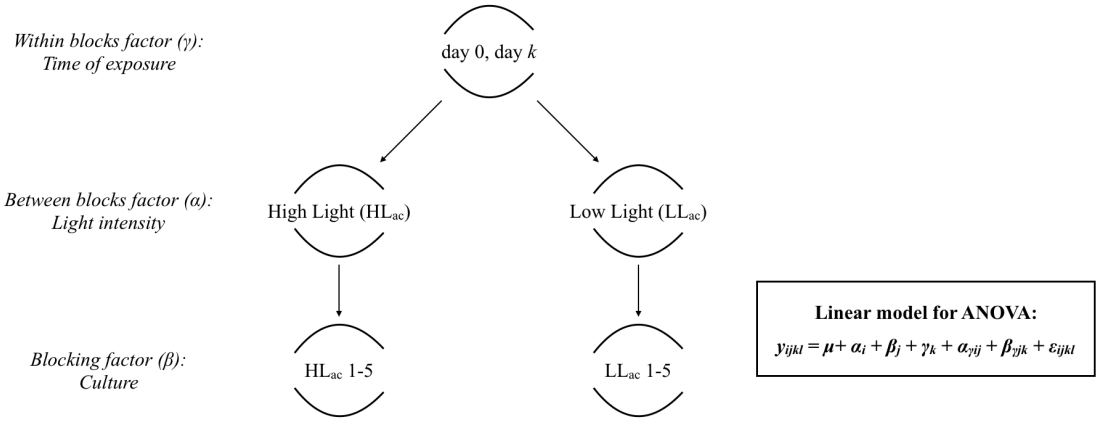


Fig. 4.4 Experimental design and linear model for ANOVA analysis used for testing differences in pigment ratios during 7 days of exposure to low/high light. Effect of light acclimation was tested using a repeated measure design, with one factor Between blocks (Light intensity, fixed with 2 levels), one factor Within blocks (Time of exposure, fixed with 6 levels) and one Blocking factor (Culture, random with 5 levels).

Differences in D1 protein content between HLac and LLac samples, with or without Lincomycin treatment were tested using a two-way analysis of variance (ANOVA, **Fig. 4.5**). Homogeneity of variance was verified with Cochran's test and pairwise post-hoc comparisons were performed using Student-Newman-Keuls (SNK) test.

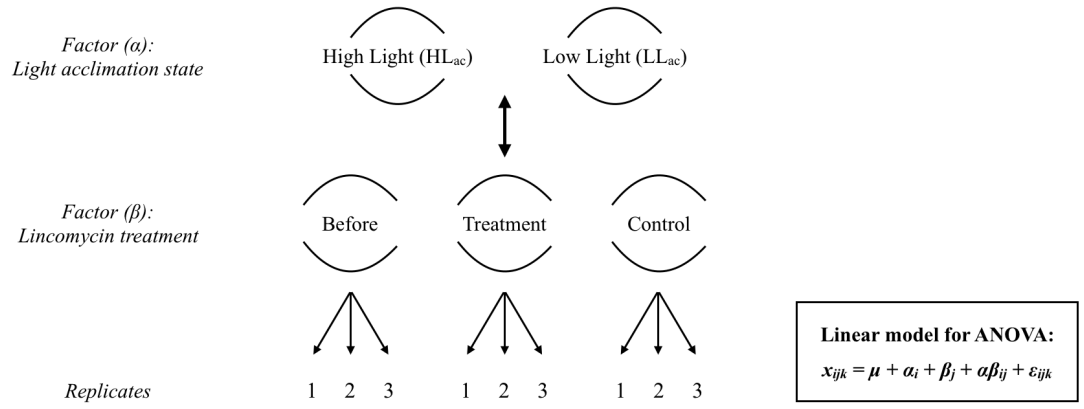


Fig. 4.5 Experimental design and linear model for ANOVA analysis used for testing differences in D1 content. Lincomycin effect was tested using a two-way design, with two crossed factors: Light acclimation state (fixed with 2 levels: HLac and LLac), Lincomycin treatment (fixed with 3 levels: before, treatment and control) and 3 random replicates for each combination

Finally, total pigment content variability was assessed with multivariate approach (Principal Component Analysis, PCA). Statistical analyses were carried out using R (*R Foundation for Statistical Computing, Vienna, Austria*; packages: "stats", "car", "ez", "ggbiplot", "GAD").

5. Results

5.1 Pigment content variation during 7 days of LLac/HLac

The two investigated species, *Codium tomentosum* and *Bryopsis plumosa* displayed a similar pigment pool, as reported in **Tab. 5.1**: Chl *a*/*b* and siphonaxanthins (Siph/Siph-do) as main light harvesting pigments and several carotenoids, including other xanthophylls (t-Neo, c-Neo, Viola, Anth, Zea) and carotenes (ε/α/β). Notably, Lut was detected only in *Codium tomentosum*.

HPLC absorbance chromatograms of the extracted pigment pool for each species and acclimation condition are reported in **Fig. 5.1**.

Pigment	*Abbreviation*	*Retention time (min)*	λ_{max} *(nm)*
Siphonaxanthin	Siph	10.41 (± 0.04)	448
all-trans-Neoxanthin	t-Neo	11.38 (± 0.08)	418; 441; 470
9'-cis-Neoxanthin	c-Neo	12.00 (± 0.06)	413; 437; 466
Violaxanthin	Viola	13.00 (± 0.09)	416; 441; 471
Antheraxanthin	Anth	14.82 (± 0.11)	426; 447; 475
Lutein*	Lut	16.63 (± 0.02)	423; 447; 475
Zeaxanthin	Zea	16.67 (± 0.12)	452; 479
Siphonaxanthin dodecenoate	Siph-do	18.53 (± 0.09)	455
Chlorophyll *b*	Chl *b*	22.63 (± 0.06)	458; 597; 645
Chlorophyll *a*	Chl a	24.22 (± 0.06)	430; 617; 662
ε-Carotene	ε-Car	28.14 (± 0.05)	418; 441; 471
α-Carotene	α-Car	28.33 (± 0.05)	447; 476
β-Carotene	β-Car	28.54 (± 0.06)	450; 474

Tab. 5.1 Pigment composition of *Codium tomentosum* and *Bryopsis plumosa* detected with HPLC. Pigments names, abbreviation, mean retention time (with standard deviation) and mean absorption maxima (λ_{max}) are reported; standard deviation for λ_{max} was always low (between 0 and 3). *: Lutein was detected only in *Codium tomentosum*.

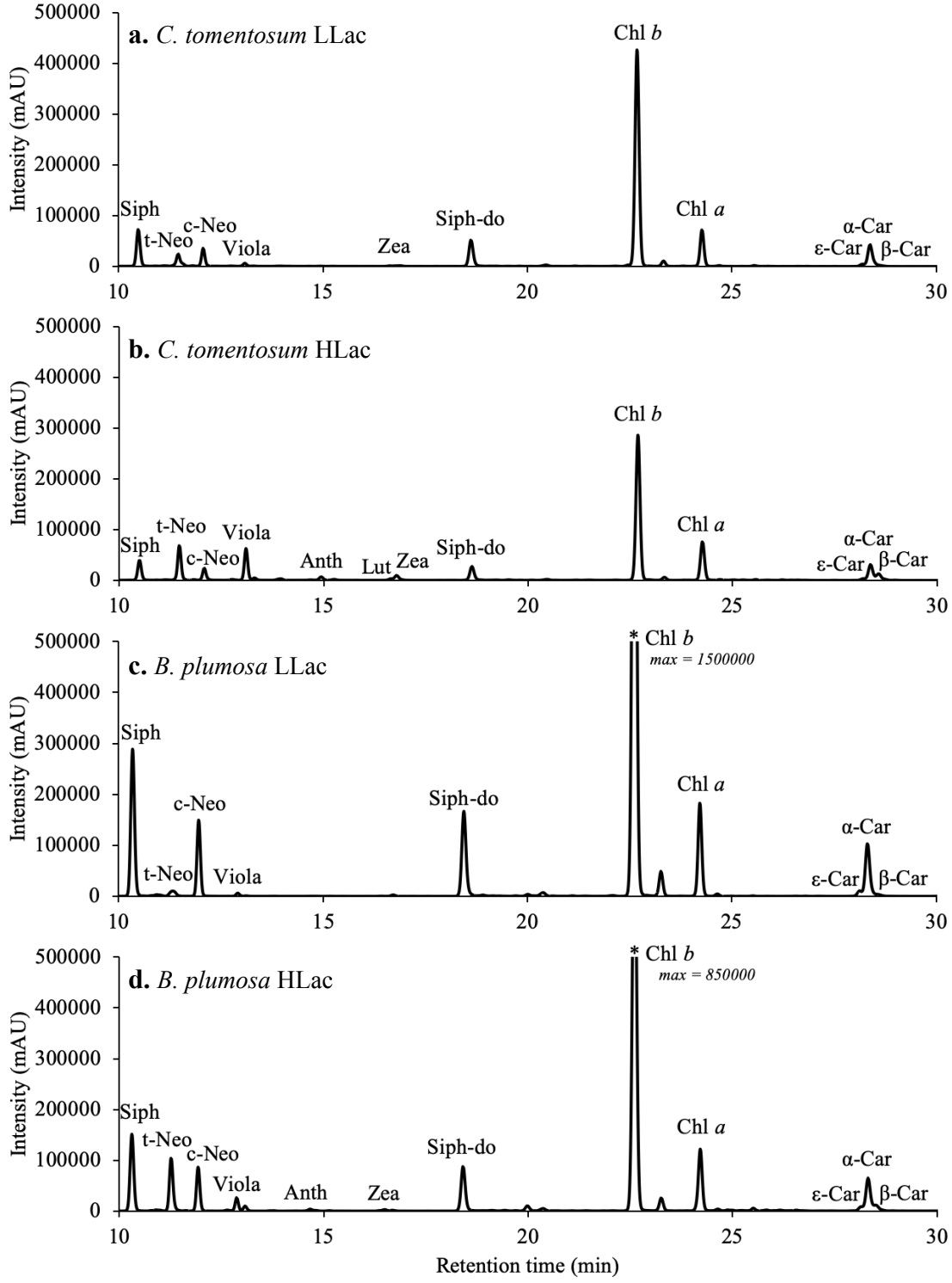


Fig. 5.1 Representative HPLC absorbance chromatograms (450 nm) of *Codium tomentosum* and *Bryopsis plumosa* after 7 days of LL/HL acclimation. **a-b.** *C. tomentosum* LLac (**a.**) and HLac (**b.)**; **c-d.** *B. plumosa* LLac (**c.**) and HLac (**d.**). Pigments abbreviations are listed in **Tab 5.1**.

In general, a similar pattern of acclimation was observed in both species (**Fig. 5.2**): while no significant differences were present at day 0, HLac cultures after 7 days displayed overall decrement of main light harvesting pigments (Chl *a*/*b*, Siph/Siph-do) and alterations of carotenoid composition, including highly significant accumulation of t-Neo and Viola.

Despite a general common pattern, slight differences in pigment content were found between the two species. First, pigment concentration per dry weight were in general higher in *Bryopsis plumosa*; after 7 days HLac culture of this species displayed a significant decrement of c-Neo, ε- and α-Car, while this alteration was not observed in *C. tomentosum*. As previously mentioned, Lut was never found in *B. plumosa.*

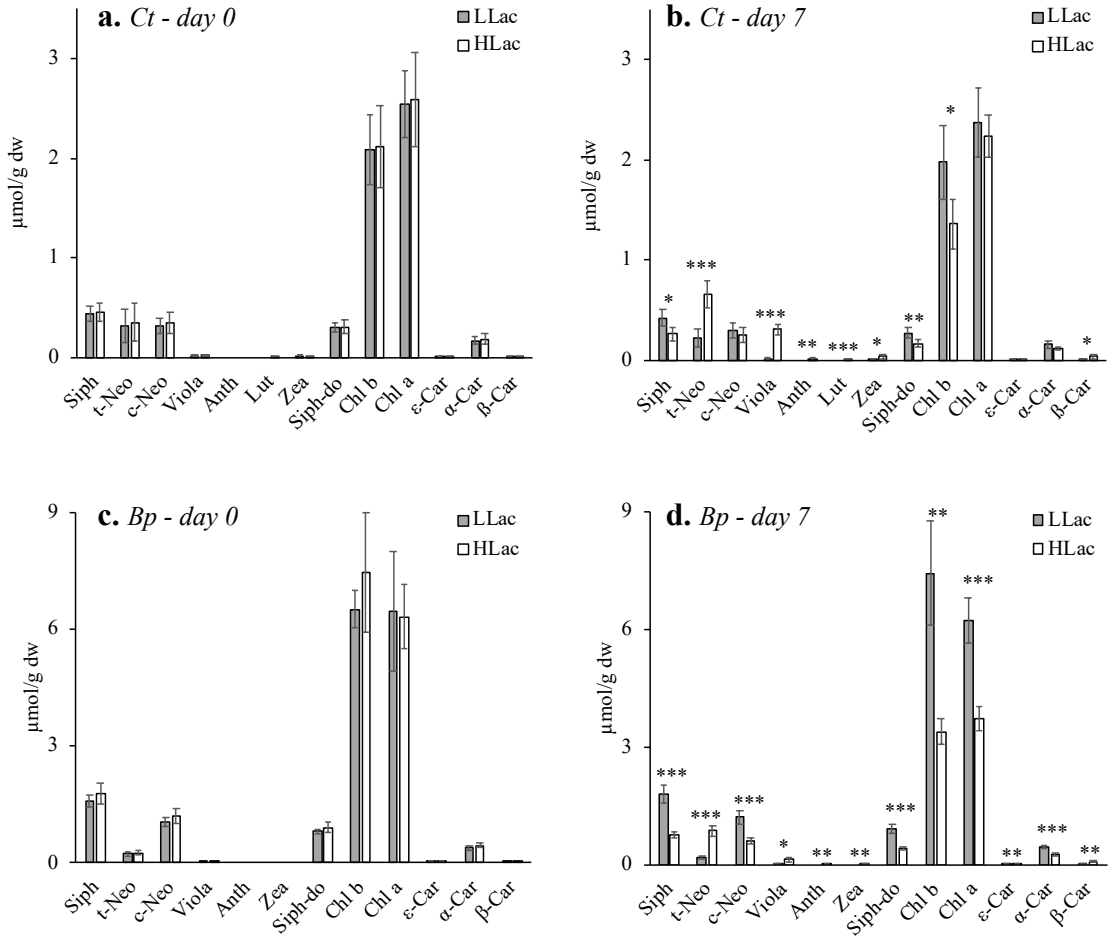


Fig. 5.2 Pigment concentration per dried weight of Low Light (LLac, 20 µmol photons m^{-2} s^{-1}) and High Light (HLac, 1000 µmol photons m^{-2} s^{-1}) acclimated *C. tomentosum* (*Ct*, **a.** at day 0; **b.** at day 7) and *B. plumosa* (*Bp*, **c.** at day 0; **d.** at day 7), with standard deviation (n=5). Asterisks indicate significant differences between LLac and HLac (t-tests): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Interestingly, the two species showed differences in the production of Chl *a* (**Fig. 5.3**). ANOVA analysis showed no significant differences between HLac and LLac *C. tomentosum*, while for *B. plumosa* significant effects of irradiance ($p = 0.00003$) and Time ($p = 0.007$) were observed. Post-hoc comparisons (t-test) revealed that the content of Chl *a* in *B. plumosa* was affected by relevant fluctuations, despite a general trend of decrement in HLac cultures.

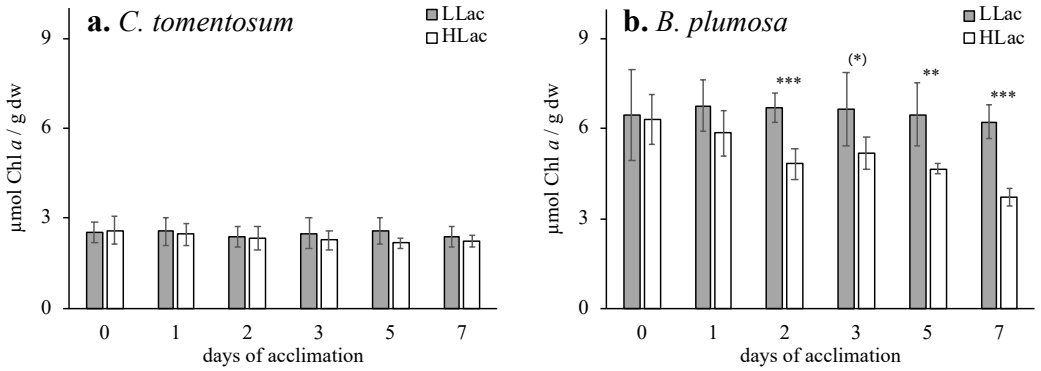


Fig. 5.3 Chlorophyll *a* concentration per dried weight of LLac and HLac *C. tomentosum* (**a.**) and *B. plumosa* (**b.**), with standard deviation (n=5). Asterisks indicate significant differences between LLac and HLac cultures (t-test): (*) $p = 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Since Chl *a* is considered to be the main photosynthetic pigment, pigment concentrations were normalised per Chl *a* (Pigment:Chl *a* ratios, **Fig. 5.4**) to analyse relative changes in composition. The reported significant differences in ε- and α-Car in *Bryopsis plumosa* were no longer observed when pigment content was normalised per Chl *a*.

Pigment:Chl *a* ratios confirmed the general High Light acclimation pattern described above: significant decrement of light harvesting pigments (Chl *b*, Siph, Siph-do) and increases in other carotenoids. Additionally, the decrease in light harvesting pigments concentration Chl *b*, Siph and Siph-do under High Light was more significant than the decrease in Chl *a*, as revealed by significantly lower pigment to Chl *a* ratios after 7 days of HL_{ac}. Regarding carotenoids, significant accumulation of t-Neo, Viola, other xanthophylls (Anth, Zea; Lut in *C. tomentosum*) and ß-Car was observed in both species. Only *B. plumosa* showed a significant decrement of c-Neo.

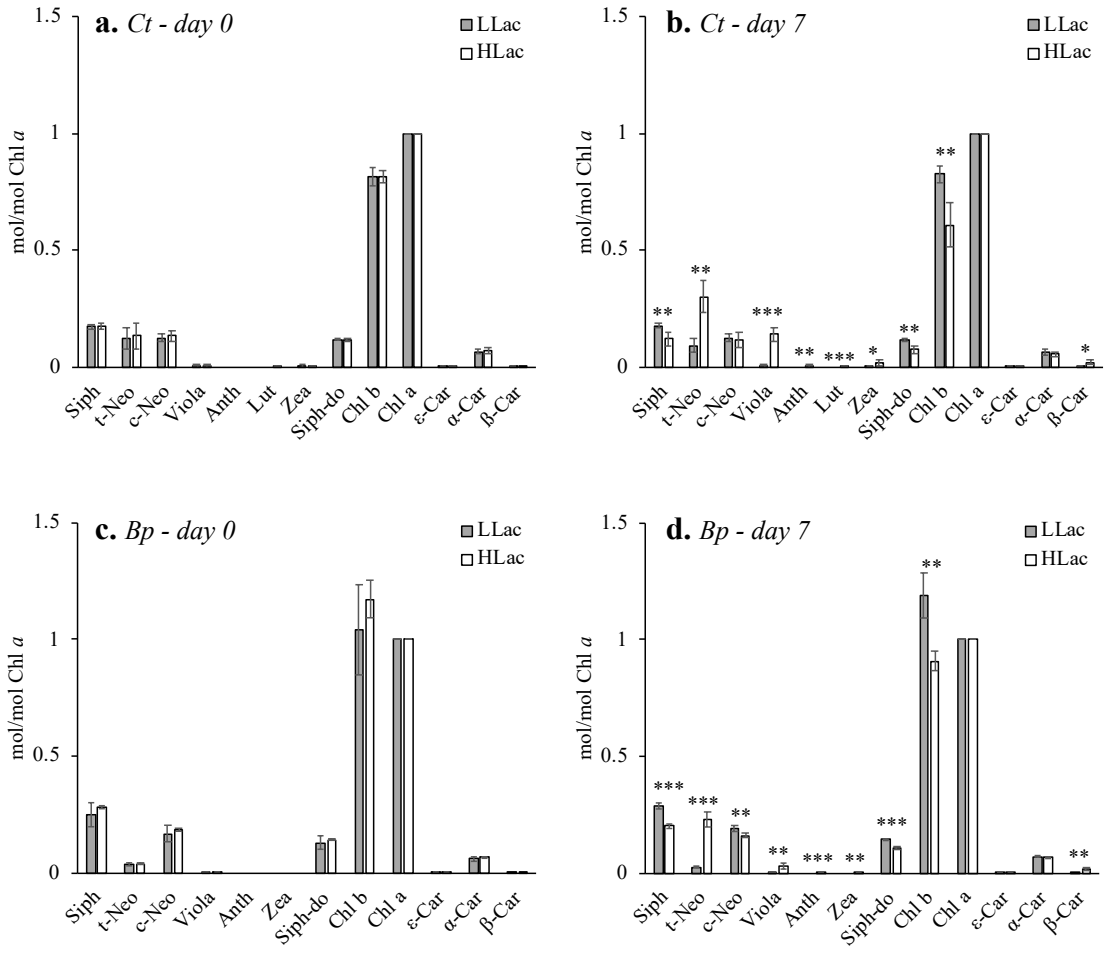


Fig. 5.4 Mean Pigment:chlorophyll *a* ratios of Low Light (LLac, 20 µmol photons m^{-2} s^{-1}) and High Light (HLac, 1000 µmol photons m^{-2} s^{-1}) acclimated *C. tomentosum* (*Ct* **a.** at day 0; **b.** at day 7) and *B. plumosa* (*Bp* **c.** at day 0; **d.** at day 7), with standard deviation. Asterisks indicate significant differences between LLac and HLac (t-tests): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Pigment:Chl *a* ratios during the 7 days of acclimation were further analysed, for investigating the patterns of pigment accumulation or loss during prolonged exposure to High Light in both species. ANOVA results for each pigment variable are summarised in **Tab. 5.2** and **5.3**.

For both species, no relevant abnormalities were found during preliminary data exploration. When Sphericity assumption was not met (*C. tomentosum*: Viola, $p = 6\ 10^{-7}$; Chl *b*, $p = 0.004$; α-Car, $p = 0.020$; ß-Car, $p = 0.002$. *B. plumosa*: Siph, $p = 0.025$; t-Neo, $p = 0.036$; c-Neo, 0.001; Viola, $p = 8\ 10^{-7}$; Zea, $p = 8\ 10^{-42}$, Siph-do, $p = 0.023$, Chl *b*, $p = 0.039$; ß-Car, $p = 0.020$) Greenhouse-Geisser ε correction was applied.

	Effect	DF_{num}	DF_{err}	*p value*	*Significance*
Siph	Light Intensity	1	8	0.020	*
	Time	5	40	$3\ 10^{-9}$	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-9}$	***
t-Neo	Light Intensity	1	8	0.0009	***
	Time	5	40	0.0001	***
	Light Intensity ✕ Time	5	40	$9\ 10^{-7}$	***
c-Neo	Light Intensity	1	8	0.080	
	Time	5	40	0.001	**
	Light Intensity ✕ Time	5	40	0.0004	***
Viola	Light Intensity	1	8	$5\ 10^{-7}$	***
	Time	5	40	$7\ 10^{-8}$	***
	Light Intensity ✕ Time	5	40	$7\ 10^{-8}$	***
Anth	Light Intensity	1	8	$1\ 10^{-5}$	***
	Time	5	40	$1\ 10^{-11}$	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-11}$	***
Lut	Light Intensity	1	8	0.047	*
	Time	5	40	0.0002	***
	Light Intensity ✕ Time	5	40	$7\ 10^{-6}$	***
Zea	Light Intensity	1	8	0.080	
	Time	5	40	0.0001	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-6}$	***
Siph-do	Light Intensity	1	8	0.005	**
	Time	5	40	$3\ 10^{-14}$	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-12}$	***
Chl b	Light Intensity	1	8	0.009	**
	Time	5	40	0.00003	***
	Light Intensity ✕ Time	5	40	$9\ 10^{-6}$	***
ε-Car	Light Intensity	1	8	0.458	
	Time	5	40	0.155	
	Light Intensity ✕ Time	5	40	0.014	*
α-Car	Light Intensity	1	8	0.406	
	Time	5	40	0.260	
	Light Intensity ✕ Time	5	40	0.135	
β-Car	Light Intensity	1	8	0.013	*
	Time	5	40	0.00007	***
	Light Intensity ✕ Time	5	40	0.0001	***

Tab. 5.2 ANOVA results for 7 days LLac and HLac *Codium tomentosum*. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Note that it was not possible to apply Sphericity test to Anth data; therefore the significance level for this variable might be inflated.

	Effect	DF_{num}	DF_{err}	*p value*	*Significance*
Siph	Light Intensity	1	8	0.0004	***
	Time	5	40	0.030	*
	Light Intensity ✕ Time	5	40	0.001	**
t-Neo	Light Intensity	1	8	$1\ 10^{-7}$	***
	Time	5	40	$1\ 10^{-9}$	***
	Light Intensity ✕ Time	5	40	$5\ 10^{-10}$	***
c-Neo	Light Intensity	1	8	0.018	*
	Time	5	40	0.51	
	Light Intensity ✕ Time	5	40	0.017	*
Viola	Light Intensity	1	8	$4\ 10^{-7}$	***
	Time	5	40	$9\ 10^{-5}$	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-4}$	***
Anth	Light Intensity	1	8	$1\ 10^{-8}$	***
	Time	5	40	$1\ 10^{-12}$	***
	Light Intensity ✕ Time	5	40	$1\ 10^{-12}$	***
Zea	Light Intensity	1	8	$4\ 10^{-7}$	***
	Time	5	40	$7\ 10^{-6}$	***
	Light Intensity ✕ Time	5	40	$7\ 10^{-6}$	***
Siph-do	Light Intensity	1	8	0.0004	***
	Time	5	40	0.066	
	Light Intensity ✕ Time	5	40	0.005	**
Chl b	Light Intensity	1	8	0.003	**
	Time	5	40	0.185	
	Light Intensity ✕ Time	5	40	0.001	**
ε-Car	Light Intensity	1	8	0.098	
	Time	5	40	0.020	*
	Light Intensity ✕ Time	5	40	0.057	
α-Car	Light Intensity	1	8	0.211	
	Time	5	40	0.011	*
	Light Intensity ✕ Time	5	40	0.020	*
β-Car	Light Intensity	1	8	$1\ 10^{-6}$	***
	Time	5	40	0.00001	***
	Light Intensity ✕ Time	5	40	0.0001	***

Tab. 5.3 ANOVA results for 7 days LLac and HLac *Bryopsis plumosa*. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Note that it was not possible to apply Sphericity test to Anth data; therefore the significance level for this variable might be inflated.

In general, both factors (Light Intensity and Time) and their interaction (Light Intensity × Time) had significant effects on the global pigment pool of *Codium tomentosum* and *Bryopsis plumosa*. In particular, for both species all the considered effects resulted highly significant ($p < 0.001$) for t-Neo and Viola, target pigments of this study. Also, high significant effects on the variance of other accessory xanthophylls (Anth, Zea; and Lut for *C. tomentosum*) were observed, with the exception of c-Neo, that displayed less significant results particularly in *B. plumosa*. Almost no effect on ε-Car and α-Car was observed, while effects on the variance of ß-Car resulted highly significant.

Regarding main light harvesting pigments (Siph, Siph-do and Chl *b*) the analysed effects resulted slightly different between species: for *Codium tomentosum* a highly significant effect ($p < 0.001$) of Time and Light Intensity × Time was observed, while the effect of Light Intensity alone resulted less significant (Siph, Chl *b*: $p < 0.01$; Siph-do: $p < 0.05$); on the contrary, for *Bryopsis plumosa* the effect of Light Intensity (Siph, Siph-do: $p < 0.001$; Chl b: $p < 0.01$) and Light Intensity × Time ($p < 0.01$) per time was stronger, while the effect of Time resulted almost null.

This observation is supported by the results of post-hoc comparisons (t-test). Considering the changes in main photosynthetic pigment content of *Codium tomentosum* during 7 days of light acclimation (**Fig. 5.5**, **I.**) the statistical significance of t-test between HLac and LLac cultures grew smoothly over time. On the contrary in *Bryopsis plumosa*, despite the appearance of significant differences from the second/ third day of acclimation, light harvesting pigments variance endured relevant fluctuations (**Fig. 5.5**, **II.**). This result is probably to be attributed to higher fluctuations in the content of all main light harvesting pigments in LLac *Bryopsis plumosa*, as already described for Chl *a* (**Fig. 5.3**).

Despite these fluctuations, it appears evident that 7 days acclimation to High Light determined a significant decrease of main light harvesting pigments in both *Codium tomentosum* and *Bryopsis plumosa*.

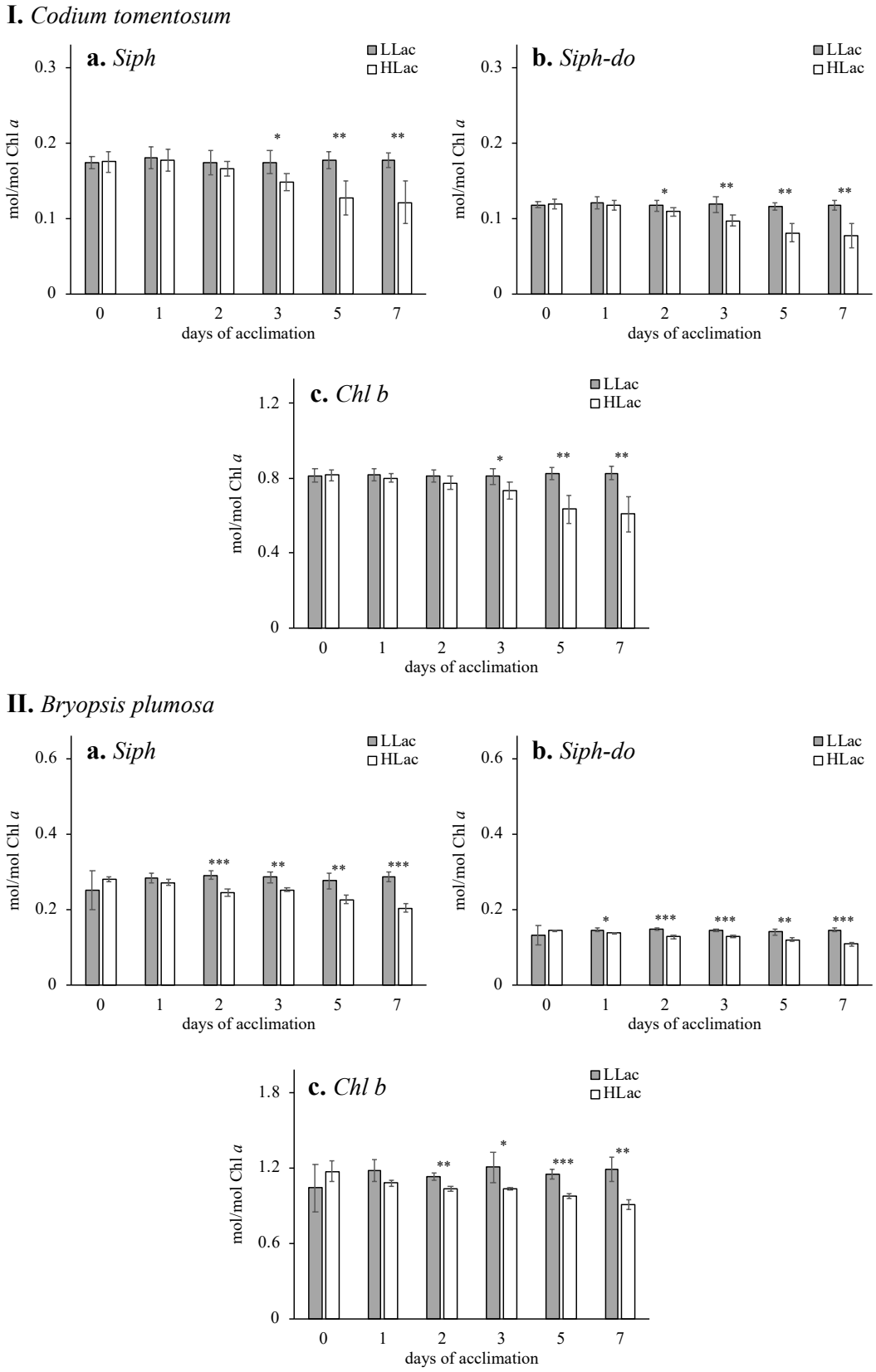


Fig. 5.5 Main light harvesting pigments content (**a.** Siph; **b.** Siph-do; **c.** Chl *b*) of LLac and HLac *Codium tomentosum* (**I.**) and *Bryopsis plumosa* (**II.**), during 7 days of light acclimation. Mean Pigment:Chl *a* ratios with standard deviation are reported (n=5). Asterisks indicate significant differences between LLac and HLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

On another note, considering the pool of accessory xanthophylls, the two species displayed similar results (with the exception of the complete absence of Lut in *B. plumosa*; it has however to be considered that this peak was particularly close to the signal-to-noise ratio).

First, post-hoc comparisons (t-test) performed on *Codium tomentosum* (**Fig. 5.6**) explained the previously described ANOVA results: for t-Neo, Viola, Anth, Lut and Zea an increasing significant difference in pigment content, from day 0 to day 7, was reported. In particular, t-Neo and Viola reached a relatively high Pigment:Chl *a* ratio in HLac cultures (around 0.3 and 0.15 respectively), while Anth, Lut and Zea were 10 times less abundant. On the contrary, differences in c-Neo were less evident and did not seem to follow a clear pattern. Similar results were obtained in *Bryopsis plumosa* (**Fig. 5.7**): differences in t-Neo, Viola, Anth and Zea content increased over time, while the amount of c-Neo did not show a clear acclimation trend.

Relative abundance of accessory xanthophylls resulted similar between the two species, with the exception of Viola (more abundant in HLac *C. tomentosum*) and Zea, that was apparently more abundant in *C.tomentosum*, but with relatively high standard deviation. Interestingly, despite the basal level of t-Neo in LLac *B. plumosa* was sensibly lower compared to *C. tomentosum*, both species displayed a similar amount of t-Neo in HLac cultures after 7 days of acclimation. Interestingly, response of both species to High Light acclimation seems to first affect Viola concentrations (within 1 day) and only later t-Neo (within 2 days).

The two species presented also a similar response regarding carotenes content (**Fig. 5.8**), coherently with the already described ANOVA results: while ε- and α-Car did not display a clear acclimation trend, post-hoc comparisons (t-test) revealed a significant increase of ß-Car after some days of High Light Acclimation. Interestingly, while the pattern was clearer in *B. plumosa*, standard deviation for ß-Car appeared sensibly higher in *C. tomentosum*.

Again, despite some differences we can conclude that High Light acclimation resulted in significant changes of carotenoid content, with coherent patterns between the two species.

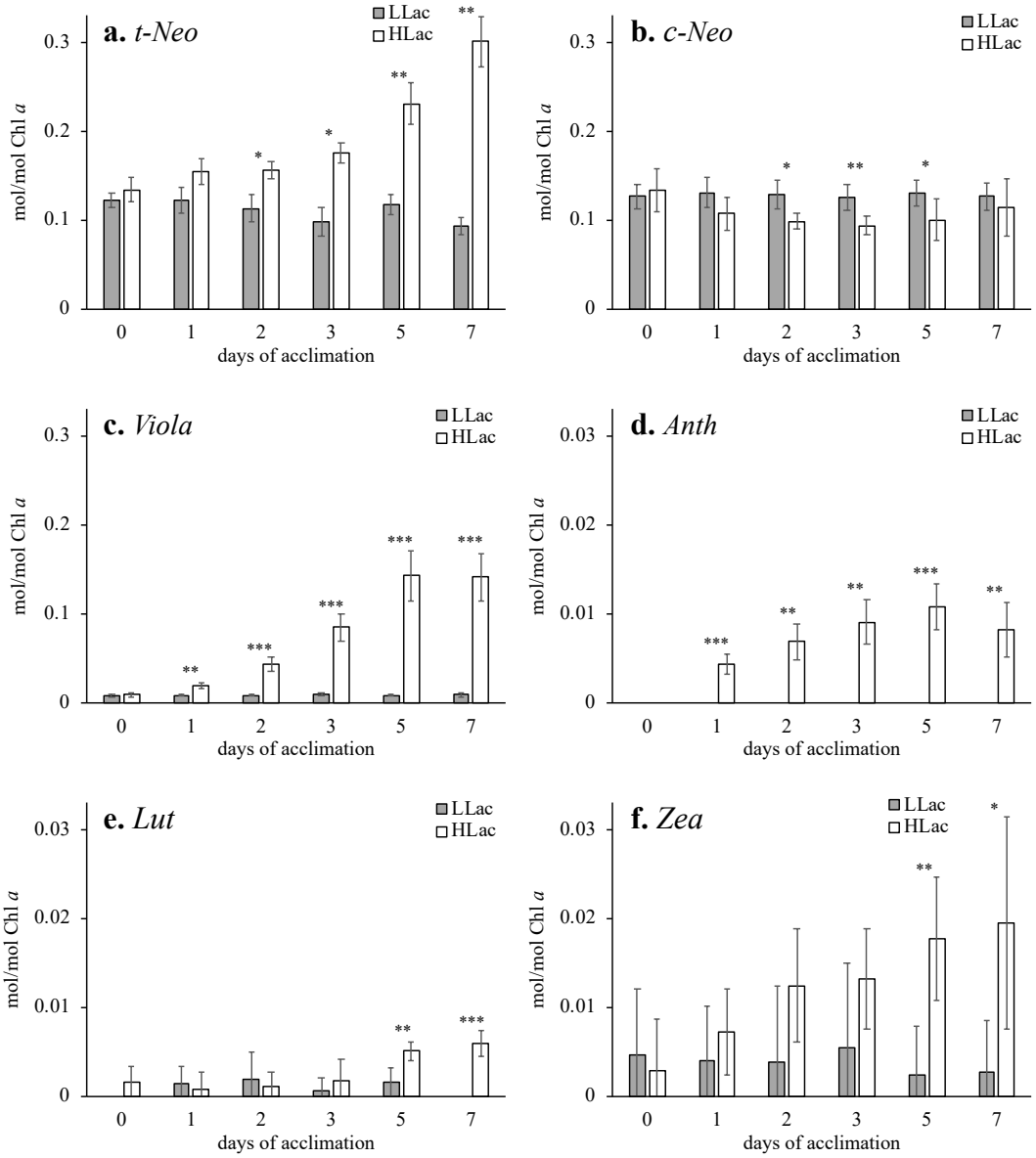


Fig. 5.6 Accessory xanthophylls content (**a.** t-Neo; **b.** c-Neo; **c.** Viola; **d.** Anth; **e.** Lut; **f.** Zea) of LLac and HLac *Codium tomentosum*, during 7 days of light acclimation. Mean Pigment:Chl *a* ratios with standard deviation are reported (n=5). Asterisks indicate significant differences between LLac and HLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

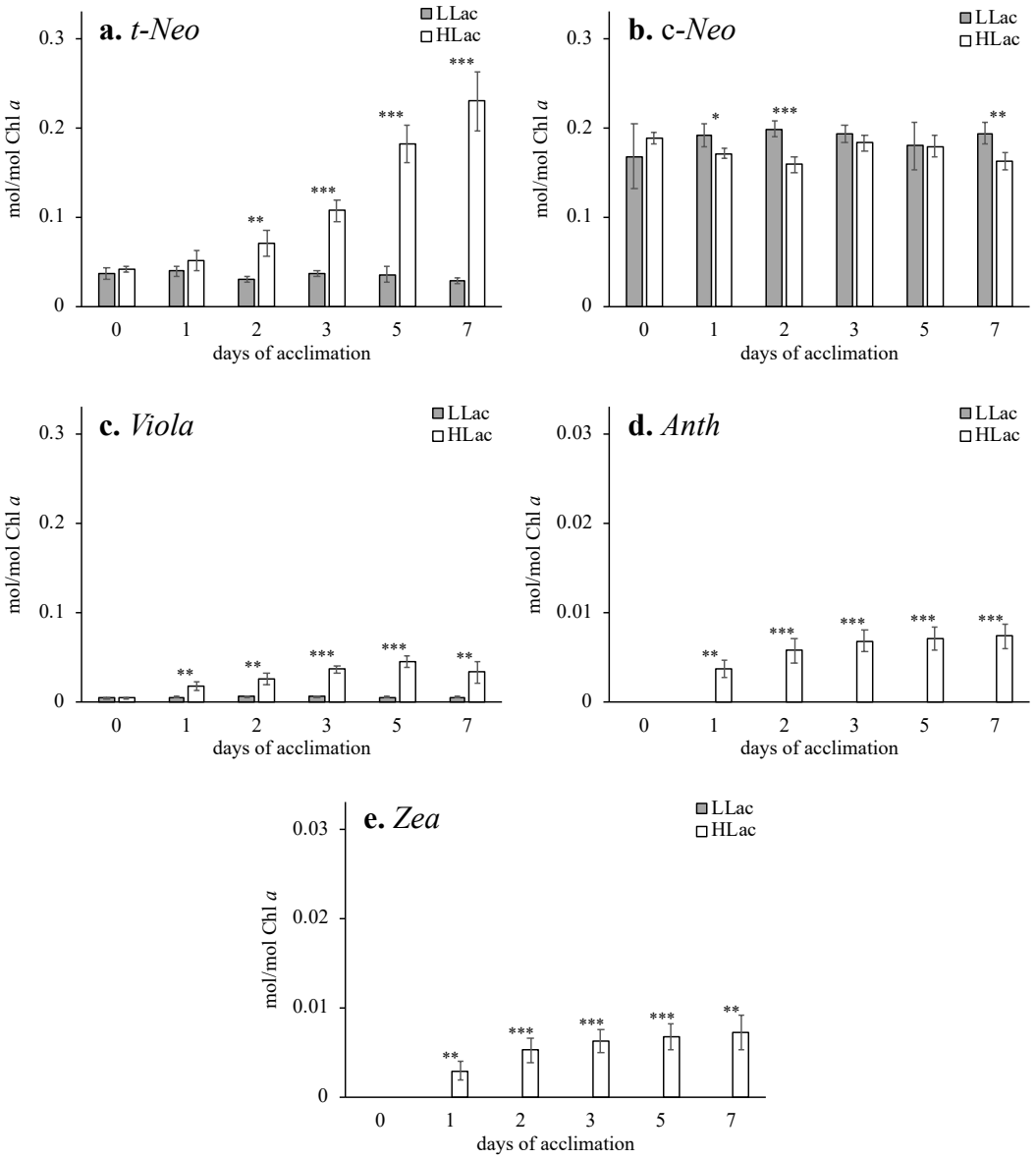


Fig. 5.7 Accessory xanthophylls content (**a.** t-Neo; **b.** c-Neo; **c.** Viola; **d.** Anth; **e.** Zea) of LLac and HLac *Bryopsis plumosa*, during 7 days of light acclimation. Mean Pigment:Chl *a* ratios with standard deviation are reported (n=5). Asterisks indicate significant differences between LLac and HLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

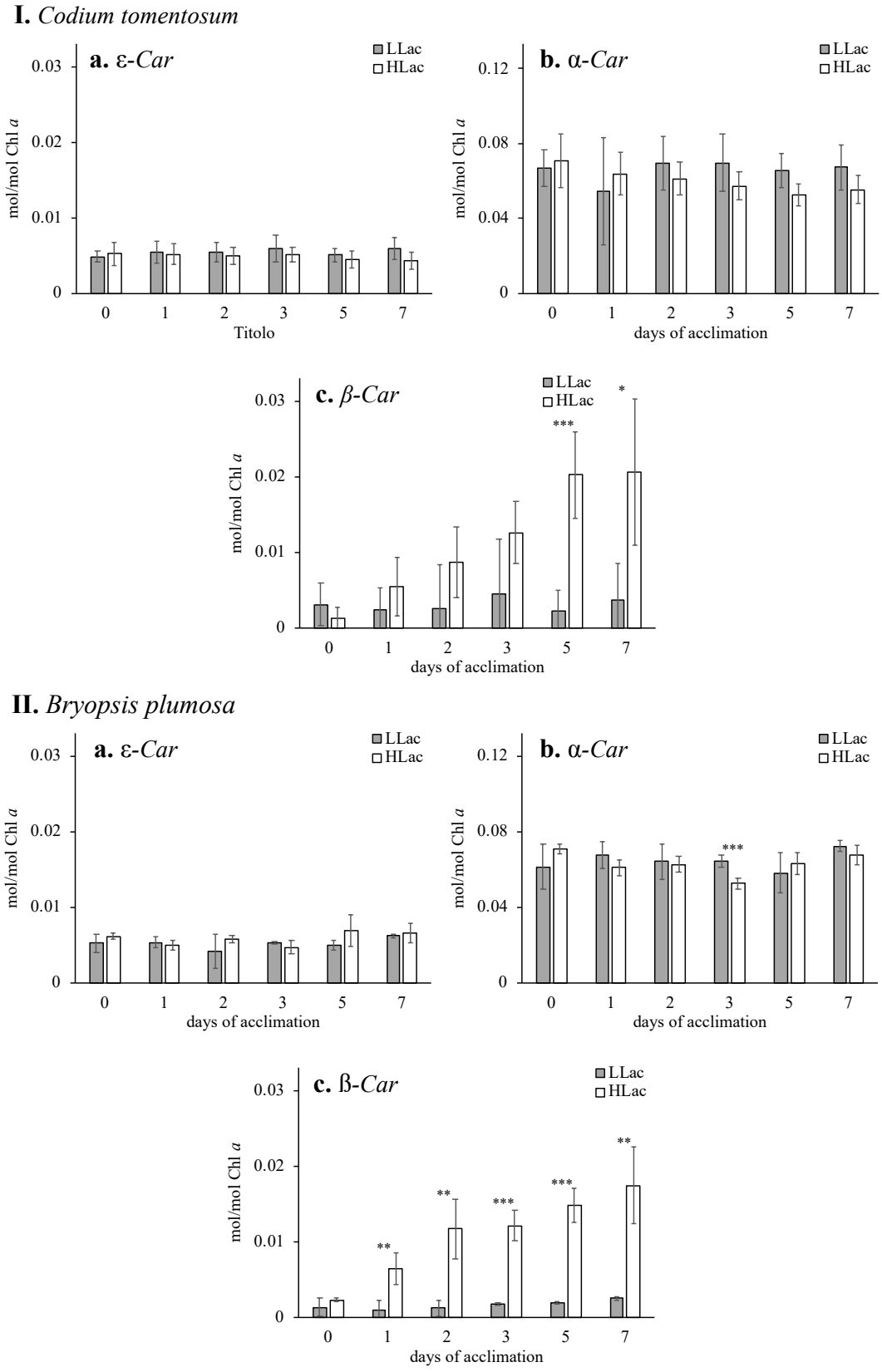


Fig. 5.8 Carotenes content (**a.** ε-Car; **b.** α-Car; **c.** ß-Car) of LLac and HLac *Codium tomentosum* (**I.**) and *Bryopsis plumosa* (**II.**), during 7 days of light acclimation. Mean Pigment:Chl *a* ratios with standard deviation are reported (n=5). Asterisks indicate significant differences between LLac and HLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

For further investigating the physiological pigment content changes driven by light acclimation, Chl *a*:Chl *b* ratios were analysed (**Fig. 5.9**). For both species, High Light acclimation determined significant increment of mean Chl *a*:Chl *b* ratio, while no difference was reported within Low Light treatment. Notably, both species displayed relatively low Chl *a*:Chl *b* ratios, with *C. tomentosum* having the highest (LLac: 1.2 ± 0.05; HLac: 1.7 ± 0.3) and *B. Plumosa* the lowest (LLac: 0.95 ± 0.07; HLac: 1.24 ± 0.06); the reported values correspond to cultures after 7 days of acclimation.

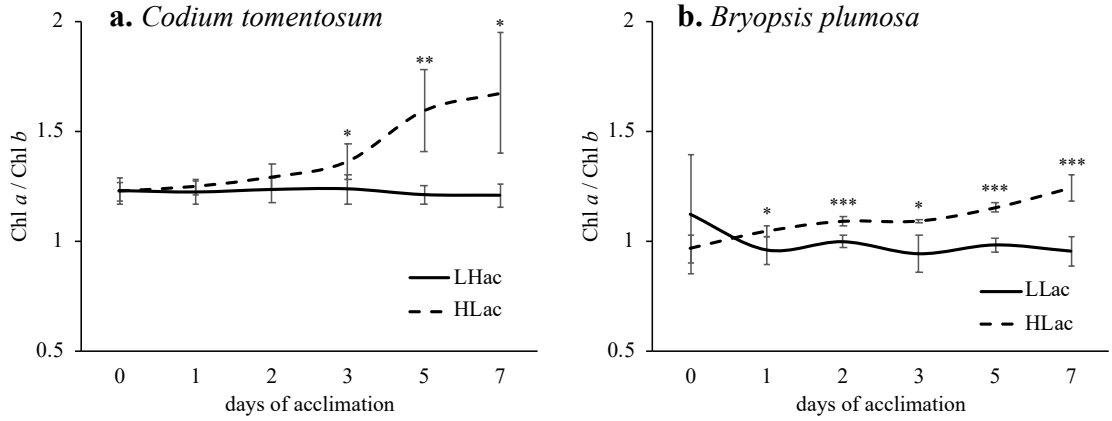
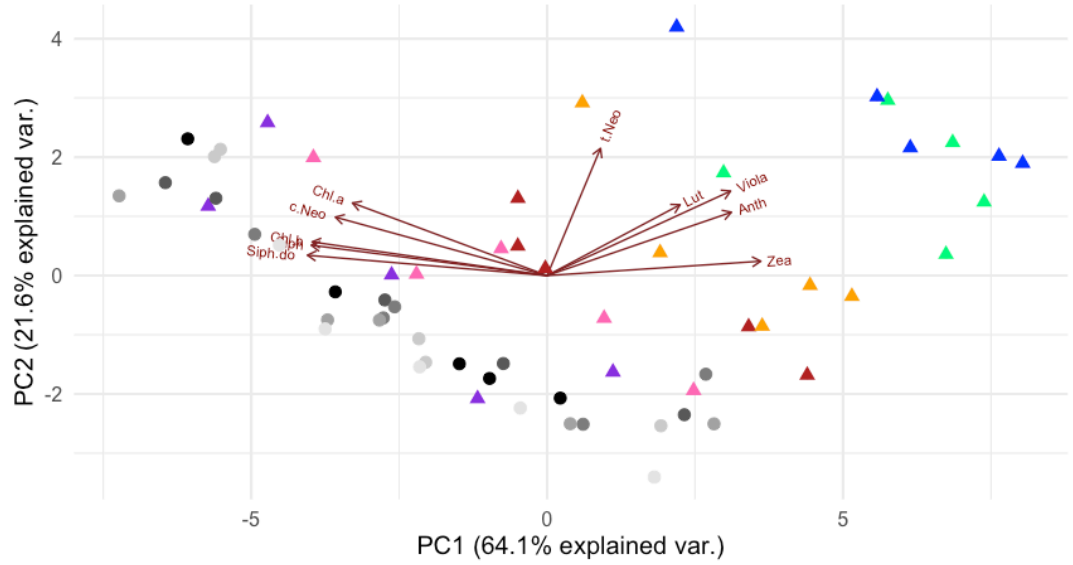


Fig. 5.9 Chl *a*:Chl *b* ratios in LLac and HLac *Codium tomentosum* (**a.**) and *Bryopsis plumosa* (**b.**), during 7 days of light acclimation. Mean ratios with standard deviation are reported. Asterisks indicate significant differences between LLac and HLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Finally, variation of the total pigment pool of *C. tomentosum* and *B. plumosa* was analysed with a multivariate approach. PCA plots gave similar results for both species (**Fig. 5.10**): LLac samples form a unique cluster, characterised by high values for main light harvesting pigments (Chl *a* and *b*, Siph, Siph-do) and c-Neo; on the contrary, HLac samples form separate clusters that gradually depart from the LLac one, with HLac day 0 being the closest and HLac day 7 the farthest. HLac clusters are characterised by higher values for xanthophylls (t-Neo, Viola, Anth, Zea; Lut for *C. tomentosum*). Notably, LLac clusters are also characterised by a relatively high variability, denoted by a relatively high dispersion of LLac points on both PC1 and PC2 axes.

a. *Codium tomentosum*



b. *Bryopsis plumosa*

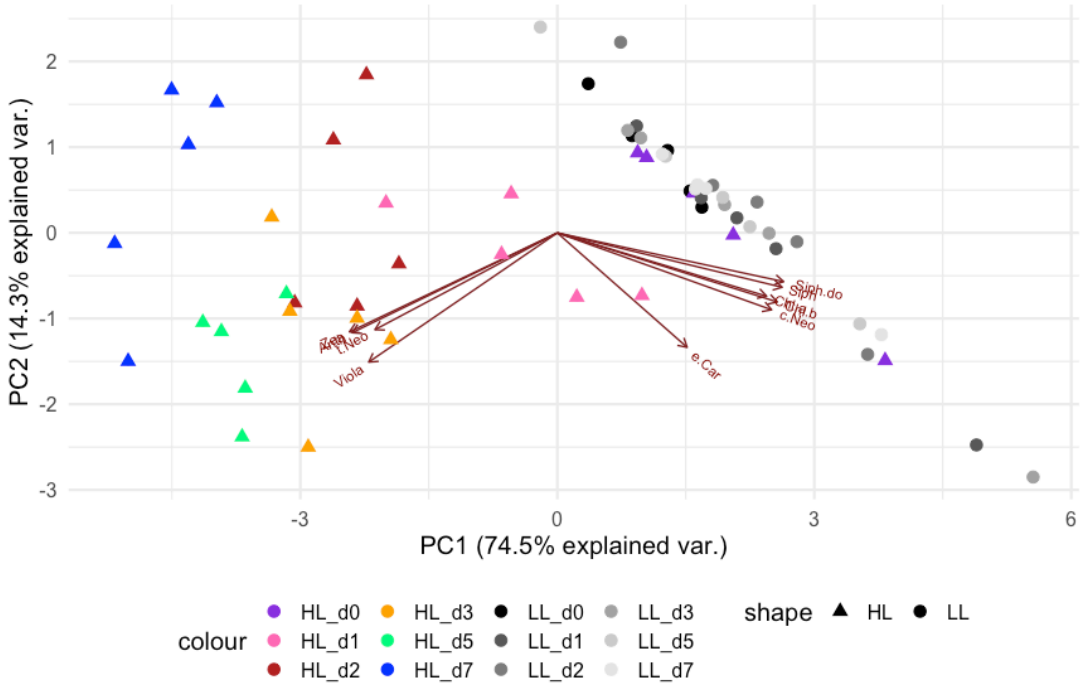


Fig. 5.10 Multivariate analysis (PCA) of LLac and HLac *Codium tomentosum* (**a.**) and *Bryopsis plumosa* (**b.**), during 7 days of light acclimation. In both cases, axes PC1 and PC2 explain most of the observed variability (> 85%). Data were analysed as Pigment concentration per dried weight. Colour and shape code are reported; contribution of each variable (pigment) is indicated with red arrows.

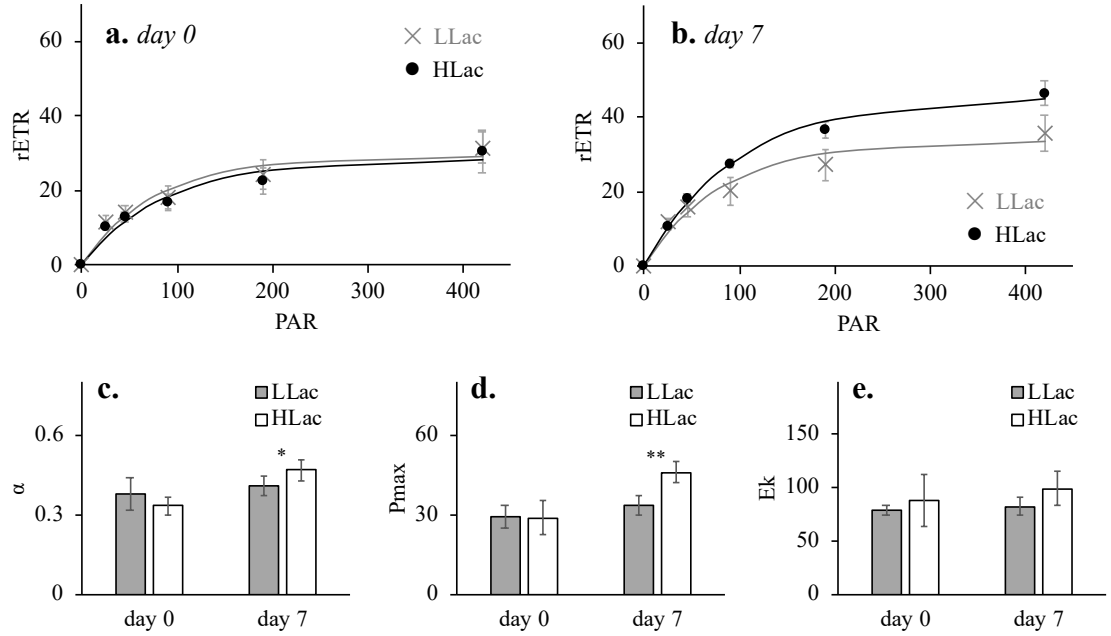
5.2 Acclimation state assessment with Steady State Light-response Curves

Steady State Light-response Curves (SSLC) were analysed for assessing the acclimation state of algal cultures (**Fig. 5.11**). *Codium tomentosum* showed a clear High Light acclimation trend after a week of exposure to 1000 µmol photons m^{-2} s^{-1}: while the LLac light curve is almost identical to the one at day 0, HLac light curve reaches an higher maximum rETR (rETRmax), indicative of High Light acclimation. HLac light curve also displays a steeper initial slope (α), meaning that despite an increase of rETRmax HLac cultures still maintained high photosynthetic efficiency under Low Light. t-tests between HLac and LLac resulted in significant differences of rETRmax ($p = 0.001$) and α ($p = 0.049$) at day 7, while no significant difference of Ek was reported.

On the contrary, *Briopsis plumosa* did not show a clearly different response between the two imposed light acclimation conditions. Indeed, an overall decrease in photosynthetic efficiency was observed: both LLac and HLac curves at day 7 are characterised by a drop of rETRmax compared to day 0. Multiple pairwise comparisons (t-test with Bonferroni adjustment) revealed that for all parameters day 0 was significantly different from day 7 (α, $p = 0.025$; rETRmax, $p = 0.001$; Ek, $p = 0.049$), but a significant difference between LLac and HLac cultures at day 7 was observed only for α ($p = 0.018$) and no significant differences were found for rETRmax and Ek. Moreover, the reported difference for α parameter at day 7 has to be attributed to a decrement under LLac conditions, rather than an increment for HLac.

Therefore, while *Codium tomentosum* showed a clear HLac/LLac response, the few differences observed in *Bryopsis plumosa* did not correspond to a high light acclimation pattern, and have to be attributed to changes that did not strictly depend on light treatment.

I. *Codium tomentosum*



II. *Bryopsis plumosa*

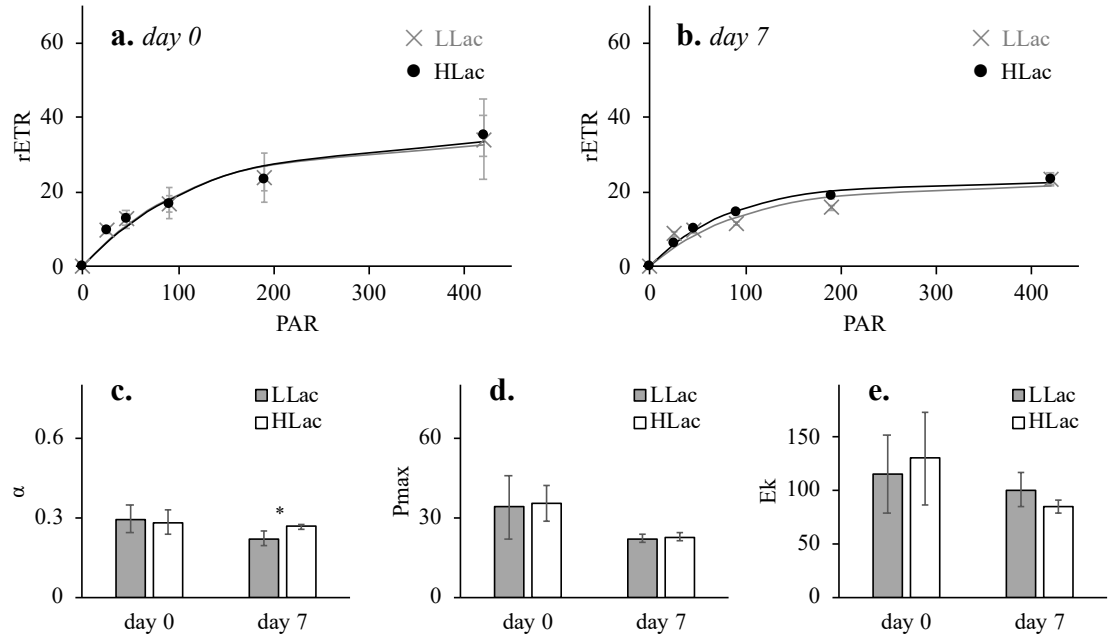


Fig. 5.11 Steady State Light-response Curves (SSLC) and related parameters from LLac/HLac *Codium tomentosum* (**I**) and *Bryopsis plumosa* (**II**). SSLC at day 0 (**a.**) and after 7 days of acclimation (**b.**) were fit using model by *Platt et al. 1980*; residuals of fit were always good ($r > 0.94$). Average points with standard deviation are reported (n=5, except n=4 for day 7 HLac *B. plumosa* where an obvious outlier was removed). Parameters α (**c.**), rETRmax (**d.**) and Ek (**e.**) with standard deviation are reported. Asterisks indicate significant differences between HLac and LLac (t-test): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5.3 Assessing photoprotective effectiveness of NPQ with Chl *a* fluorescence

For assessing photoprotective effectiveness of NPQ, a recent approach developed by *Ruban & Murchie, 2012* was applied (see **Section 4.4**). This analysis was based on the mathematical relationship between Non Photochemical Quenching (NPQ), quantum yield of Photosystem II (YII) and the photochemical quenching parameter (qP). This method allows the determination of the onset of photoinhibition, and is based on the assumption that qP corresponds to the photoprotective state of PSII reaction centres. Indeed, values for which qP is close to the theoretical line (qPd = qP th = 1) represent the maximum amplitude of NPQ at which all reaction centres remain protected. On the contrary, the moment when qPd deviates from the theoretical line (qPd ≠ qP th) represents the minimum light intensity at which photoprotective efforts are no longer sufficient and photoinhibition takes place. qPd can be plotted in parallel with YII, that follows the same pattern: experimentally measured YII is close to the theoretical calculated value (YII = YII th) as long as all reaction centres remain protected, and deviates (YII ≠ YII th) at the onset of photoinhibition.

Considering at first the results of *Codium tomentosum* (**Fig. 5.12**), no difference was observed in LLac cultures after 7 days of light acclimation; notably, calculated values of qP and YII immediately deviate from theoretical values, meaning that photoinhibition takes place already at the lowest light intensity (25 PAR). On the contrary, HLac cultures displayed a completely different response: while the pattern at day 0 correspond to what was observed for LLac, after 7 days of HLac qP and YII deviate from theoretical values only at a PAR value of 90, meaning that photoinhibition takes place only at higher light intensities. Notably, maximum YII and NPQ are sensibly lower compared to the one reached in LLac samples.

Similar results for LLac cultures and HLac cultures at day 0 were reported in *Bryopsis plumosa* (**Fig. 5.13**): qP and YII immediately deviate from theoretical values (25 PAR). However, HLac samples at day 7 present a different response if compared with *C. tomentosum*: while a similar reduction of maximum YII and NPQ was observed, qP and YII immediately deviate from theoretical values, as in LLac cultures and HLac cultures at day 0, meaning that photoinhibition already takes place at a light intensity of 25 PAR.

YII and NPQ measurements for both species are reported in **Fig. 5.14**. It is important to highlight that, despite a relatively long time of dark-adaptation (1h), YII in HLac samples remained low (0.51 ± 0.07 for *C. tomentosum* and 0.40 ± 0.05 for *B. plumosa*) at the onset of assessment of photoprotective effectiveness of NPQ. Implications of this phenomenon will be discussed in **Chapter 6**.

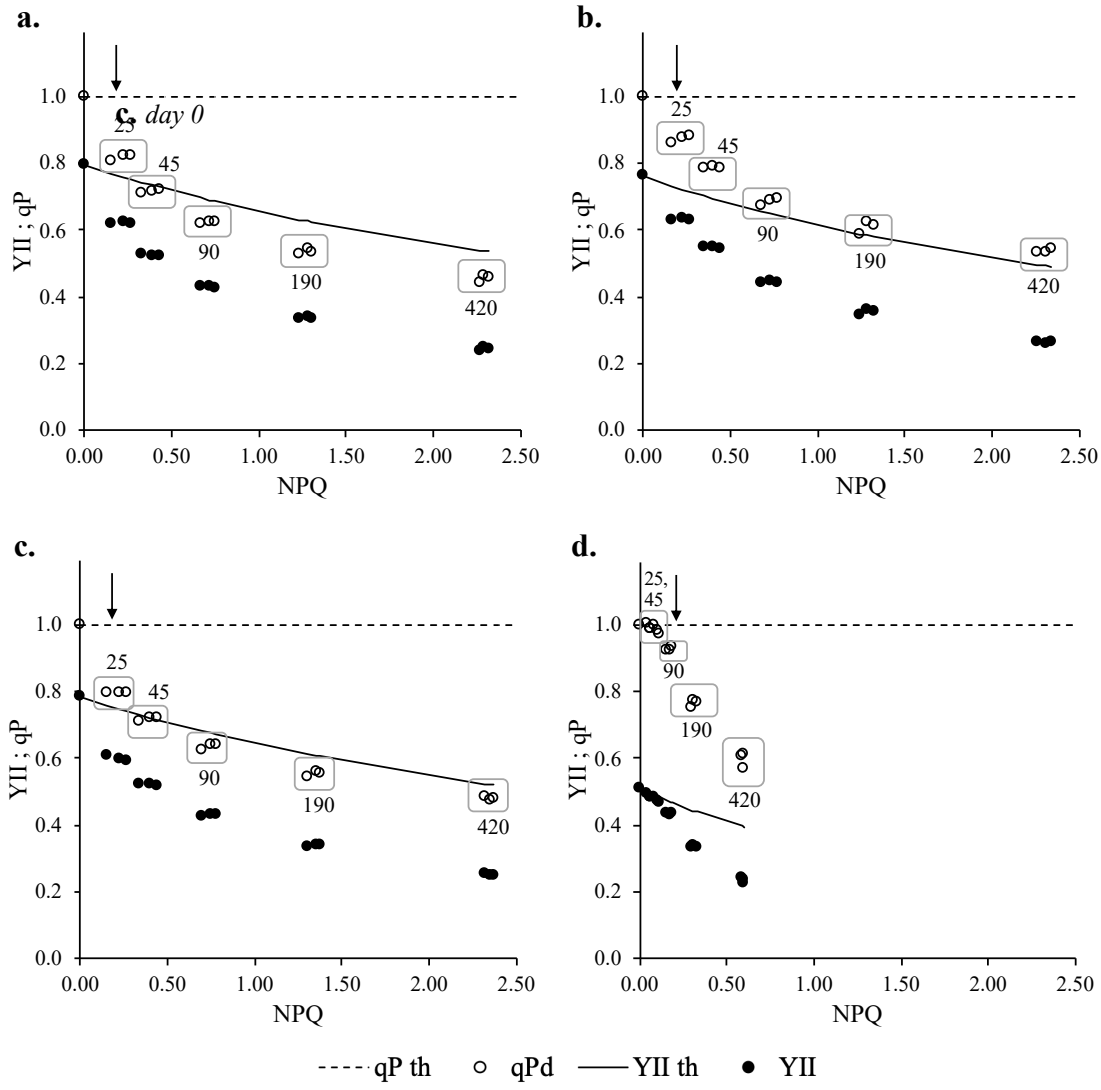


Fig. 5.12 Photoprotective NPQ (pNPQ) assessment of LLac and HLac *Codium tomentosum*, at day 0 and after 7 days of light acclimation. **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Plots represent the relationship between PSII quantum efficiency (YII) and photochemical quenching measured under far red light (qPd) in function of NPQ. Grey boxes enclose data points that correspond to each illumination phase (AL intensity in PAR is indicated by numbers below the boxes); black vertical arrows indicate the onset of photoinhibition. For the sake of graphic clarity standard deviation was not plotted, but was relatively low in all cases.

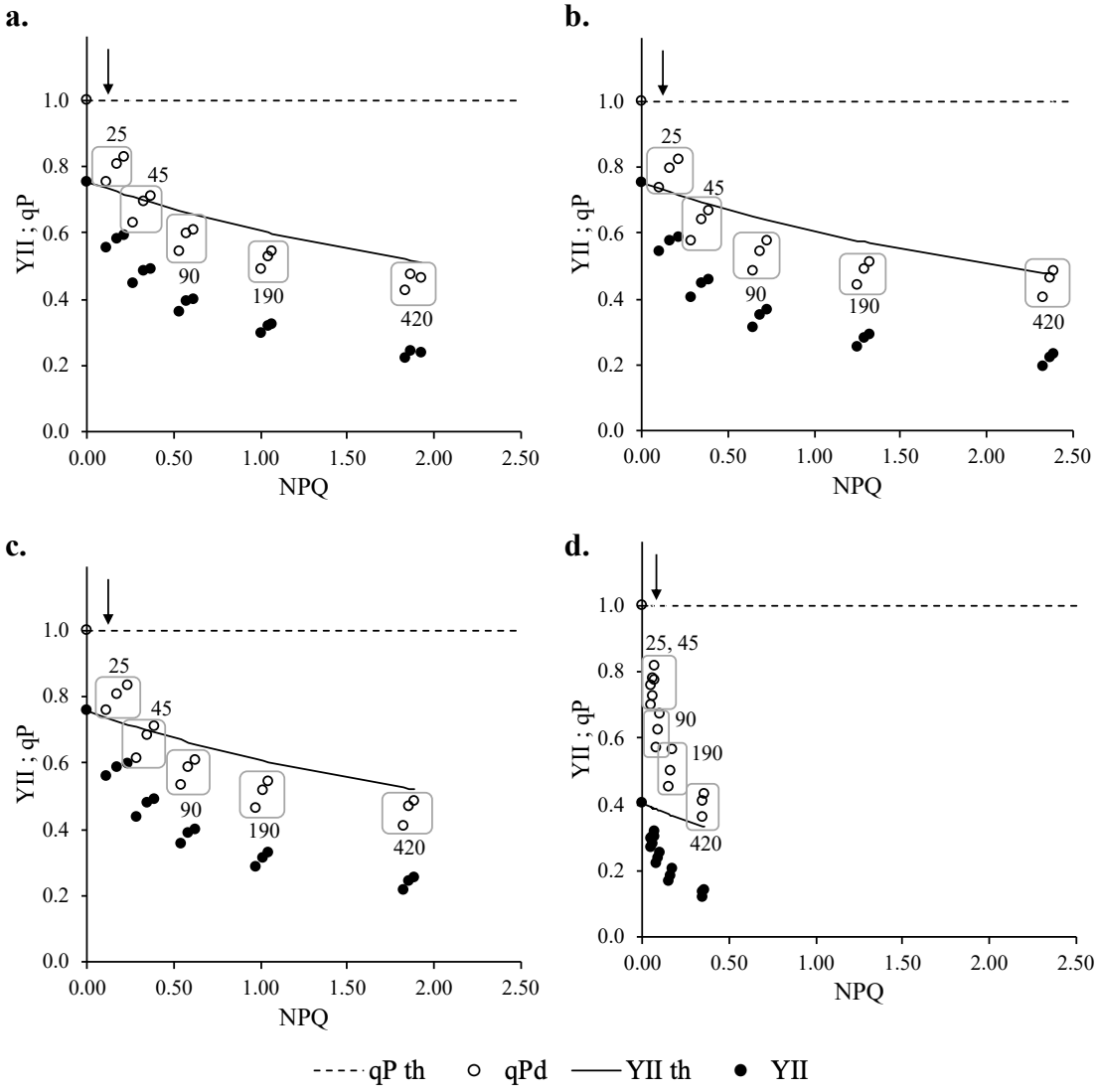


Fig. 5.13 Photoprotective NPQ (pNPQ) assessment of LLac and HLac *Bryopsis plumosa*, at day 0 and after 7 days of light acclimation. **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Plots represent the relationship between PSII quantum efficiency (YII) and photochemical quenching measured under far red light (qPd) in function of NPQ. Grey boxes enclose data points that correspond to each illumination phase (AL intensity in PAR is indicated by numbers below the boxes); black vertical arrows indicate the onset of photoinhibition. For the sake of graphic clarity standard deviation was not plotted, but was relatively low in all cases.

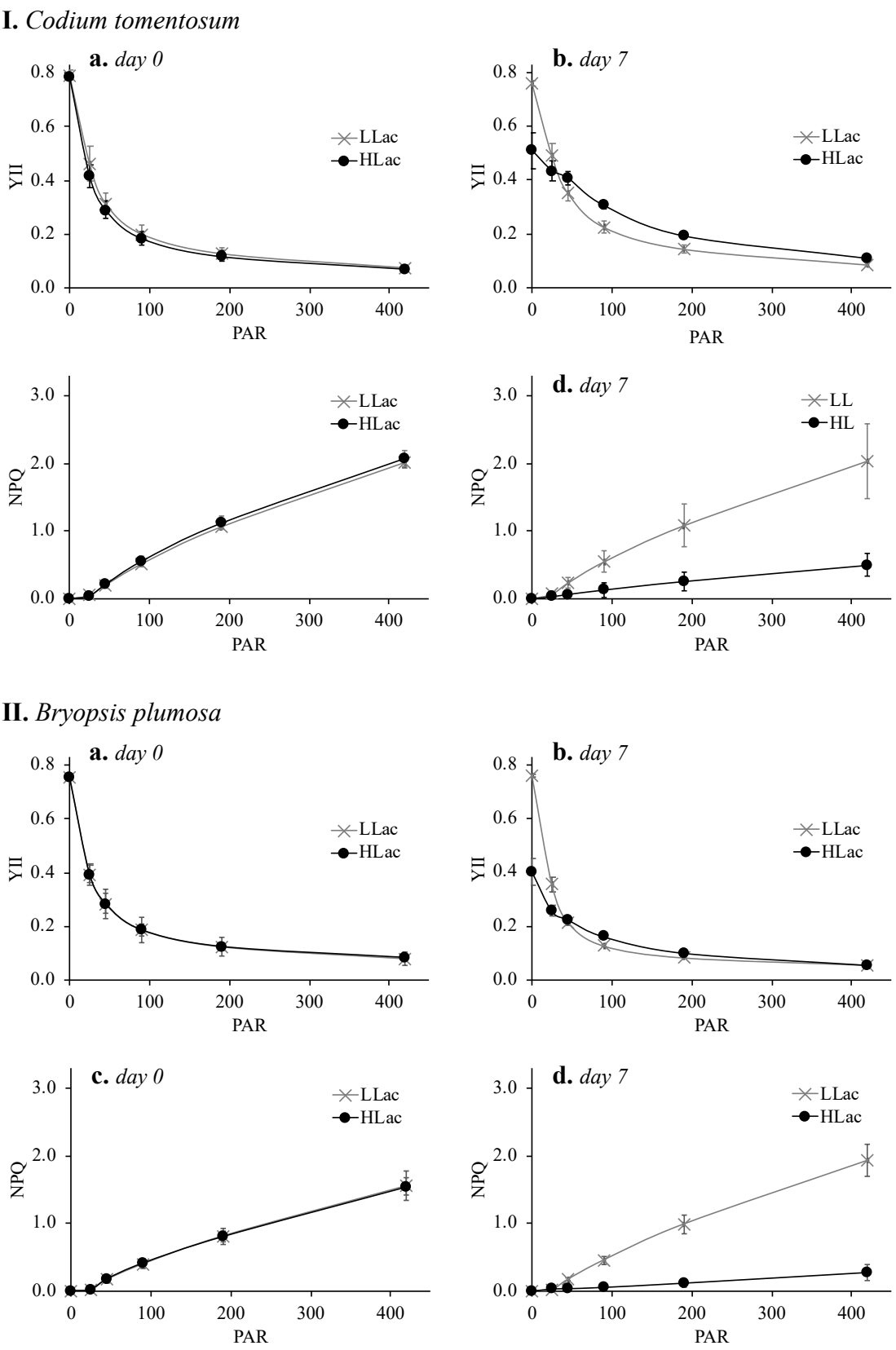


Fig. 5.14 YII and NPQ plots from SSLC of LLac and HLac *Codium tomentosum* (**I**) and *Bryopsis plumosa* (**II**). Plots represent YII and NPQ in function of PAR at day 0 (**a.**; **c.**) and day 7 (**b.**; **d.**); average points with standard deviation are reported (n=5).

Due to theoretical issues linked to the pNPQ method and its application on Bryopsidales algae (further discussed in **Chapter 6.**) photoprotective capacity was also evaluated with complementary quantum yield parameters: YII, Y(NPQ), and Y(NO). As explained by *Klughammer & Schreiber, 2008*, these parameters describe the fate of excitation energy in PSII and allow deep insights into the plant's capacity to cope with excess of energy. In particular, YII (quantum yield of PSII) corresponds to the fraction of excitation energy chemically converted in PSII; the remaining fraction (energy loss) is composed by Y(NPQ) (quantum yield of regulated non-photochemical energy loss in PSII) and Y(NO) (quantum yield of non-regulated non-photochemical energy loss in PSII), and reflects the photosynthetic performance of the target organism. The difference between Y(NPQ) and Y(NO) is characterised by the way excess of energy is dissipated: while Y(NPQ) relies on finely regulated NPQ mechanisms, Y(NO) corresponds to the energy passively dissipated as heat and fluorescence, therefore representing the organism inability of protecting itself against photodamage. For this reason, successful energy regulation is generally characterised by high YII values and minimum energy loss (Yloss), with high Y(NPQ)/Y(NO) ratio.

Complementary quantum yield parameters and Y(NPQ)/Y(NO) ratios for *Codium tomentosum* (**Fig. 5.15**) and *Bryopsis plumosa* (**Fig 5.16**) are reported. Both species displayed similar trends.

First of all, at day 0 and LLac cultures at day 7 high YII with low energy loss was observed in the dark. Under increasing light, YII dropped and Yloss increased; at the beginning, Y(NPQ)/Y(NO) ratio was quite low, but at higher light intensities an increase of Y(NPQ) was observed, with a final high Y(NPQ)/Y(NO) ratio. On the contrary, HLac cultures at day 7 showed low YII in the dark, with high value of Yloss; under increasing light intensity steps Y(NO) always represented the main portion of Yloss, with relatively low Y(NPQ)/Y(NO) ratios, meaning that HLac algae display suboptimal capacity of photoprotective reaction, with high passive energy losses.

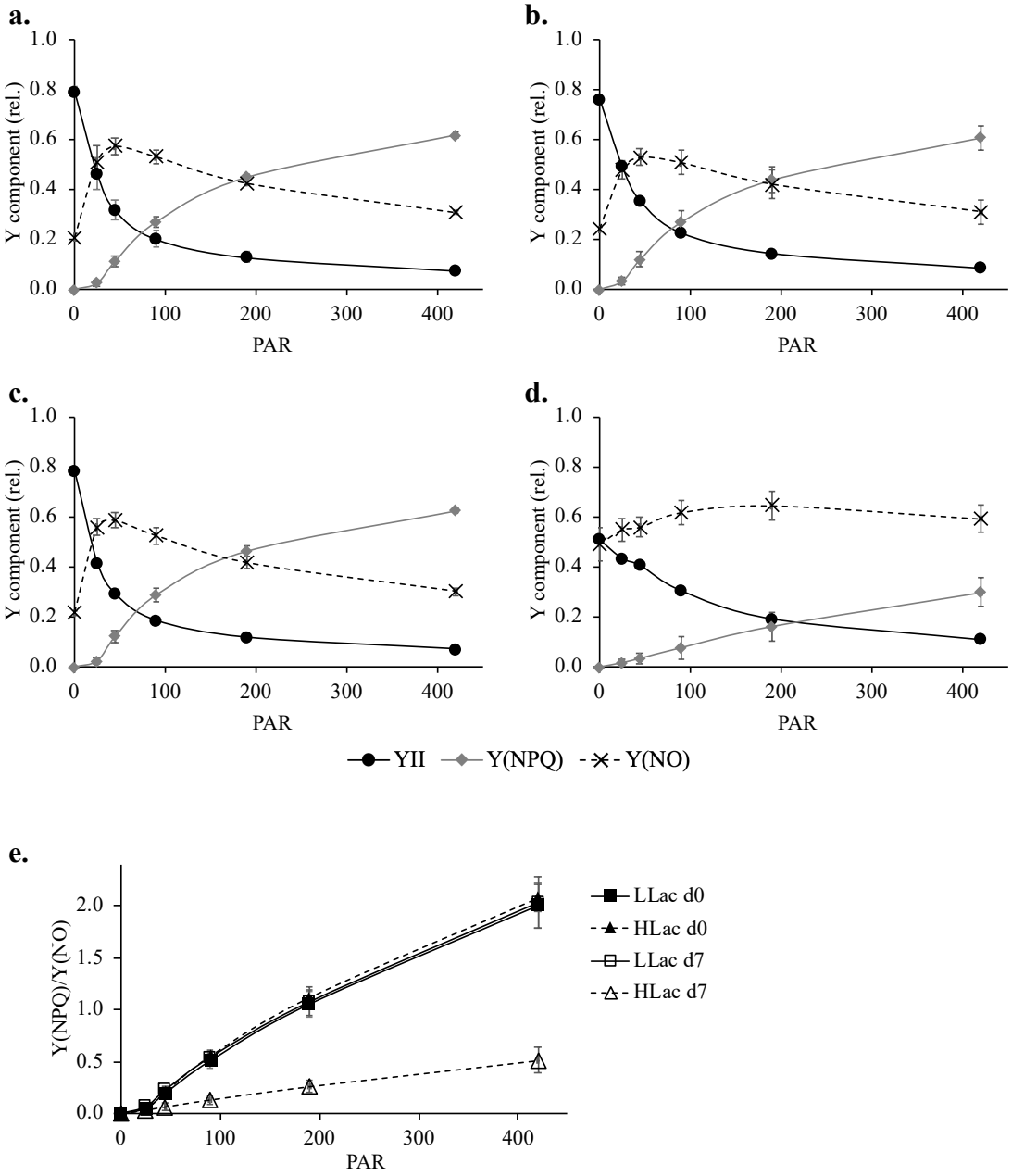


Fig. 5.15 Light intensity response curves of complementary quantum yields of LLac and HLac *Codium tomentosum*. (**a-d.**) relationship between YII, Y(NPQ) and Y(NO) in function of PAR; **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Key is reported at the bottom. (**e.**) Y(NPQ)/YNO ratio. Average points with standard deviation are reported (n=5).

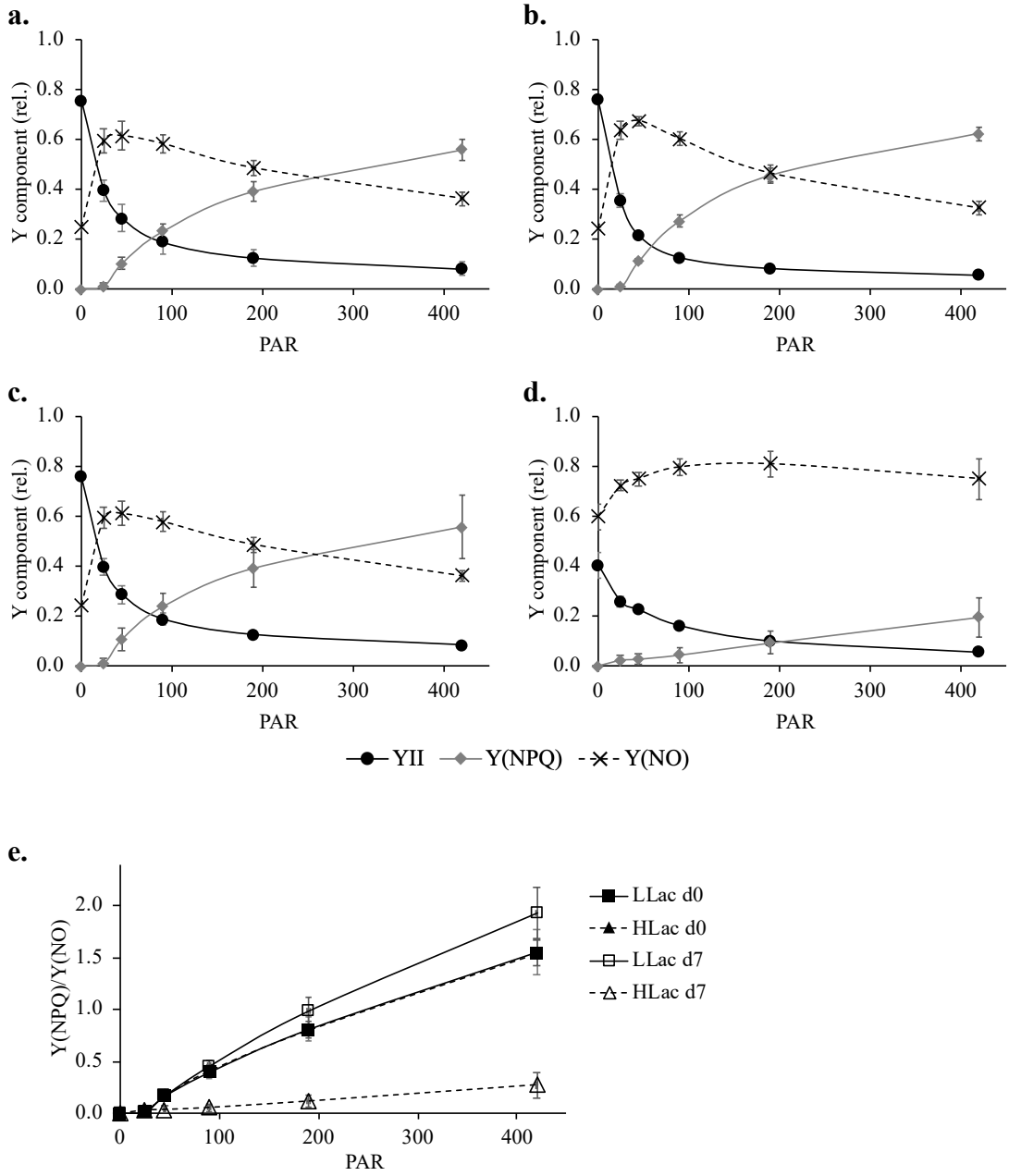


Fig. 5.16 Light intensity response curves of complementary quantum yields of LLac and HLac *Bryopsis plumosa*. (**a-d.**) relationship between YII, Y(NPQ) and Y(NO) in function of PAR; **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Key is reported at the bottom. (**e.**) Y(NPQ)/YNO ratio. Average points with standard deviation are reported (n=5).

5.4 Second round: validation of previous results

As mentioned in material and methods (**Section 4.5**) the 7 days Low Light/High Light acclimation was repeated for achieving a new set of LLac and HLac cultures for further experiments. Results of HPLC and fluorescence analysis on this new set of cultures are reported in the present section.

Pigment quantification confirmed what was previously reported (**Fig 5.17**): both species presented significant decrement of light harvesting pigments (Chl *b*, Siph/Siph-do; Chl *a* only in *Bryopsis plumosa*) and alterations of carotenoid composition (significant accumulation of t-Neo, Viola, Anth, Zea, Lut and ß-Car). Only *B. plumosa* showed a significant decrement of c-Neo and increment of ε-Car. Detected pigments with related parameters are reported in **Tab. 5.4**.

Pigment	*Abbreviation*	*Retention time (min)*	λ_{max} *(nm)*
Siphonaxanthin	Siph	10.40 ± 0.06	448
all-trans-Neoxanthin	t-Neo	11.41 ± 0.06	418; 442; 471
9'-cis-Neoxanthin	c-Neo	11.98 ± 0.05	413; 437; 476
Violaxanthin	Viola	13.06 ± 0.06	416; 441; 471
Antheraxanthin	Anth	14.93 ± 0.07	426; 445; 474
Lutein *	Lut	16.59 ± 0.07	423; 446; 475
Zeaxanthin	Zea	16.79 ± 0.07	453; 479
Siphonaxanthin dodecenoate	Siph-do	18.52 ± 0.07	455
Chlorophyll *b*	Chl *b*	22.30 ± 0.09	458; 597; 645
Chlorophyll *a*	Chl a	23.95 ± 0.07	430; 617; 662
ε-Carotene	ε-Car	27.86 ± 0.08	419; 441; 471
α-Carotene	α-Car	28.04 ± 0.08	448; 476
β-Carotene	β-Car	28.25 ± 0.08	452; 479

Tab. 5.4 Pigment content of *Codium tomentosum* and *Bryopsis plumosa* detected with HPLC (validation of initial results). Pigments names, abbreviation, mean retention time (with standard deviation) and mean absorption maxima (λ_{max}) are reported; standard deviation for λ_{max} was always low (between 0 and 2). *In this second experiment Lut was detected in 7 days HLac cultures of both species. Although, in *B. plumosa* Lut signal was always close to the detection limit; therefore, conclusions on the presence of this pigment must be taken with caution.

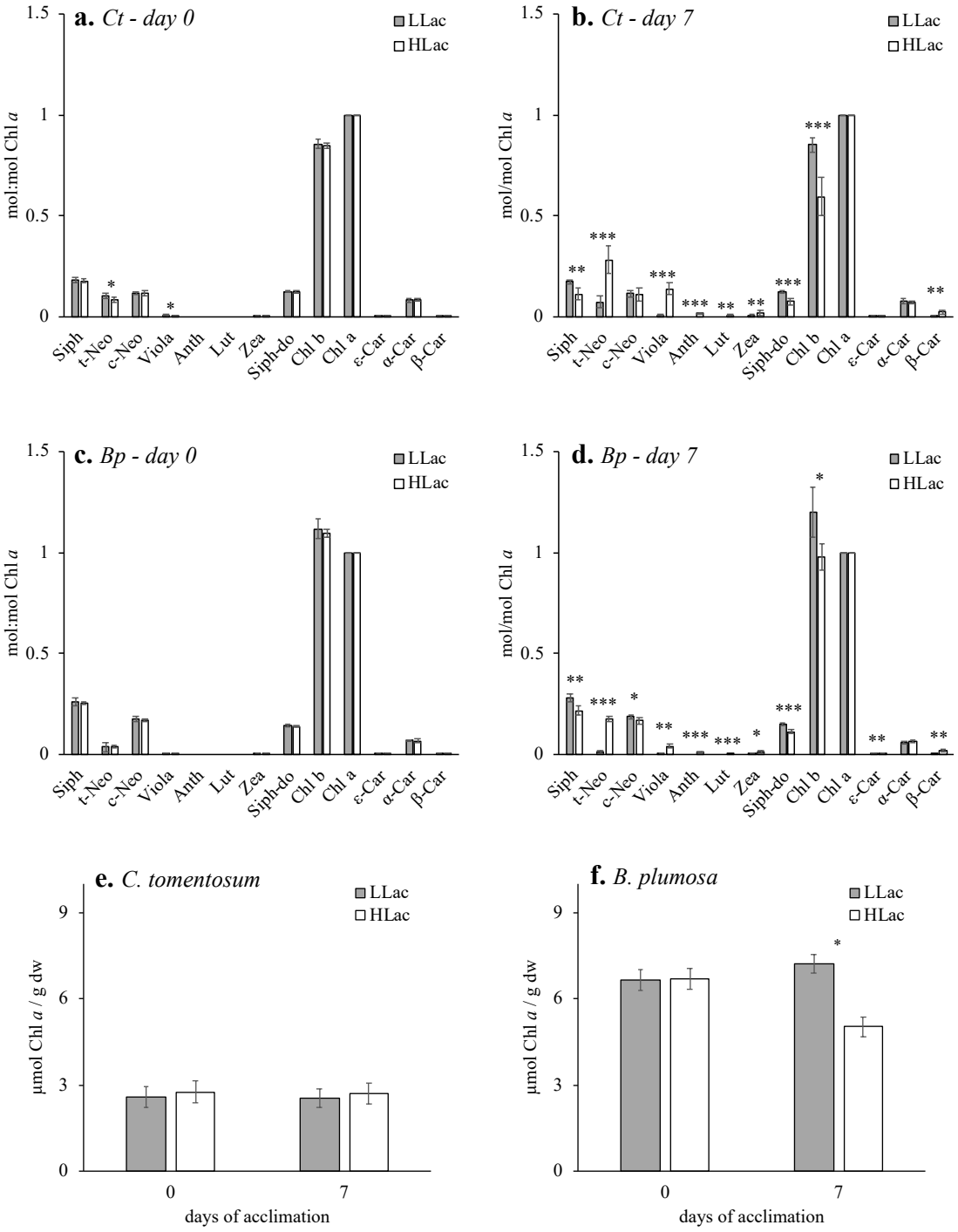


Fig. 5.17 Pigment content of LLac and HLac acclimated *Codium tomentosum* and *Bryopsis plumosa* (validation of initial results). (**a-d.**) Mean Pigment:Chl *a* ratios of *C. tomentosum* (*Ct*, **a.** day 0; **b.** day 7) and *B. plumosa* (*Bp,* **c.** day 0; **d.** day 7). (**e-f.**) Chlorophyll *a* concentration per dried weight of *C. tomentosum* (**e.**) and *B. plumosa* (**f.**). Mean values with standard deviation are reported. Asterisks indicate significant differences between LLac and HLac (t-tests): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. An obvious outlier for Zea was removed from 7 days HLac *B. plumosa*.

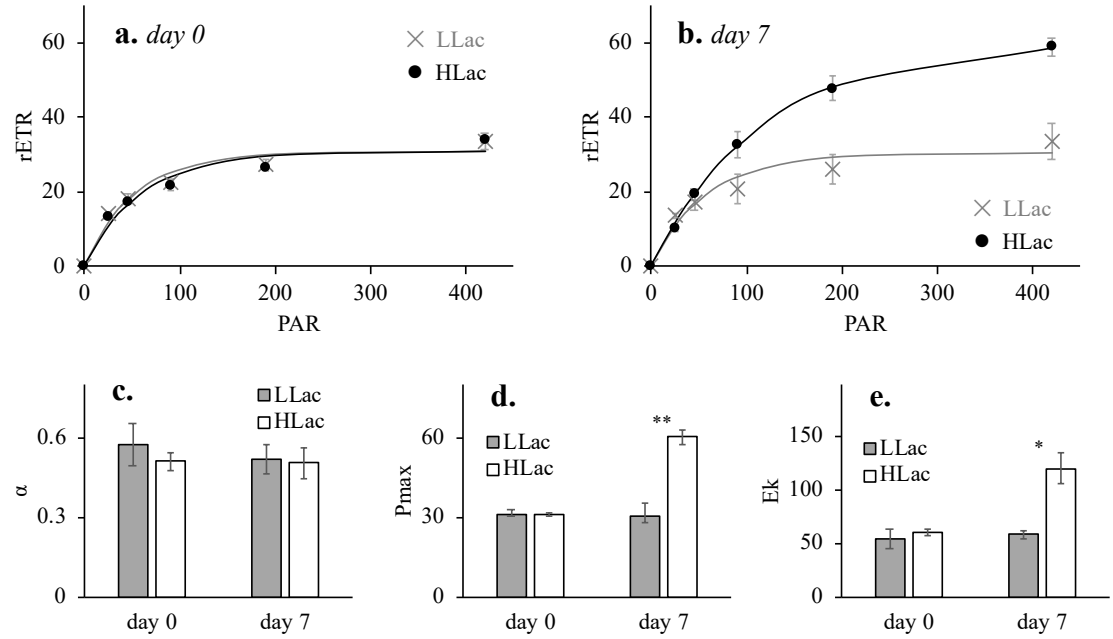
Note that in *Codium tomentosum* a significant difference between HLac and LLac cultures at day 0 was detected, with HLac having slightly significant lower amount of t-Neo ($p = 0.047$) and Viola ($p = 0.012$). Despite this fact, HLac cultures at day 7 accumulated a significantly higher amount of t-Neo ($p = 0.0004$) and Viola ($p = 0.0002$). Therefore, the natural difference present at day 0 should not have exercised a determinant effect on the experiment.

Overall, also fluorescence analysis confirmed what previously reported. Although, some differences in the results are present between the two experiments, and will be described as follows. Note that while for HPLC analysis the same number of replicates of the previous experiment (5) was maintained, for PAM analysis it was reduced to 3, as mentioned in Materials and Methods (**Section 4.6**).

For what concerns Steady State Light Curves, *Codium tomentosum* showed a clear High Like acclimation response, like in the previous experiment; this time, not only a difference in rETRmax but also a significant increment of Ek was reported in 7 days HLac cultures, while α showed no significant variation, meaning that HLac cultures acclimated efficiently to High Light intensities, but still maintained a high photosynthetic efficiency under Low Light. This result confirmed an even more decisive capacity of *Codium tomentosum* to acclimate at the extreme high light intensity of 1000 µmol photons m^{-2} s^{-1} after 7 days of exposure.

On the contrary, *Bryopsis plumosa* did not show a clear High Light acclimation response: no difference between light curves of the two acclimation conditions were reported; multiple comparison pairwise t-test (with Bonferroni correction) revealed a statistically significant difference between day 0 and day 7 for α and Ek ($p = 0.036$ and $p = 0.019$ respectively) but no significant difference emerged with direct comparison (two sample t-test) between HLac and LLac at any time point. The only significant difference was observed for Ek between at day 0 and day 7 HLac cultures ($p = 0.038$). Therefore, the observed difference was not enough for stating the presence of a clear High Light acclimation event in *B. plumosa*. Notably, in this experiment both HLac and LLac cultures after 7 days did not show a drastic decrement of photosynthetic efficiency, that was indeed reported in the previous experiment.

I. *Codium tomentosum*



II. *Bryopsis plumosa*

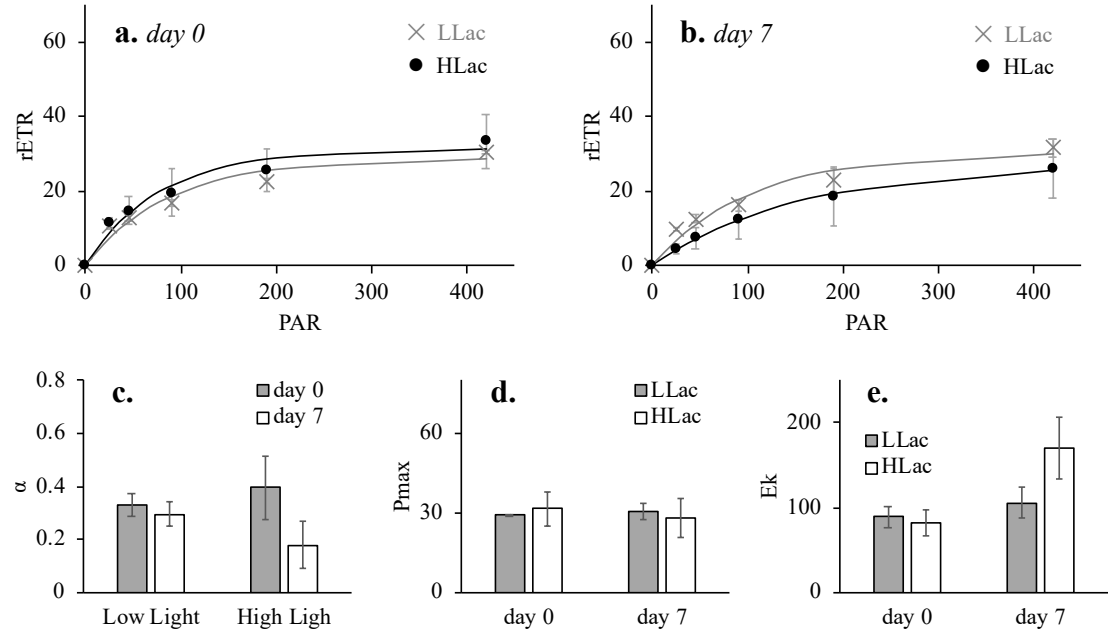


Fig. 5.18 Steady State Light Curves (SSLC) and related parameters from LLac/HLac *Codium tomentosum* (**I**) and *Bryopsis plumosa* (**II**) (validation of initial results). SSLC at day 0 (**a.**) and after 7 days of acclimation (**b.**) were fit using model by *Platt et al. 1980*; residuals of fit were always good ($r > 0.95$). Parameters α (**c.**), rETRmax (**d.**) and Ek (**e.**). Average points with standard deviation are reported (n=3). Asterisks indicate significant differences between HLac and LLac (t-test): * $p < 0.05$; ** $p < 0.01$.

Assessment photoprotective effectiveness of NPQ with pNPQ protocol (**Fig. 5.19-20**) confirmed the results reported the previous experiment. 7 days HLac *Codium tomentosum* showed a different response if compared with day 0 and LLac cultures: qP and YII deviated from theoretical values only at higher PAR, meaning that photoinhibition took place at higher light intensities, compared to what observed in LLac cultures. Interestingly, qP and YII deviated from the theoretical values at an intensity of 190 PAR, two times higher if compared to the one of the previous experiment (90 PAR); therefore, this result is coherent with what stated after the analysis of SSLC: in this second experiment *Codium tomentosum* seemed to display an even more decisive capacity of acclimating to the extreme high light intensity of 1000 µmol photons m^{-2} s^{-1}. As happened during the previous experiment, this response was not observed in *Bryopsis plumosa*.

Although, it has to be noted that both species presented a sensible decrease of maximum YII and NPQ after 7 days of High Light acclimation, particularly evident in *Bryopsis plumosa*, as in the previous experiment. Moreover, complementary quantum yield analysis (**Fig. 5.21**) confirmed previously reported results: contrarily to what observed in LLac, HLac cultures at day 7 showed low YII in the dark, with high value of Yloss; under increasing light intensity steps Y(NO) always represented the main portion of Yloss, with relatively low Y(NPQ)/Y(NO) ratios, meaning that HLac algae displayed suboptimal capacity of photoprotective reaction, with high passive energy losses, particularly high in *Bryopsis plumosa*.

The relationship between pNPQ protocol results and complementary quantum yield analysis and their relative implications will be further discussed in **Chapter 6**.

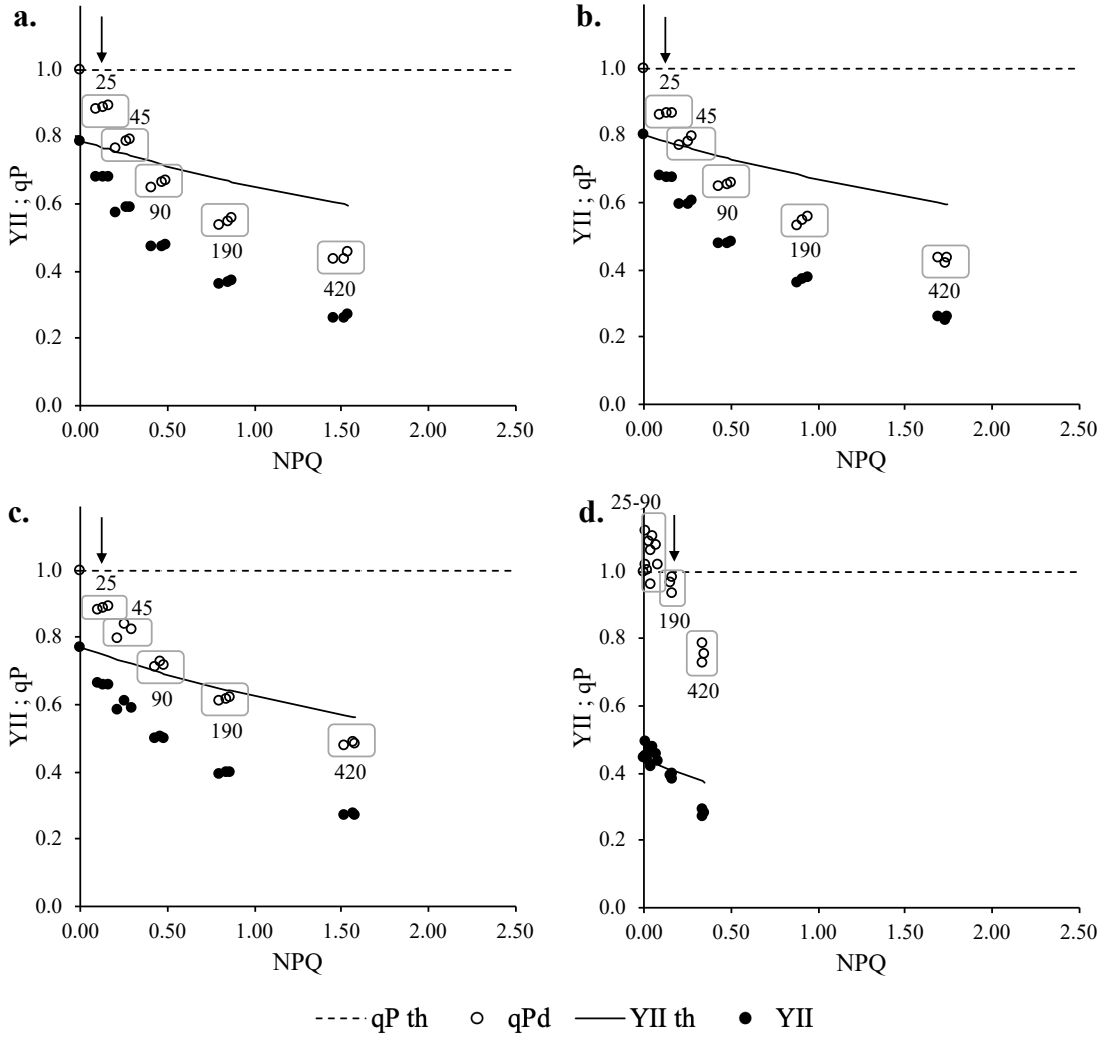


Fig. 5.19 Photoprotective NPQ (pNPQ) assessment of LLac and HLac *Codium tomentosum*, at day 0 and after 7 days of light acclimation (validation of initial results). **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Plots represent the relationship between PSII quantum efficiency (YII) and photochemical quenching measured under far red light (qPd) in function of NPQ. Grey boxes enclose data points that correspond to the each illumination phase (AL intensity in PAR is indicated by numbers below the boxes); black vertical arrows indicate the onset of photoinhibition. For the sake of graphic clarity standard deviation was not plotted, but was relatively low in all cases.

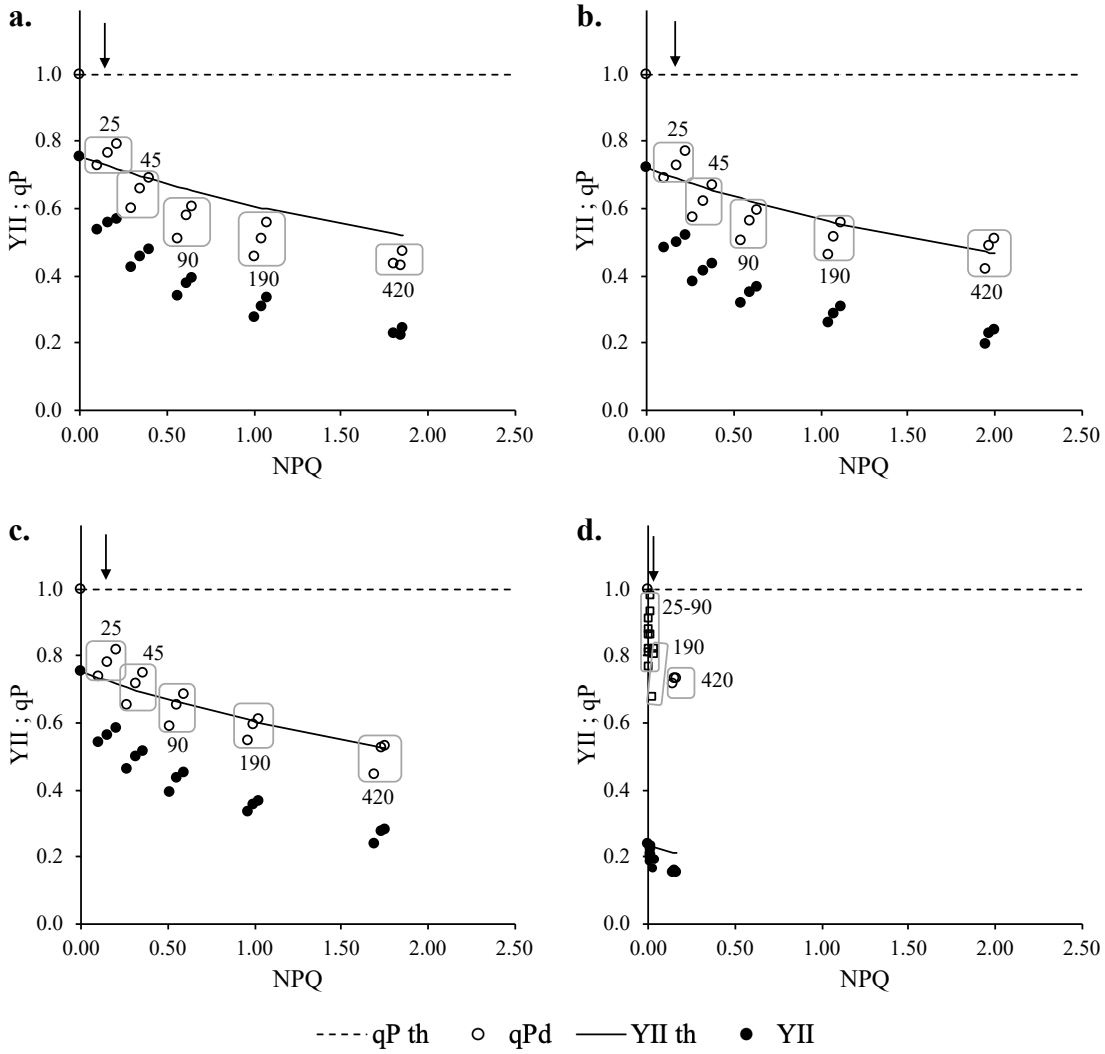


Fig. 5.20 Photoprotective NPQ (pNPQ) assessment of LLac and HLac *Bryopsis plumosa*, at day 0 and after 7 days of light acclimation (validation of initial results). **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Plots represent the relationship between PSII quantum efficiency (YII) and photochemical quenching measured under far red light (qPd) in function of NPQ. Grey boxes enclose data points that correspond to the each illumination phase (AL intensity in PAR is indicated by numbers below the boxes); black vertical arrows indicate the onset of photoinhibition. For the sake of graphic clarity standard deviation was not plotted, but was relatively low in all cases.

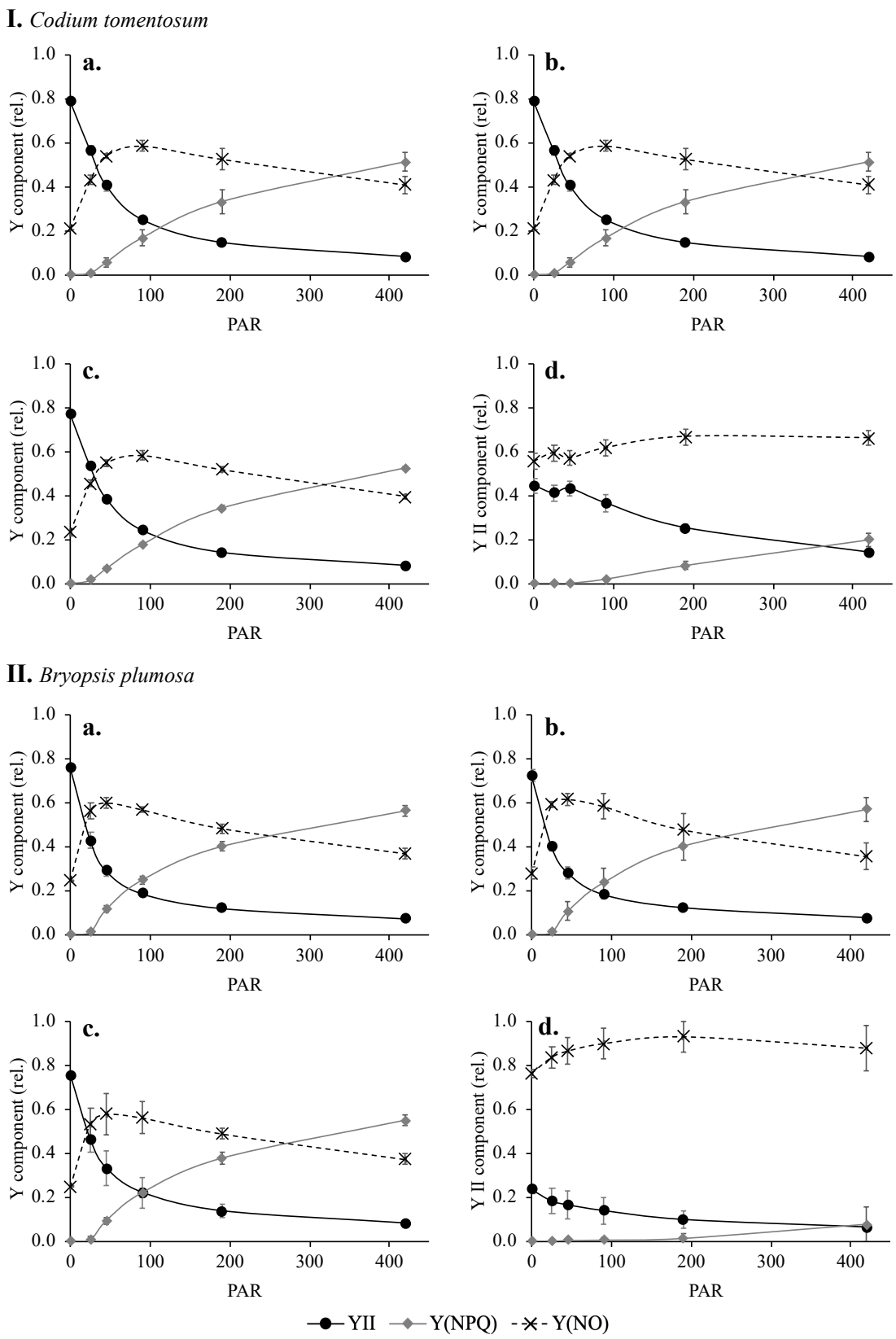


Fig. 5.21 Light intensity response curves of complementary quantum yields of LLac and HLac *Codium tomentosum* (**I**) and *Bryopsis plumosa* (**II**) (validation of initial results). **a.** LLac at day 0; **b.** LLac at day 7; **c.** HLac at day 0; **d.** HLac at day 7. Average points with standard deviation are reported.

5.5 Seeking for signs of a xanthophyll cycle

As mentioned in Materials and Methods (**Section 4.5**) during the second acclimation experiment pigment samples at day 7 were taken at 3 different time points: immediately after the night (6:00), at late morning (10:00, normal sampling time) and at the end of the light period (21:00), for assessing the presence/absence of a xanthophyll cycle. Differences in Pigment content between HLac and LLac cultures before and after the week of acclimation were analysed above (**Fig 5.17**). First of all, no evidences of the conventional VAZ cycle (Viola, Ant, Zea) were reported in both species (**Fig. 5.22**).

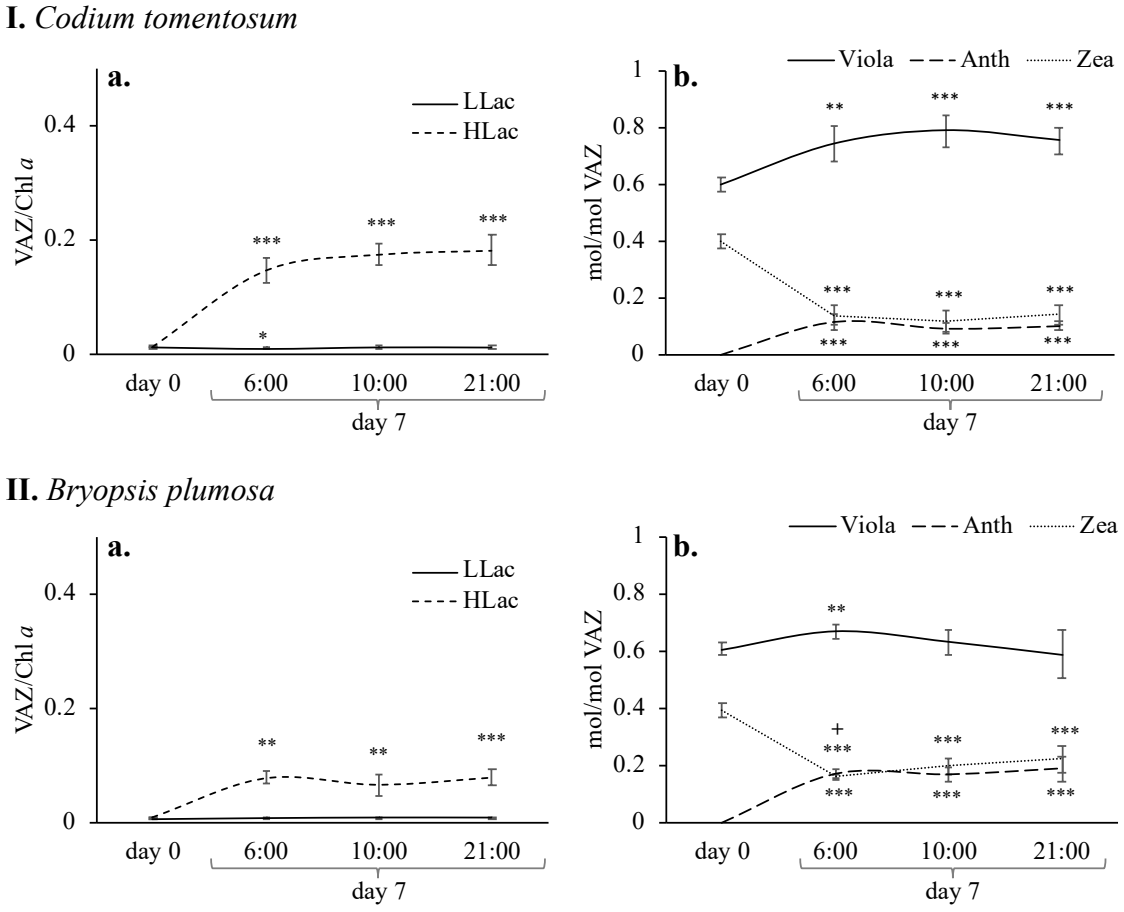


Fig. 5.22 Mean VAZ (Viola, Anth, Zea) pool size and Viola:VAZ, Anth:VAZ, Zea:VAZ ratios from LLac and HLac *Codium tomentosum* (**I.**) and *Bryopsis plumosa* (**II.**). (**a.**) VAZ:Chl *a* ratios in LLac and HLac cultures; (**b.**) Pigment:VAZ ratios in HLac cultures. Values at day 0 and day 7 (h6:00, h10:00, h21:00) with standard deviation are reported. Statistical significance (t-test): */+ $p < 0.05$; **/++ $p < 0.01$; ***/+++ $p < 0.001$. Meaning of statistical significance code: +, day 7 value at h 6:00 or at h 10:00 is significantly different from h 21:00; *, value is significantly different from day 0.

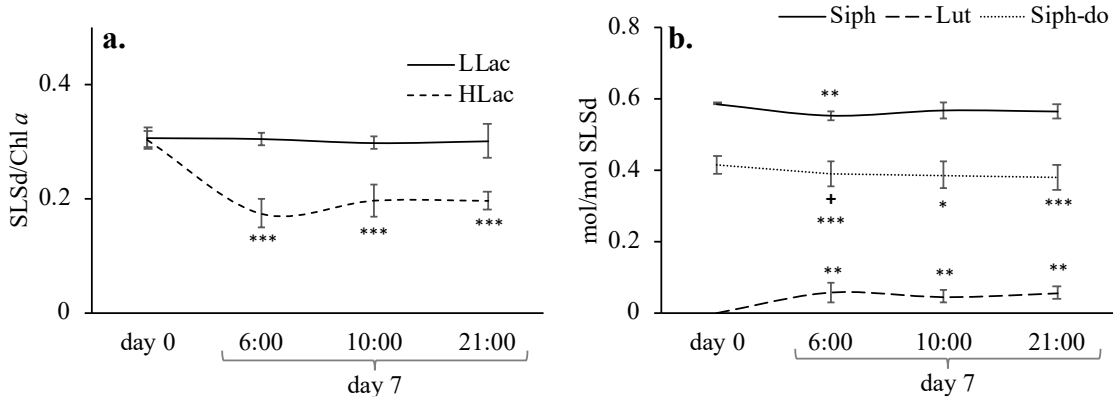
In particular, expected significant differences in Viola:VAZ, Anth:VAZ and Zea:VAZ ratios between day 0 and day 7 HLac cultures were present; however, no significant differences between day 7 samples collected at the beginning of the day (h6:00), at late morning (h10:00) and at the end of the day (h21:00) were reported, with the exception of a single and not highly significant difference between Zea:VAZ ratio at h6:00 and h21:00 in HLac *Bryopsis plumosa*. Comprehensively, these results suggest that an evident trend of interconversion from Viola to Ant/Zea was absent in both target species.

Moreover, the presence of the siphonaxanthin cycle, involving an interconversion between Siph and Lut (SL), was also investigated. As reported for VAZ, no evidence of SL cycling was observed in both *Codium tomentosum* and *Bryopsis plumosa* (**Fig. 5.22**). Indeed, no significant variations between S:SLSd and L:SLSd ratios at different time points were observed in HLac cultures, while a significant difference between day 0 and day 7 was always present.

As expected, in both species the total VAZ (**Fig 5.22.a**) and SLSd (**Fig 5.23.a**) pool size varied significantly between day 0 and day 7, but remained constant during the day (day 7: h6:00, h10:00, h21:00). In general, coherently with the already discussed pigment accumulation results (**Section 5.1**) HLac determined a significant increase of VAZ pool size and an opposite significant decrease of SLSd pool size. Indeed, despite Lut is accumulated in HLac cultures, its amount in Bryopsidales algae is significantly lower compared with those of Siph and Siph-do, additional light harvesting pigments that decrease when the algae acclimate to High Light.

Globally, the total VAZ pool size was observed to be higher in HLac *C. tomentosum* (VAZ:Chl *a* ratio around 0.2), compared with *B. plumosa* (VAZ:Chl *a* ratio around 0.1), while an opposite trend was observed for the total pool of Siph, Lut and Siph-do (SLSd:Chl *a* ratio around 0.2 in HLac *C. tomentosum*; 0.3 in *B. plumosa*).

I. *Codium tomentosum*



II. *Bryopsis plumosa*

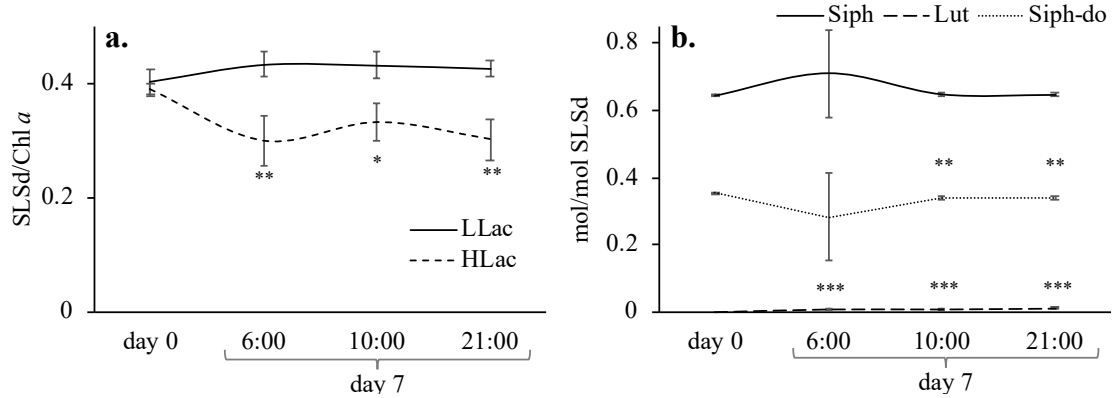


Fig. 5.23 Mean SLSd (Siph, Lut, Siph-do) pool size and Siph:SLSd, Lut:SLSd, Siph-do:SLSd ratios f from LLac and HLac *Codium tomentosum* (**I.**) and *Bryopsis plumosa* (**II.**). (**a.**) SLSd:Chl *a* ratios in LLac and HLac cultures; (**b.**) Pigment:SLSd ratios in HLac cultures. Values at day 0 and day 7 (h6:00, h10:00, h21:00) with standard deviation are reported. Statistical significance (t-test): */+ $p < 0.05$; **/++ $p < 0.01$; ***/+++ $p < 0.001$. Meaning of statistical significance code: +, day 7 value at h 6:00 or at h 10:00 is significantly different from h 21:00; *, value is significantly different from day 0.

Finally, due to the reported presence of specific accumulation of t-Neo and Viola in HLac Bryopsidales algae, signs of interconversion between these two pigments, possibly revealing the presence of a peculiar xanthophyll cycle, were investigated.

Also in this case, no signs of a clear xanthophyll interconversion was reported for both target species (**Fig. 5.24**): despite a significant increase of t-Neo+Viola (tNV) pool size between day 0 and day 7 under High Light acclimation, no significant differences in t-Neo:tNV and Viola:tNV ratios were reported between 7 days HLac cultures at different time of the day.

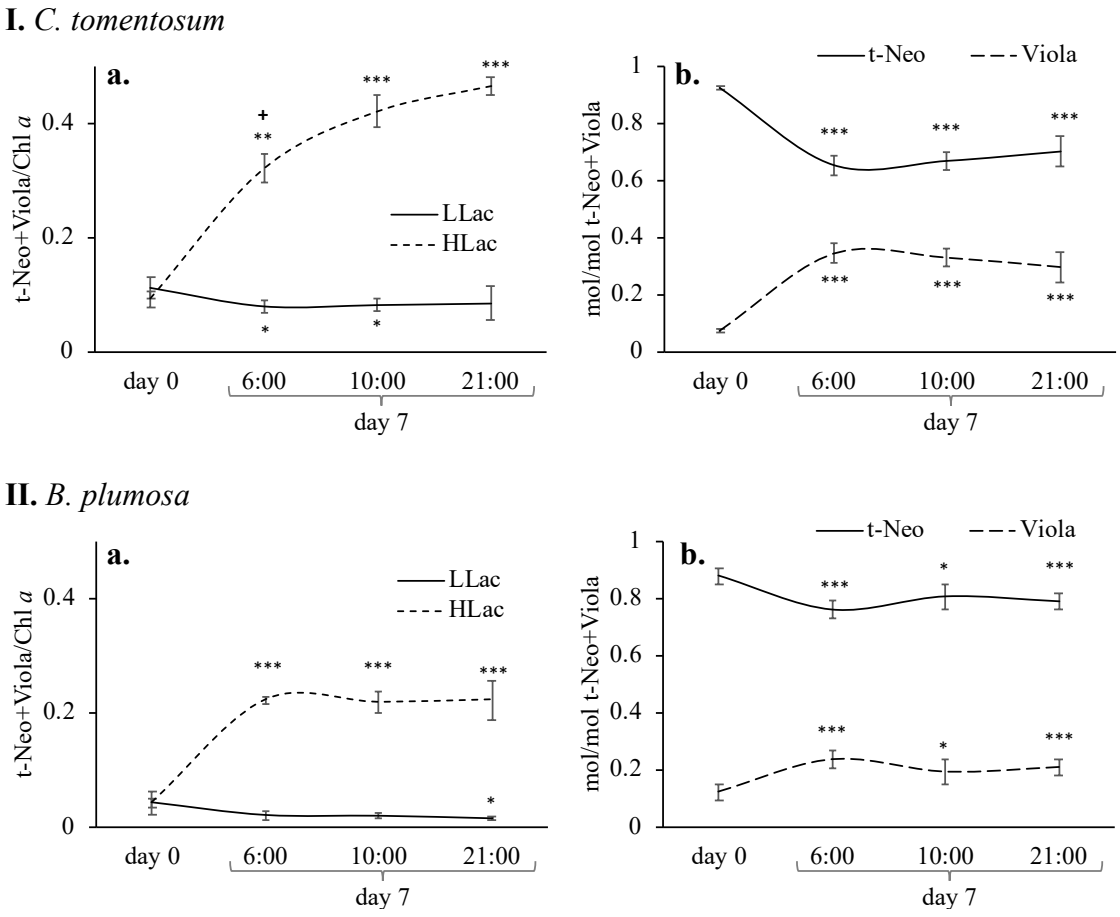


Fig. 5.24 Mean t-Neo+Viola pool size and t-Neo:t-Neo+Viola, Viola:t-Neo+Viola ratios from LLac and HLac *Codium tomentosum* (**I.**) and *Bryopsis plumosa* (**II.**). (**a.**) t-Neo+Viola:Chl *a* ratios in LLac and HLac cultures; (**b.**) Pigment:t-Neo+Viola ratios in HLac cultures. Values at day 0 and day 7 (h6:00, h10:00, h21:00) with standard deviation are reported. Statistical significance (t-test): */+ $p < 0.05$; **/++ $p < 0.01$; ***/+++ $p < 0.001$. Meaning of statistical significance code: +, day 7 value at h 6:00 or at h 10:00 is significantly different from h 21:00; *, value is significantly different from day 0.

In conclusion, strong evidences of VAZ, SL, and of an hypothesised new cycle involving t-Neo and Viola, were not found in both *Codium tomentosum* and *Bryopsis plumosa.*

5.6 D1 repair quantification

Finally, for assessing the global physiological capacity of the algae of avoiding photodamage, the state of D1 protein repair cycle in HLac/LLac *Codium tomentosum* and *Bryopsis plumosa* cultures was investigated. As explained in Materials and Methods (**Section 4.6**), cultures from both species and acclimation conditions were exposed to 1h of light stress in the presence or absence of Lincomycin, an antibiotic known for being able to block the D1 turnover cycle. Therefore, the use of Lincomycin allows the indirect quantification of the extent of photodamage that hits the photosystems, in terms of D1 protein drop.

From a preliminary data analysis, average D1 protein content, quantified with ELISA method (**Fig. 5.25**), the response of the two species appeared similar. Although, ANOVA analysis and post hoc SNK-test yielded different results.

First of all, according to ANOVA analysis (**Tab. 5.5**), D1 protein content of *Codium tomentosum* samples was characterised by highly significant effect of all considered factors: experimental Condition, Light Acclimation and their interaction (Light Acclimation × Condition). A less clear response was reported in *Bryopsis plumosa*: ANOVA analysis showed the presence of a highly significant effect only for factor Condition, together with a slightly significant effect of the interaction Light Acclimation × Condition. Indeed, SNK-test showed that while some significant differences beteen HLac and LLac *Codium tomentosum* were present, no such differences were reported in *Bryopsis plumosa*. Interestingly, in both species no differences between HLac samples exposed to different experimental conditions were observed, while LLac Control samples displayed a significant increment of D1 content, compared to both Before and Treat conditions.

It has however to be noted that several difficulties were encountered during the reported experiment, therefore results must be interpreted with caution; problems related to the chosen experimental approach, and their implications for the present study will be further discussed in **Chapter 6**.

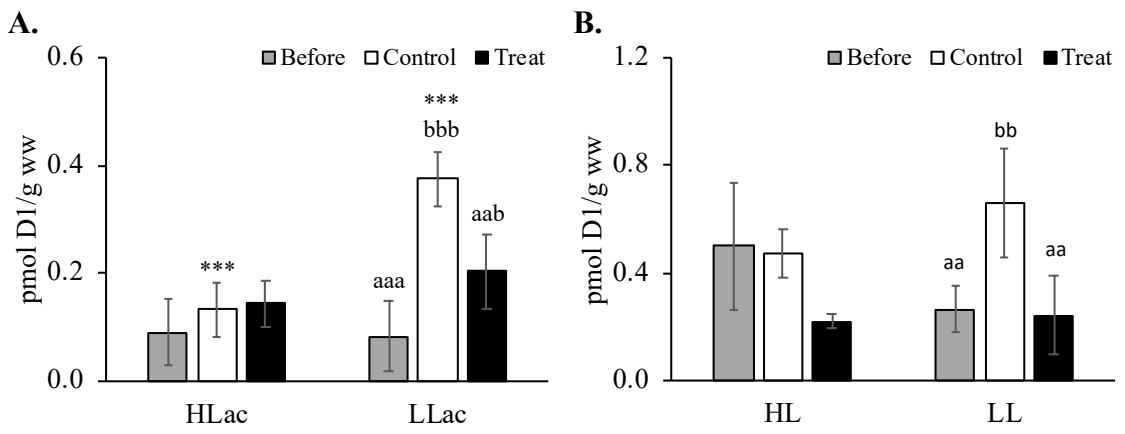


Fig. 5.25 Results of D1 protein quantification of *Codium tomentosum* (**A.**) and *Bryopsis plumosa* (**B.**) cultures acclimated to different light conditions (HLac, LLac) and subjected to Lincomycin treatment experiment. Living samples from each acclimation condition were taken at the beginning (Before), and after 1h of light stress; before the light stress, samples were either incubated Lincomycin+ASW (Treat) or just ASW (Control). Mean values from each biological replicate with standard deviation are reported (n=3). Meaning of statistical significance code (SNK-test): different letters indicate differences between experimental conditions (Before, Control and Treatment), based on SNK-test ranking; asterisks indicate differences beween HLac and LLac cultures, under same experimental condition. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; null: not significant.

Species	*Effect*	*DF*	*Sum Sq*	*Mean Sq*	*F value*	*Prob. >F*	*Stat. Sign.*
C.t.	Condition	2	0.084	0.042	14.40	0.0006	***
	Light acclimation	1	0.043	0.043	14.75	0.002	**
	Light acclimation ✕ Condition	2	0.050	0.025	8.47	0.005	**
	Residual	12	0.035	0.003			
B.p.	Condition	2	0.337	0.169	7.50	0.008	**
	Light acclimation	1	0.0003	0.0003	0.015	0.9	
	Light acclimation ✕ Condition	2	0.137	0.068	3.04	0.085	°
	Residual	12	0.270	0.022			

Tab 5.5 ANOVA results for D1 protein quantification of *Codium tomentosum* (*C.t.*) and *Bryopsis plumosa* (*B.p.*) cultures acclimated to different light conditions (HLac, LLac) under different experimental conditions (Before, Treatment, Control). Cochran test resulted not significant ($p > 0.1$). Statistical significance code: ° $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; null: not significant.

6. Discussion

This study started with a focus on understanding High Light acclimation patterns in Bryopsidales algae, with particular attention on the accumulation of xanthophyll pigments. Therefore, with a dense set of experiments at first the main light acclimation patterns involving different target species of Bryopsidales (*Codium tomentosum* and *Bryopsis plumosa*) were characterised, trying to answer two main questions: what is the trigger for accumulation of *trans*-neoxanthin and violaxanthin, and how is their accumulation carried on over prolonged high light exposition? Also, do these algae eventually display alternative xanthophyll cycle-like mechanisms that might be responsible for photoprotection?

Considering the global light acclimation pattern, the two studied species presented some differences in pigment content from a quantitative point of view: *Codium tomentosum* had a lower amount of pigments, if compared with *Bryopsis plumosa* (around 2 times lower, as can be seen from mean pigment concentrations per dried weight). This quantitative difference might be explained by morphological differences between the two species: while *Bryopsis plumosa* is composed by dense filamentous branches, *Codium tomentosum* has a spongy, thick tallum, with an internal colourless region (medulla) free of chloroplasts (*van den Hoek, 1995*; *Lee, 2018*); for this reason, chloroplast:weight ratio should be higher in *B. plumosa*, explaining the higher pigment concentration per dried weight observed. However, it must be noted that direct quantitative comparisons between the two species must be addressed with caution, since differences in pigment extraction yield might be present.

Considering relative Pigment:Chl *a* ratios, the two species displayed a similar pigment pool, either in low light or high light conditions. In both algae, acclimation to excessive light determined a general decrement of light harvesting pigments and alterations of carotenoid composition, with significant accumulation of t-Neo, Viola, target pigments of the present study, and other xanthophylls. Interestingly, c-Neo content in both species did not seem to be correlated with acclimation to high light.

Although, despite the reported general common pattern few differences were reported: Chl *a* decrease in HLac cultures was observed only in *Bryopsis plumosa*, that also displayed a generally lower amount of Viola; moreover, in this species Lut was almost absent. The differences in Chl *a* patterns were at first surprising: while in both species all other light harvesting pigments (Chl *b*, Siph, Siph-do) decreased under High Light acclimation, Chl *a* amount remained unchanged between LLac and HLac *Codium tomentosum*, but decreased significantly in HLac *Bryopsis plumosa*. However, when taking into consideration the relative amounts of Chlorophylls, a significative increase of Chl *a*:Chl *b* ratio was observed as a High Light response in both species, meaning that High Light reduced the abundance of Chl *b* more than that of Chl *a*. Similar responses have been reported for *Arabidopsis thaliana* leaves and have been correlated with a reduction of the PSII antenna size, a known High Light acclimation response in plant physiology (*Anderson et al. 1995*; *Pfündel et al. 2018*). Also, in a study by *Chen et al. 2005*, the decrease of Chl *b* molecules associated with PSII antenna complexes was identified as a possible photoprotective mechanisms in a Bryopsidales species (*Bryopsis corticulans*). Indeed, these authors postulated that the dissociation of Chl *b* from the antenna complex and the following alteration of the protein structural conformation would reduce the ability of light absorption in the algae, therefore providing photoprotection from High Light irradiation. However, the lack of sufficient proteomic data in our study does not allow to further exploring this hypothesis.

In general, Chl *a*:Chl *b* ratios reported for *Codium tomentosum* (LLac: 1.2 ± 0.05; HLac: 1.7 ± 0.3) and *Bryopsis Plumosa* (LLac: 0.95 ± 0.07; HLac: 1.24 ± 0.06) were relatively low, in all tested acclimation conditions. As well known in plant biology and shown in the present study, this ratios are not constant, but directly correspond to the light acclimation state of the algae; in general, Chl *b* is more abundant than Chl *a* in shaded or low light plants (*Anderson et al. 1995*), and high Chl *b*:Chl *a* ratios have been reported in Bryopsidales algae. In particular, *Giovagnetti et al. 2018* reported a low Chl a/b comparable to the one of the present study (1.26 ± 0.03) in shaded talli of *Bryopsis corticulans*. These results are coherent with Bryopsidales photobiology: these algae normally live in deep intertidal habitats, and therefore need a pigment pool able of enhancing their photosynthetic efficiency in low light. In the case of *Codium*

tomentosum and *Bryopsis plumosa* we proved that this acclimation events involve not only a relative decrease of Chl *b*, but also of their characteristic light harvesting pigments, Siph and Siph do, responsible for enhancing light absorption in those green-blue wavelengths that penetrate deeper in the water column (*Anderson, 1983*; *Wang et al 2013*).

Qualitatively, our results are coherent with previously reported studied carried out *Codium tomentosum* (*Cartaxana et al. 2018*); it has to be noted that minor differences in pigment content between different experiments on this same species might derive from previously existing acclimation conditions: since *C. tomentosum* samples were always directly harvested from the wild, the initial culture conditions of each experimental batch might be different, depending on the complex set of environmental and biological variables characterising the natural population. Different should be the case of *Bryopsis plumosa*, for which controlled laboratory strains are easier to achieve/find and maintain; however, we were not able to find in literature similar studies on this species, that would have been useful for qualitative comparison with our work.

As mentioned above, our main interest during this work was not only to achieve a clear High Light acclimation state, but also to determine the condition for a highly significant accumulation of t-Neo and Viola, pigment with a speculated photoprotective function (*Uragami et al. 2014*; *Cartaxana et al. 2018*). The establishment of a HLac culture with these characteristics in our case required prolonged exposure to considerably High Light: no significant acclimation events were observed during preliminary experiments (2 weeks exposure to 200 µmol photons m^{-2} s^{-1}; 10h exposure to 1000 µmol photons m^{-2} s^{-1}), while 7 days of exposure to intense High Light (1000 µmol photons m^{-2} s^{-1}) determined a clear high light acclimation trend when compared with Low Light cultures (20 µmol photons m^{-2} s^{-1}), with significant accumulation of t-Neo, Viola and other related xanthophylls.

Interestingly, as expected the accumulation of these pigments seemed to be directly correlated with exposure time, as clearly demonstrated by analysis of pigment content during the 7 days of light acclimation and related ANOVA results. Overall, the xanthophyll accumulation observed in this study is coherent with the known

biochemical pathways of production of this pigments: t-Neo is produced from Viola, in a cascade of partially reversible reaction that starts from β-Car, and involves Zea and Anth (**Fig. 6.1**); for this reason, the fact that a significant accumulation of t-Neo and Viola is observed only when also the other related pigments are present, is coherent with the biochemical pathway of production of these pigments. Moreover, this also explains why high light acclimation first affected Viola concentrations (significant accumulation within 1 day) and only later t-Neo (within 2 days).

This also consolidated the hypothesis that the presence of Zea and Anth xanthophylls in Bryopsidales algae is vestigial. Indeed, the relative amount of these pigments seemed too low for justifying a crucial photoprotective function, and our results showed that during the day no variations in relative Viola:VAZ, Anth:VAZ and Zea:VAZ content were present, confirming the absence of light dependent interconversion between these pigments, and therefore of a direct quenching function, as reported in previous studies (*Cruz et al. 2015*; *Christa et al. 2017*). Moreover, from the analysis of relative pigment ratios we were also able to prove the absence of the lutein-siphonaxanthin cycle, another possible xanthophyll-dependent photoprotection mechanism, found in *Caulerpa racemosa*, Bryopsidales (*Raniello et al. 2006*). This result was not surprising considering that, while Siph and Siph-do were always relatively abundant in the target algae, Lut was present in relatively low concentration even in HLac cultures, and in *Bryopsis plumosa* was only sporadically present. For this reason, we concluded that also the role of this pigment in High Light acclimation response in Bryopsidales algae is probably either complementary or vestigial, and should not have a direct photoprotective function.

Finally, with our results we demonstrated that no significant changes in relative content of t-Neo and Viola were present during the day, rejecting the hypothesis of existence of a peculiar xanthophyll cycle-like mechanisms involving directly these two pigments.

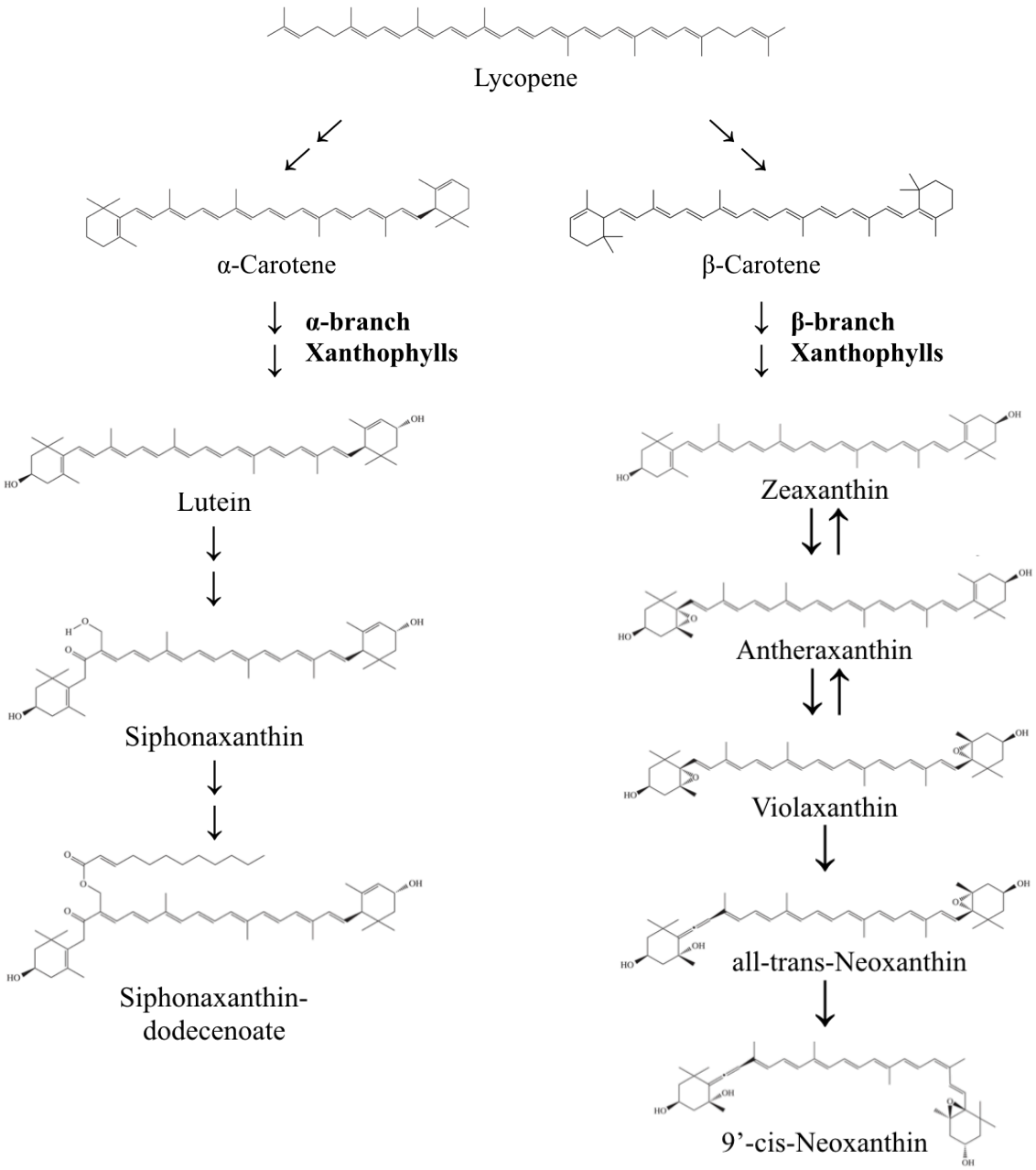


Fig. 6.1 General biosynthetic pathway of α- and ß-branch carotenoids in cyanobacteria and algae, adapted from *Roy et al. 2011*. Note that the present scheme is highly simplified and includes only the pigments of interest.

Another foretold goal of the present study was to set the ground for the solution of an ulterior major question: does *trans*-neoxanthin and violaxanthin really have a role in photoprotection of Bryopsidales algae? For doing so, we tried to asses the global photoprotective capacity and the extent of damage to the PSII machinery in HLac *Codium tomentosum* and *Bryopsis plumosa*, containing significant amounts of the target xanthophylls.

The first approach we used for determining the overall photoprotective capacity in HLac cultures was based on chlorophyll *a* fluorescence. Firs of all, for assessing the general photoacclimation state of the experimental cultures we considered the results from SSLC and related parameters, calculated following the model of *Platt et al. 1980*. *Codium tomentosum* displayed a similar pattern in both first and second acclimation experiment: HLac cultures maintained relatively high efficiency under low light intensities (comparable to the one of LLac cultures) while increasing their maximum photosynthetic rates at light saturation (rETRmax) and light saturation coefficient (Ek), known signs of acclimation to High Light (*Björkman, 1981; Beer et al. 2014*).

On the contrary, *Bryopsis plumosa* results were less clear: while in the first experiment a clear decrease of photosynthetic efficiency was observed in both HLac and LLac cultures, during the second experiment this deficiency was not reported, but no evidence of a clear High Light acclimation event was observed. Notably, during the second experiment the sample dimension of both species was reduced, due to a lower need of biological samples. Therefore, a lack of space or nutrients might have affected the overall health state of *B. plumosa* cultures during the previous experiment; indeed, it has to be considered that this species, due to its filamentous morphology, has specific space requirements, and has a tendency of accumulating oxigen bubbles, that cause the culture to float and might inhibit photosynthesis. Therefore, abiotic factors might have affected negatively the photosynthetic efficiency of these cultures; this observation must be taken into account during future experiments: the choice of using bigger culture volume or supplementing the medium with bicarbonate (to prevent lack of CO_2) or nutrients might contribute to achieve better experimental cultures of this particular species.

Also, these observations posed questions regarding the extent to which the tallum morphology influences photoacclimation responses. This concept is not unfamiliar in photophysiology; considering a Bryopsidales example, *Giovagnetti et al. 2018* reported that morphology was able to affect light harvesting modulation in *Bryopsis corticulans*, with clear functional segregation between shaded and exposed filaments. Therefore, despite this was not the main goal of our study, we cannot avoid to take into consideration that different morphological features, between and within the two target species, might play a key role in physiological mechanisms related to photoacclimation, and might have influenced our study.

Interestingly, despite in *Bryopsis plumosa* SSLC a clear acclimation response was absent, photoacclimation-related changes in pigment content were reported as in *Codium tomentosum*, indicating that both species responded to the imposed light intensity. To assess if this photoacclimation events (including significative accumulation of t-Neo and Viola) had some measurable effects on the photoprotective ability of our target species, we analysed the results from the "pNPQ protocol", a novel approach published by *Ruban & Murchie, 2012* and recently reviewed by the same author (*Ruban, 2017*). This approach has the enormous advantage of allowing the assessment the photoprotective power of NPQ, obtaining detailed information about the extent of photoinhibition and photoprotection in target organisms, with a relatively fast and easily applicable technique. As reported in **Chapter 5**, with the application of this protocol we observed that, in HLac *Codium tomentosum*, the onset if photoinhibition seemed to take place at higher light intensities compared to LLac. This response was never reported for *Bryopsis plumosa*.

At first glance, this observation would lead to the conclusion that *C. tomentosum* displayed stronger photoprotective capacity compared to *B. plumosa*. However, giving this conclusion would be hasty and not completely appropriate. Indeed, it has to be considered that for both species a great decrease of maximum YII and NPQ was reported, as a consequence of High Light acclimation. Normally, when Chl *a* fluorescence protocols are applied, photosynthetic organisms are subject to dark-

adaptation[2], a procedure that allows Photosystems recovery; if complete recovery is achieved, PSII yield values measured in the dark express the maximum YII (*Murchie & Lawson, 2013*). However, maximum YII in HLac *Codium tomentosum* and *Bryopsis plumosa* were significantly lower compared to LLac cultures, and far from the theoretical maximum. This lack of complete recovery after light stress is not new for Bryopsidales: *Cruz et al. 2015* reported that in light-stressed *Codium tomentosum* PSII maximum quantum yield did not reach complete recovery even after 2 h of dark relaxation, with presence of sustained NPQ in the dark. The authors concluded that this phenomenon might be either caused by extensive photoinhibition or by the action of other photoprotective mechanisms, responsible for the observed sustained NPQ.

This phenomenon posed significative challenge to the application of the chosen fluorescence protocol: if NPQ relaxation is not complete, maximum YII is underestimated, with consequent alteration of all the derived parameters. It has to be noted that dark-adaptation time could not be overextended until complete PSII relaxation was reached, due to the extreme velocity of some photoprotective responses (e.g. D1 turnover enhancement, pigment cycles); for this reason, an overextension of the dark-adaptation step could determine partial or complete loss of the achieved photoacclimation state, making the whole experimental procedure futile. Therefore, the impossibility of achieving a fully dark-adapted state in our algae samples presented a not negligible limit to the application of the pNPQ protocol and relative result interpretation: since maximum YII was underestimated owing to physiological reasons, mathematical problems occurred and NPQ might not represent a true indicator of the total quenching (*Tietz et al. 2017*).

Moreover, another criticality of the proposed approach resides in the hugely debated qP equation. *Joliot and Joliot, 1964*, first observed that the proposed relationship was affected by the exchange of excitation energy between the antennae of different PSII reaction centres, a process now known as “connectivity” or “grouping”. Indeed, the parameter qP is based on the so-called “puddle” model, that ignores connectivity

[2] Note that here the use of the term “adaptation” assumes a peculiar meaning; in the field of Chl *a* fluorescence techniques, a “dark-adapted state” is defined as that physiological state in which all PSII centres are open and no NPQ is present, and can usually be achieved by keeping a photosynthetic organisms in the dark for a reasonable period of time (*Murchie & Lawson, 2013*).

(*Kalaji et al. 2017*). In contrast, *Kramer et al., 2004* developed a new parameter (called qL), based on the so called "lake" or "connected units" models, that takes into account the postulated connection between the reaction centres, given by shared antennae. However, as stated by the author, the novel qL is not applicable to all marine species, which might require the use of more complex parameters due to their distinct antenna architecture. It is far from the purpose of the present study (and from my expertise) to discuss the attainability of this two different models and of the derived equations; however, the reader should be aware that such conflict currently exists in this field, in order to be able to take his own considerations.

Due to the exposed criticalities, we implemented our analysis with the determination of complementary quantum Yields, an ulterior set of parameters useful for the evaluation of photoprotective capacity in photosynthetic organisms. The low Y(NPQ)/Y(NO) ratios reported for both target species suggested that HLac algae displayed suboptimal photoprotective capacity, with high passive energy losses. This result is in contrast with what determined by pNPQ protocol in *Codium tomentosum*, but is coherent with the low observed maximum YII: the incomplete recovery of PSII yield following 1h of dark-adaptation, suggested the presence of a strong light stress, confirmed by the reported complementary quantum Yield results. However, it cannot be overlooked that also complementary quantum Yield parameters are based on the measurement of maximum YII; therefore, this results are subject to the same mathematical problems already discussed for pNPQ.

Despite the discussed criticalities of the applied fluorescence protocol, a question still rises: why did we observe a pNPQ response only in HLac *Codium tomentosum* and not in *Bryopsis plumosa*? Was this result a random artefact given by related mathematical problems, or are there some possible biological explanations for this phenomenon? The fact that the two species yielded partially different results is not surprising, nor unexpected. Light adaptation is driven by an extremely complex set of physiological responses, some of which might most likely be species-specific. A fact that immediately catches attention are the differences in SSLC acclimation responses of the two species: as discussed above, *Codium tomentosum* displayed a clearer High Light acclimation state, while in *Bryopsis plumosa* acclimation seemed to be negatively affected by not considered external factors, possibly nutrient and/or CO_2 shortage. Those abiotic

stresses might be the cause of the inferior photosynthetic performances under High Light acclimation observed in this species. Moreover, the attainability of our results is enforced by the fact that our pNPQ protocol output corresponds to those of *Arabidopsis thaliana* mutants with impaired qE (*Ruban & Murchie, 2012*). Therefore, despite the attainability of this protocol cannot be assured as discussed above, it is evident that our result is coherent with the physiology of these organism, lacking a functional xanthophyll cycle.

Another conclusion was driven by the observation of relative pigment content in the two species: in LLac *Codium tomentosum* a higher basal level of t-Neo was reported; moreover, HLac cultures of this species seemed to accumulate higher levels of Viola compared to the ones of *Bryopsis plumosa*. Without forgetting that quantitative comparisons between the two species must be taken with caution, this observation lead us to the conclusion that *C. tomentosum* might be able to exert a faster high light acclimation response, allowing this species to cope better with sudden light changes, and therefore really displaying an higher photoprotective capacity. This is also supported by the fact that while *B. plumosa* was obtained from a laboratory culture, originally harvested in 2002 and therefore acclimated to stable artificial condition for several years, *C. tomentosum* was harvested from the wild, in the Portuguese rocky shore, a highly variable intertidal habitat where tidal excursions can reach up to 3m. This would perfectly explain the ability of this species to better cope with light changes.

Considering Chl *a*:Chl *b* ratios we are driven to a similar conclusion: *Bryopsis plumosa* always displayed a lower Chl *a*:Chl *b* ratio, sign of an acclimation to Low Light. This might lead to more difficulties when acclimating to High Light, therefore explaining the better photosynthetic performances observed in *Codium tomentosum*.

With our data is impossible to prove any of these hypotheses. However, appears evident that some not unlikely biological reason might have been responsible of a better acclimation to High Light in *Codium tomentosum*, possibly determining higher photoprotective capacity, responsible of the reported pNPQ results. In the end, both species presented evident signs of chronic photoinhibition, a drastic reduction of maximum PSII quantum Yield and low Y(NPQ)/Y(NO) ratios, meaning that the algae

were not able to maintain full photosynthetic efficiency under the imposed High Light. However, both algae thrived under the imposed experimental condition, maintaining an adequate health state, meaning that despite the evident permanent damage to the photosynthetic machinery, they must still possess mechanisms responsible for their survival to prolonged light stress.

Considering the results of D1 protein repair quantification, our results suggested that Bryopsidales algae acclimated to extreme light conditions might suffer from less D1 damage, and therefore possibly display enhanced photoprotection (either in the terms of photodamage avoidance or active photoprotection mechanisms). Ineed, in both species no differences in D1 content between HLac differently treated samples were reported, while LLac samples incubated with Lincomycin, inhibitor of D1 repair cycle, displayed significantly lower amount of undamaged D1 compared with control condition, meaning that LLac cultures would have undergone more direct damage to the PSII machinery, compared with HLac. Also, in *Codium tomentosum* significant differences between the two light acclimation conditions, unrelated to treatment, were reported, meaning that some differences between HLac and LLac acclimated cultures might be present. This could have two possible meanings: on one hand, HLac cultures might be so stressed, that they are not able to fully reconstitute their basal D1 level, determining a significative decrease of this protein under chronic exposition to extreme High Light; on the contrary, the constitutive reduction of D1 protein in HLac cultures, possibly together with other components of the photosynthetic machinery, might be an active photoacclimation response to light excess, determining stronger High Light acclimation ability in *Codium tomentosum*.

These results acquire more relevance considering that the presence of a fast PSII repair has been suggested as one of the possible photoprotective mechanisms responsible for photoprotective NPQ in Bryopsidales species, together with transient inactivation of the photosystem (*Giovagnetti et al. 2018*). It is therefore not absurd to hypothesise that these algae, lacking the conventional fast NPQ response (qE, given by the xanthophyll cycle) could compensate this deficiency simply with an increased rate of photosynthetic machinery repair, especially considering that these organisms are exposed to extreme

high lights only for short periods of time, during low tides, and these mechanisms might therefore sufficient for guaranteeing minimisation of photodamage.

However, the reader should be aware that these conclusions are drawn with some uncertainty, driven by technical difficulties encountered during the experimental procedure, including the adaptation of the protocol for thylakoid protein extraction and the ELISA quantification itself. Also, the results interpretation is not assisted by the fact that in LLac algae under control condition displayed higher D1 content after light stress compared with before treatment condition. It's important to note that we chose to take the samples for this experiment at the end of the night, immediately before the beginning of the light photoperiod. This choice was driven by the knowledge that our HLac algae were subject to a permanent stress condition, given by not completely efficient acclimation to extreme High Light revealed by the presence of permanent damage to photosynthetic efficiency. Therefore, we chose to take our experimental samples in the moment of the day when they would have been the less stressed possible, identified as the end of the night. However, D1 post transcriptional regulation and the translation of the corresponding *psbA* mRNA sequence, are known to be light-induced processes (*Järvi et al. 2015*); therefore, the choice of sampling the algae at the end of the night, although led by physiological considerations, influence our experiments and their interpretation. For these reasons, the results discussed in the present study represent only a preliminary assessment of D1 repair capacity in these algae, which will definitely need to be further validated with additional techniques and explored with future and more complete proteomic studies.

In the end, several hypotheses on the extent of photoprotection mechanisms in HLac *Codium tomentosum* and *Bryopsis plumosa* are still open. Regarding the direct role of t-Neo and Viola, we were not able to prove a direct correlation between increased photoprotective activity and the presence of these pigments, but none of the reported results contradicted the previously postulated model (*Uragami et al, 2014*; *Cartaxana et al. 2018*). Considering the results of both preliminary and final experiments, we can at least confirm that t-Neo and Viola are for sure not implicated in any short-term

photoprotection response, since their accumulation requires several days of High Light acclimation.

The long-term nature of this response is coherent with the hypothesis of *Uragami et al, 2014*, according to which the additional t-Neo and Viola would promote the SCP (Siphonaxanthin-Chlorophyll-Protein) complex oligomerization and regulate the light energy transfer to the reaction centres of PSII, thanks to week and flexible pigment-protein interactions. In addition, these molecules might as well work as antioxidants, dissolved in the lipid matrix, as known for several xanthophylls in diatoms (*Lepetit et al. 2010*). Coherently with this hypothesis, in *Arabidopsis thaliana* mutants with impaired qE, exposed to continuous light stress, it has been shown that lack of NPQ can be partly compensated by higher levels of antioxidants (*Müller-Moulé et al. 2004*; *Golan et al. 2006*).

Following a different hypothesis, the accumulation of these pigments under high light might also be a metabolical response to extreme stress; these processes have been extensively studied in green microalgae: when cultured under extreme environmental stress (including both light and nutrient stress), these microorganisms synthesise and accumulate several secondary metabolites, including carotenoids (e.g. astaxanthin, *Haematococcus pluvalis*; β-carotene, *Dunaliella salina*; lutein, *Chlorella sp.*) and lipids (e.g. PUFAs) in some cases with a direct positive correlation. The synthesis of these secondary metabolites is believed to function as attempt to retain growth rates or to increase the survival rate under unfavourable environmental conditions (*Markou & Nerantzis, 2013*).

Finally, it would not be realistic to look for a single mechanism responsible for photoprotection in any photosynthetic organism. Therefore, the lack of a functional xanthophyll cycle in Bryopsidales algae like *Codium tomentosum* and *Bryopsis plumosa* is probably compensated by a complex set of photoprotective mechanisms. As previously mentioned, chloroplasts are known for being able of migrating through different regions of the Bryopsidales' siphons following certain light stimuli (*Graham & Wilcox, 2000*). Light avoidance determined by chloroplast movement is today believed to be one of the photoprotective mechanisms commonly present in algae and higher

plants (*Takahashi & Badger, 2011*; *Pfündel et al. 2018*). Therefore, this mechanism might be of fundamental importance in the Bryopsidales order, that display the ideal morphology (coenocytic) for allowing organelles movement. Due to the fact that these organisms are exposed regular short periods of intense high light (low tides), it's conceivable to think that this fast, not metabolical response might be essential in allowing algae to minimise photodamage. Assessing the presence of these mechanisms would be decisive for photo-physiology research on this particular group of algae: it has been reported that the chloroplast movement could modify NPQ without contributing to the quenching process, by decreasing light absorption and consequently determining and underestimation of several Chl *a* fluorescence parameters (*Cazzaniga et al. 2013*).

Other mechanisms putatively involved in Bryopsidales photoprotection are PSI cyclic electron transport and PSII state transitions. Indeed, in green microalgae like *Chlamydomonas reinhardtii*, with relatively low qE capacity under normal light conditions, it has been reported that such mechanisms have higher importance compared to other photoprotection strategies (*Kukuczka et al. 2014*; *Rochaix, 2014*) and a similar role can be postulated in Bryopsidales. Also the presence and the role of Lhcx-family proteins, involved in qE formation e.g. in diatoms (*Bailleul et al. 2010*; *Zhu & Green, 2010*; *Buck et al. 2019*) has not been fully assessed in these algae; we cannot therefore exclude that proteins of this family might also be playing a particular role in Bryopsidales photoprotection.

7. Conclusions and Future remarks

In the end, with the results achieved in the present work a significant milestone was reached: we extended available information on the pigment production profiles of *Codium tomentosum* and *Bryopsis plumosa* under high light acclimation, and we depended our knowledge on the physiological implication of such acclimation events, following several interesting cues. However, after all the conducted analysis, a question remained still open: if no xanthophyll cycle is present in these species, do t-Neo and Viola have a role in photoprotection? Or which other mechanisms are responsible for photoprotection in this particular branch of algae? Indeed, we were not able to give strong confirmation of photoprotective activity in these algae and to prove the hypothesised correlation between production of these pigments and photoprotection.

Also, while in the present study we focused only of the effect of light intensity, preliminary experiments suggested that the light spectrum might influence photoacclimation ability in Bryopsidales algae, leading to additional questions regarding how these species utilise the available photosynthetically active radiation. For these reasons, I believe that the questions assessed in this experiment could be further expanded, investigating both the effects of light quantity and quality of the photoacclimation events that happen in Bryopsidales algae. Since production of target xanthophylls resulted to be directly correlated with light intensity and time of exposure, novel studies that take into account a higher number of light variables, could provide additional useful details. Research on this topic is currently being carried on under the CtLight project and we expect it to complete the currently collected information.

In the end, our study gave interesting results but the road is still long: it's clear that ulterior experiments, including improved molecular and fluorescence approaches, will be able of overcoming the limitations encountered in the present study, allowing to move forward in this field. In this respect, the identification of experimental conditions responsible of t-Neo and Viola production, without determining a permanent light driven stress should be of primary importance for evading in particular those problems involving Chl *a* fluorescence measurements. Also, examination of ROS metabolism

under High Light acclimation should complete the information regarding the ability of these algae of responding to light excess.

Moreover, new hypothesis explaining the accumulation of *trans*-neoxanthin and violaxanthin in high light acclimated Bryopsidales algae should be tested. As previously suggested, the accumulation of such secondary metabolites could be a stress response comparable to the one reported in microalgae, and positively correlated with lipid metabolism. For this reason, the application of lipidomic and metabolomic approaches should be highly considered for future studies.

Finally, we discussed the possibility that different photoprotection mechanisms, not related to *trans*-neoxanthin and violaxanthin accumulation, might be of key importance in the photophysiology of Bryopsidales algae, including chloroplast movement, enhancement of PSII repair, transient PSII inactivation, state transitions and PSI cyclic electron transport, and Lhcx proteins. Therefore, complete studies with a strong molecular and proteomic approach will be needed in the future for moving forward in this field.

In conclusion, Bryopsidales is a peculiar and fascinating order of algae, with high ecological and commercial relevance, on whose photophysiology we still do not know enough. The unique features of this group are of high interest for modern photosynthesis research, with several questions still waiting to be answered.

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"*Fantasy is a natural human activity. It certainly does not destroy or even insult Reason; and it does not either blunt the appetite for, not obscure the perception of, scientific verity. On the contrary. The keener and the clearer is the Reason, the better Fantasy will it make."*

J.R.R. Tolkien, On Fairy-stories

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Supplementary material

Appendix A. f/2 medium components

Composition of f/2 medium (*Guillard and Ryther*, 1962; *Guillard, 1975*), as modified by *Andersen, 2005*, is reported.

Component	*Stock Solution* ($g*L^{-1}$ dH_2O)	*Quantity used (for 1L Medium)*	*Final concentration*
$NaNo_3$	75	1 mL	$8.82*10^{-4}$
NaH_2PO_4	5	1 mL	$3.62*10^{-5}$
TM sol.	(see following recipe)	1 mL	-
Vit. sol.	(see following recipe)	0.5 mL	-

f/2 Trace Metals solution (TM sol.)

Component	*Stock Solution* ($g*L^{-1}$ dH_2O)	*Quantity used (for 1L TM sol.)*	*Final concentration (in Medium)*
$FeCl_3*6H_2O$	-	3.15 g	$1.17*10^{-5}$
$Na_2EDTA*2H_2O$	-	4.36 g	$1.17*10^{-5}$
$MnCl_2*4H_2O$	180	1 mL	$9.10*10^{-7}$
$ZnSO_4*7H2O$	22	1 mL	$7.65*10^{-8}$
$CoCl_2*6H_2O$	10	1 mL	$4.20*10^{-8}$
$CuSO_4*5H_2O$	9.8	1 mL	$3.93*10^{-8}$
$Na_2MoO_4*2H_2O$	6.3	1 mL	$2.60*10^{-8}$

f/2 Vitamin solution (Vit. sol.)

Component	*Stock Solution* ($g*L^{-1}$ dH_2O)	*Quantity used (for 1L Vit. sol.)*	*Final concentration (in Medium)*
Thiamine*HCl (Vit. B_1)	-	200 mg	$2.96*10^{-7}$
Biotin (Vit. H)	1	1 mL	$2.05*10^{-9}$
Cyanocobalamin (Vit. B_{12})	1	1 mL	$3.69*10^{-10}$

Appendix B. Fluorescence parameters and formulas

S*tandard parameters (Logan, 2005; Murchie & Lawson, 2013)*

Parameter	*Definition*	*Formula*
Fo	Minimum fluorescence yield in the dark adapted state.	-
Fm	Maximum fluorescence yield in the dark adapted state.	-
Fv	Variable fluorescence (effective level of fluorescence) during a saturating pulse in the dark adapted state.	$Fv = Fm - Fo$
Fv/Fm	Quantum yield of Photosystem II (PSII) in the dark adapted state: maximum quantum efficiency of PSII photochemistry.	-
F	Minimum fluorescence yield during steady state illumination (under actinic light).	-
Fm′	Maximum fluorescence yield during steady state illumination (under actinic light).	-
Fv′	Variable fluorescence (effective level of fluorescence) during steady state illumination (under actinic light).	$Fv' = Fm' - F$
YII	Quantum yield of Photosystem II (PSII) during steady state illumination (under actinic light): maximum quantum efficiency of PSII photochemistry.	$YII = \frac{Fv'}{Fm'}$
rETR	Relative Electron Transfer Rate.	$rETR = PAR * YII$
NPQ	Non Photochemical Quenching: estimates the rate constant for heat loss from PSII.	$NPQ = \frac{Fm - Fm'}{Fm'}$

Steady State Light Curve model (Platt et al. 1980)

Parameter	*Definition*	*Model equation*
P^B	Photosynthetic rate	$P^B = P_s^B(1 - e^{-\alpha I}/P_s^B)e^{-\beta I}/P_s^B$
P_sB	Light-saturated photosynthetic rate in the absence of photoinhibition	-
α	Initial slope of the light curve	-
I	Quantum scalar irradiance	-
β	Index of photoinhibition	-
rETRmax	Maximum relative Electron Transport Rate at light saturation (μmol e− m^{-2} s^{-1})	-
Ek	Light saturation coefficient (PAR)	-

pNPQ analysis (Oxborough & Baker, 1997; Ruban & Murchie, 2012)

Parameter	*Definition*	*Formula*
Fo′	Minimum fluorescence yield in the dark (under far red light), immediately after steady state illumination.	-
Fm′d	Maximum fluorescence yield in the dark (under far red light), immediately after steady state illumination.	-
Fo'_{cal}	Estimates the true value of Fo′, without photoinhibition.	$Fo'cal = \frac{Fo}{\frac{Fv}{Fm} + \frac{Fo}{Fm'd}}$
qP	Photochemical quenching: relates PSII maximum efficiency to operating efficiency. Non-linearly related to proportion of PSII centres that are open.	$qP = \frac{Fm' - F}{Fm' - Fo'}$
qPd	Values of qP in the dark (under far red light), immediately after steady state illumination.	$qPd = \frac{Fm'd - Fo'}{Fm'd - Fo'cal}$
qP th	Theoretical value of qP in the dark (under far red light), after turning off actinic light, assuming the absence of photoinhibition.	$qPth = \frac{Fm'd - Fo'}{Fm'd - Fo'} = 1$
YII th	Theoretical yield of Photosystem II, assuming the absence of photoinhibition.	$YIIth = qPth * \frac{Fv/Fm}{1 + (1 - Fv/Fm) * NPQ}$

Complementary quantum yields (Klughammer & Schreiber, 2008)

Parameter	*Definition*	*Formula*
	Complementarity equation	$YII + Y(NPQ) + Y(NO) = 1$
Y(NPQ)	Quantum yield of regulated non-photochemical energy loss in PS II, equivalent to Y(NPQ)	$Y(NPQ) = \frac{F}{Fm'} - \frac{F}{Fm}$
Y(NO)	Quantum yield of non-regulated non-photochemical energy loss in PS II, equivalent to Y(NO)	$Y(NO) = \frac{F}{Fm}$
Y(loss)	Total quantum yield of all loss processes	$Y(loss) = 1 - YII$
		$Y(loss) = Y(NPQ) + Y(NO)$
		$Y(loss) = \frac{F}{Fm'}$
NPQ	Non Photochemical Quenching: estimates the rate constant for heat loss from PSII.	$NPQ = Y(NPQ)/Y(NO)$
		$NPQ = \frac{Fm - Fm'}{Fm'}$

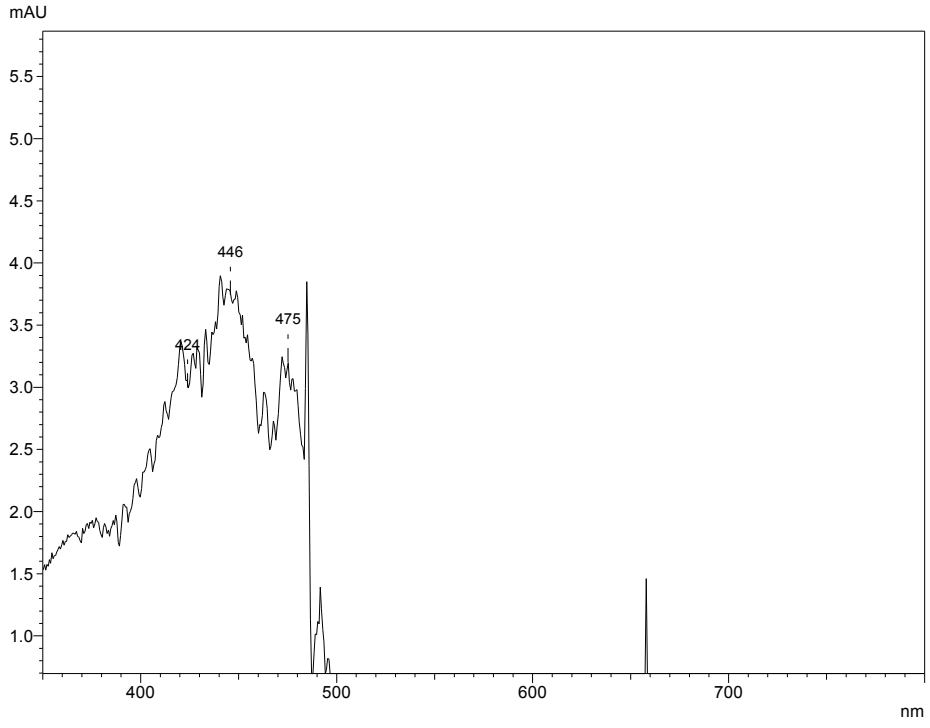
Appendix C. Pigment PDA absorption spectra

Example pigment spectra extracted from *Codium tomentosum* samples and detected with HPLC. Peaks from the PDA detector (wavelength: 450 nm) were compared to the one obtained with pure cristalline standards used for calibration curves (DHI, Denmark) and information available in literature (*Roy et al. 2011*).

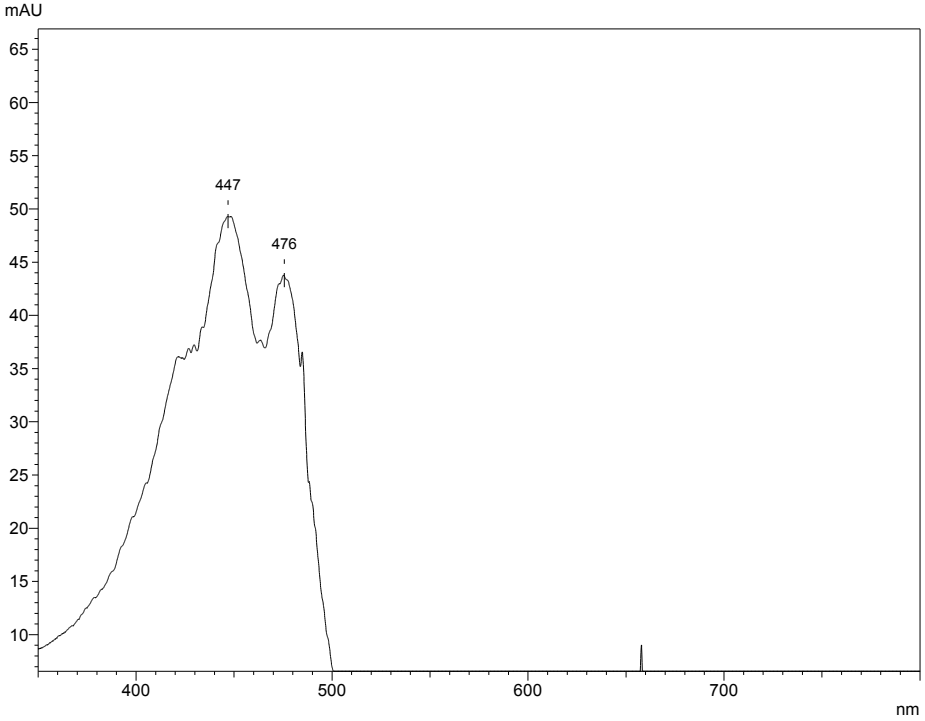
Graphs show the raw pigment spectra (after background compensation) as extracted from the equipment's program (LabSolutions, Shimadzu). λ max (nm) are indicated with numbers; note that these values can variate slightly between samples and that the presence of noise is higher in lower concentrated pigments.

Pigments are reported in alphabetic order, with abbreviations. For Carotenes, both trivial name and IUPAC name are reported.

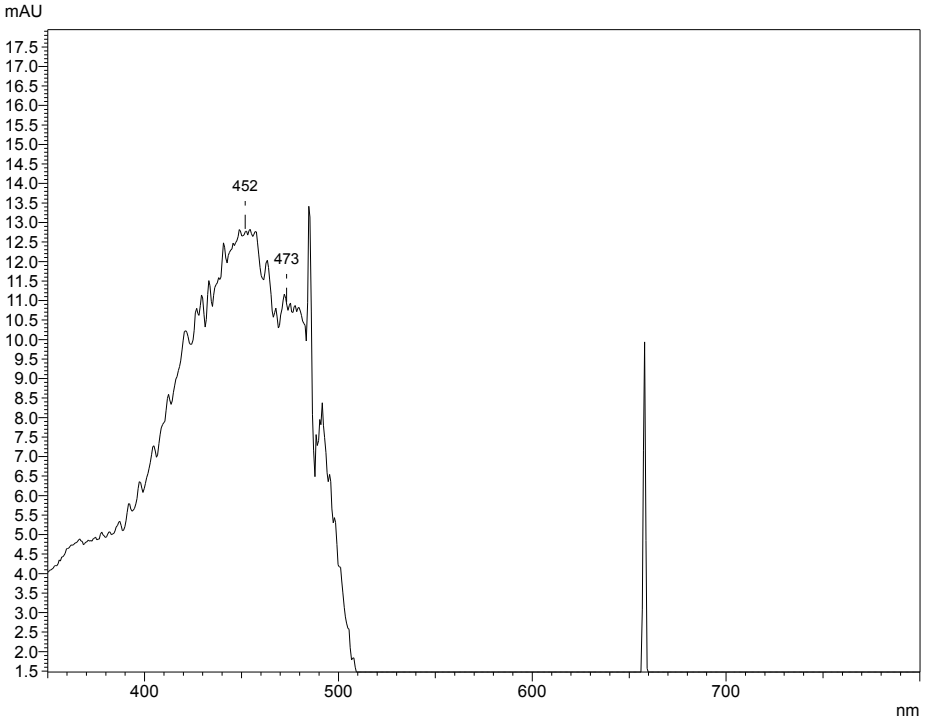
Antheraxanthin (Anth)



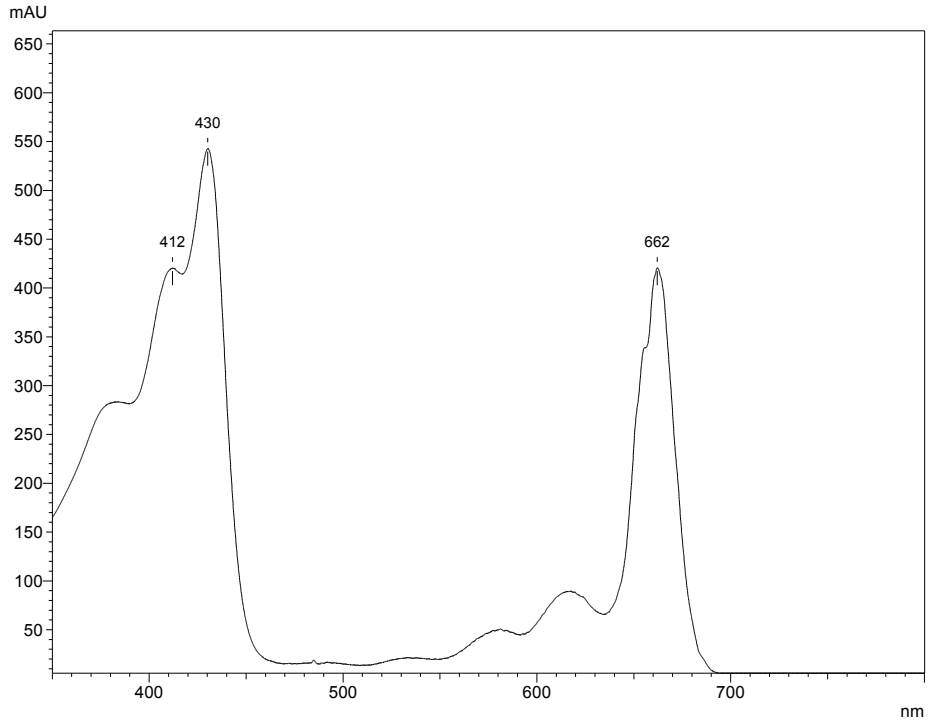
α-Carotene (α-Car) IUPAC: β,ε-Carotene



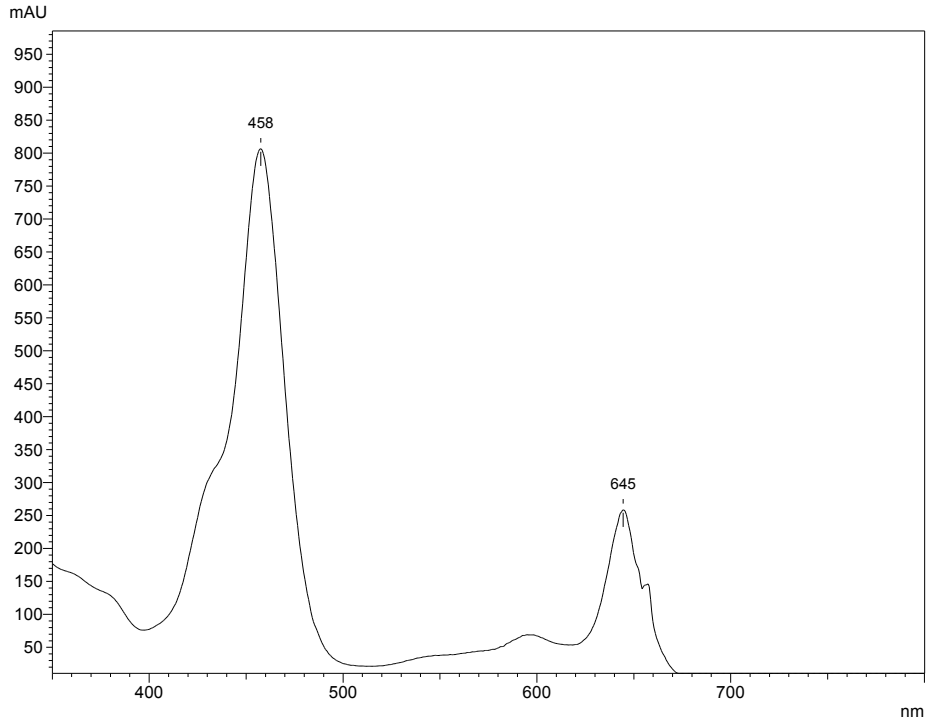
β-Carotene (β-Car) IUPAC: β,β-Carotene



Chlorophyll a (Chl a) [5]

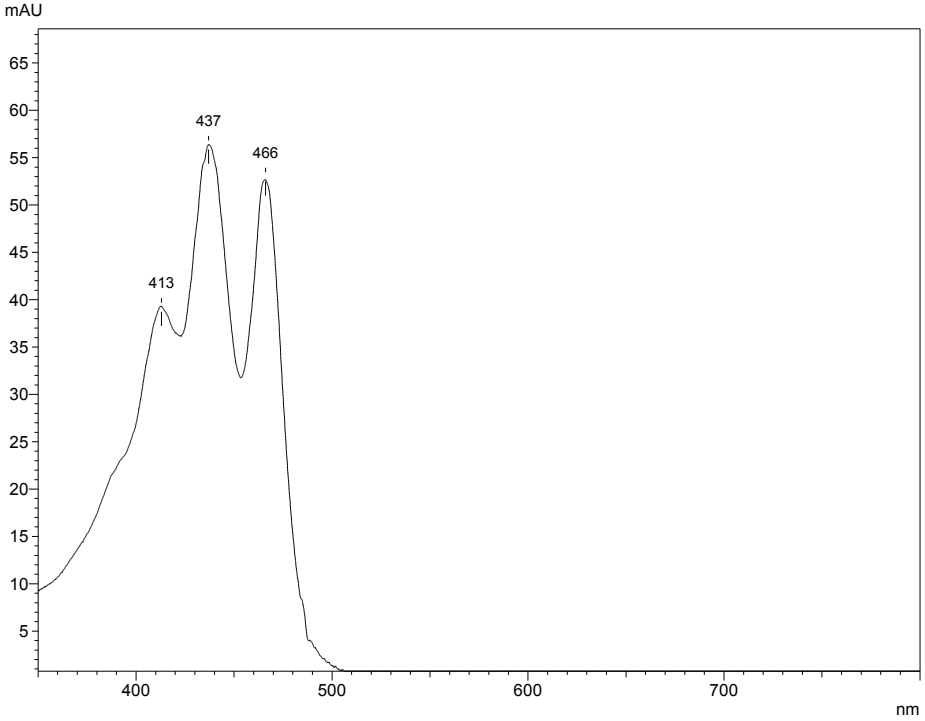


Chlorophyll b (Chl b) [5]

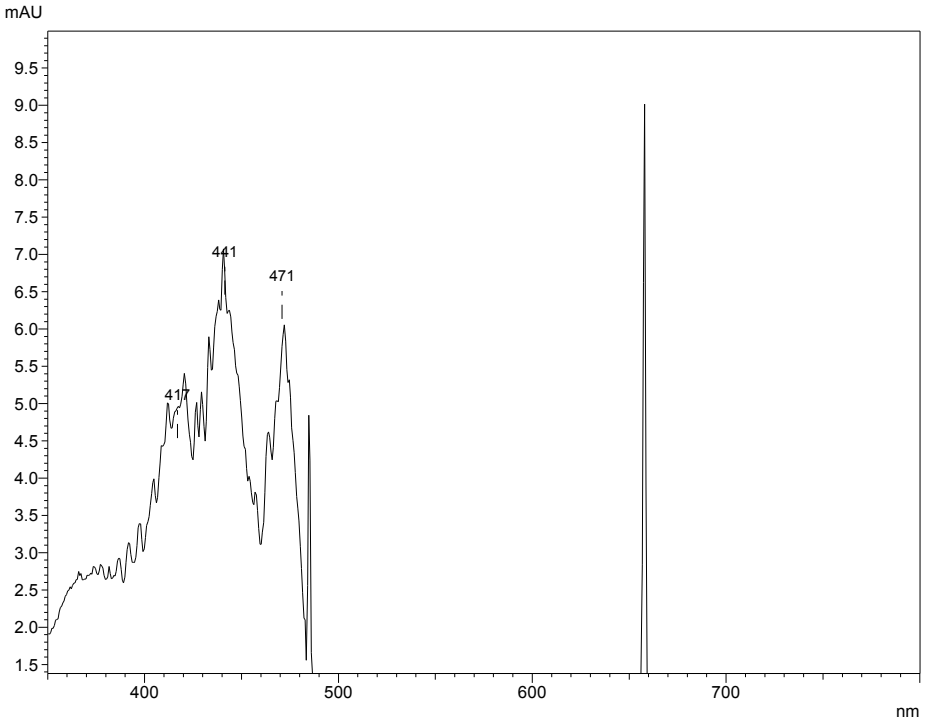


[5] For the Chlorophylls, the program did not show on graph the conventionally denoted λ max around 617 nm for Chl *a* and 597 nm for Chl *b*. These λ max value were extracted manually.

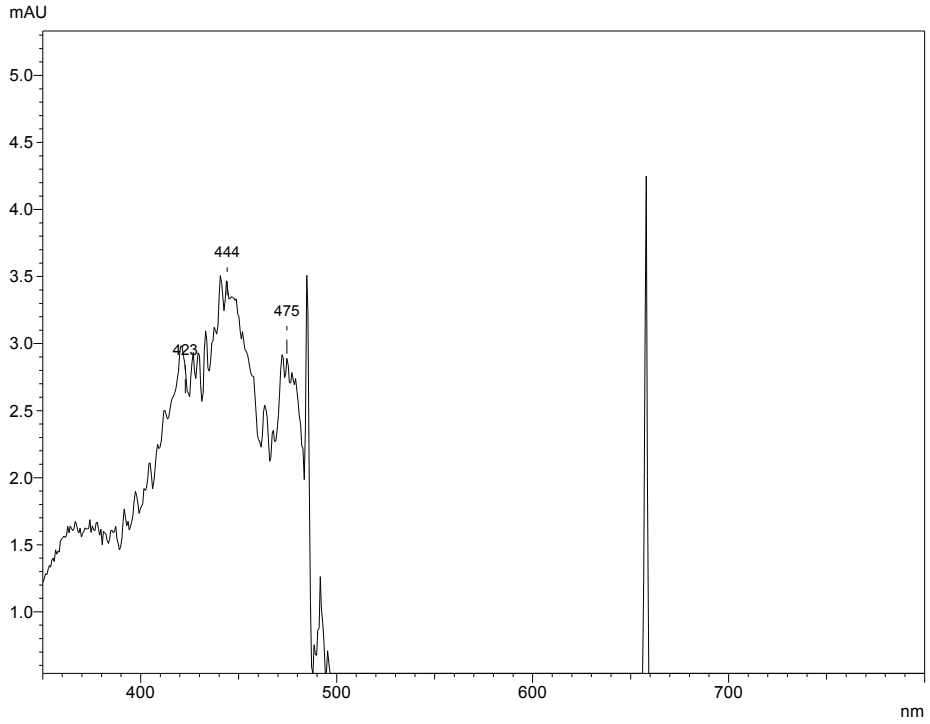
cis-Neoxanthin (c-Neo)



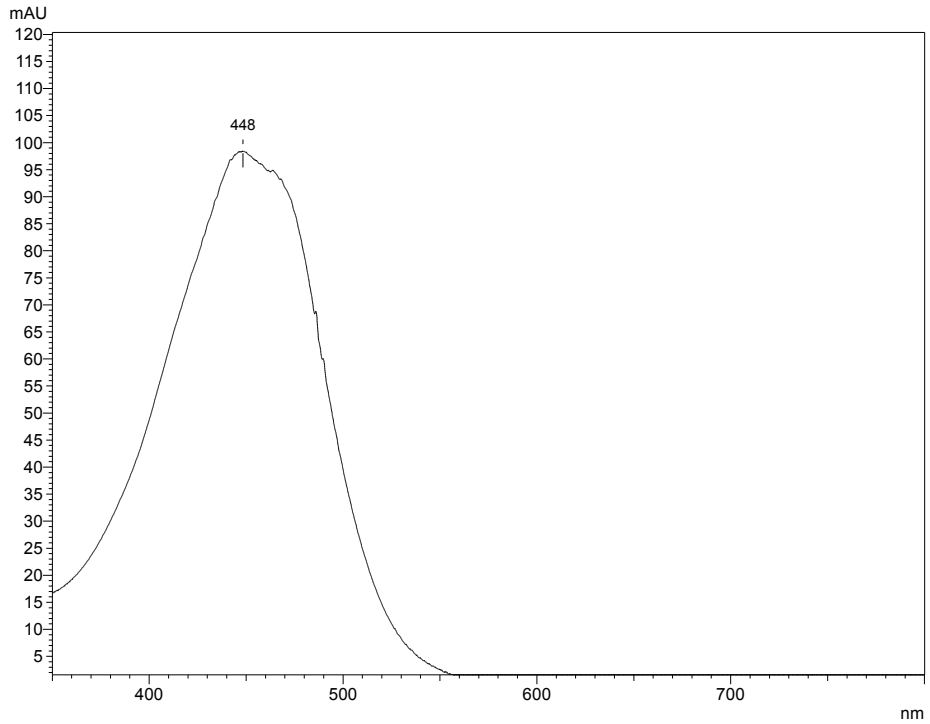
ε-Carotene (ε-Car) IUPAC: ε,ε-Carotene



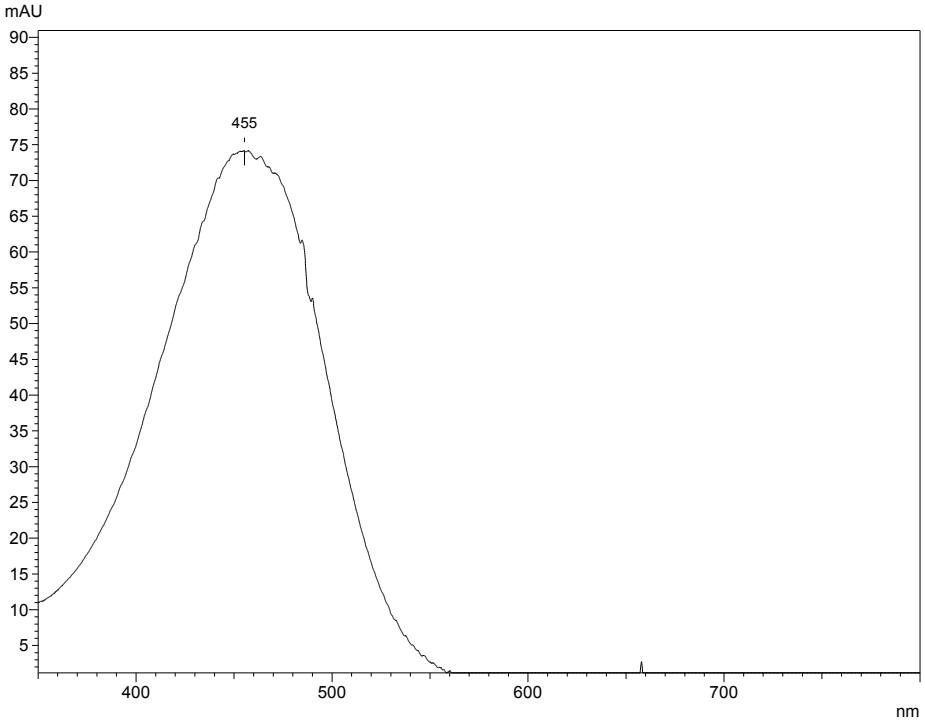
Lutein (Lut)



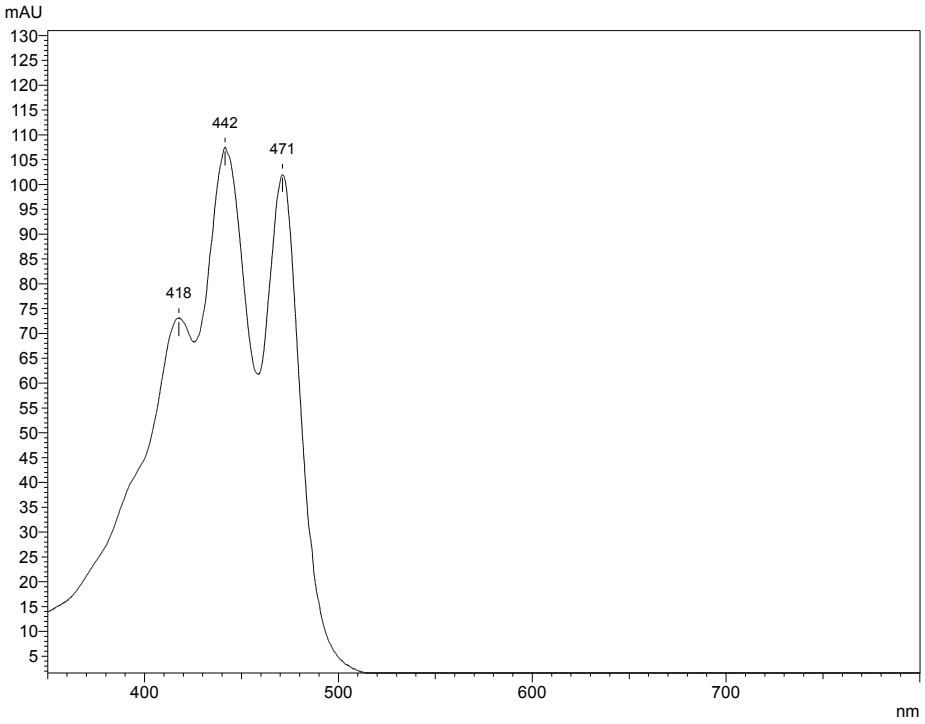
Siphonaxanthin (Siph)



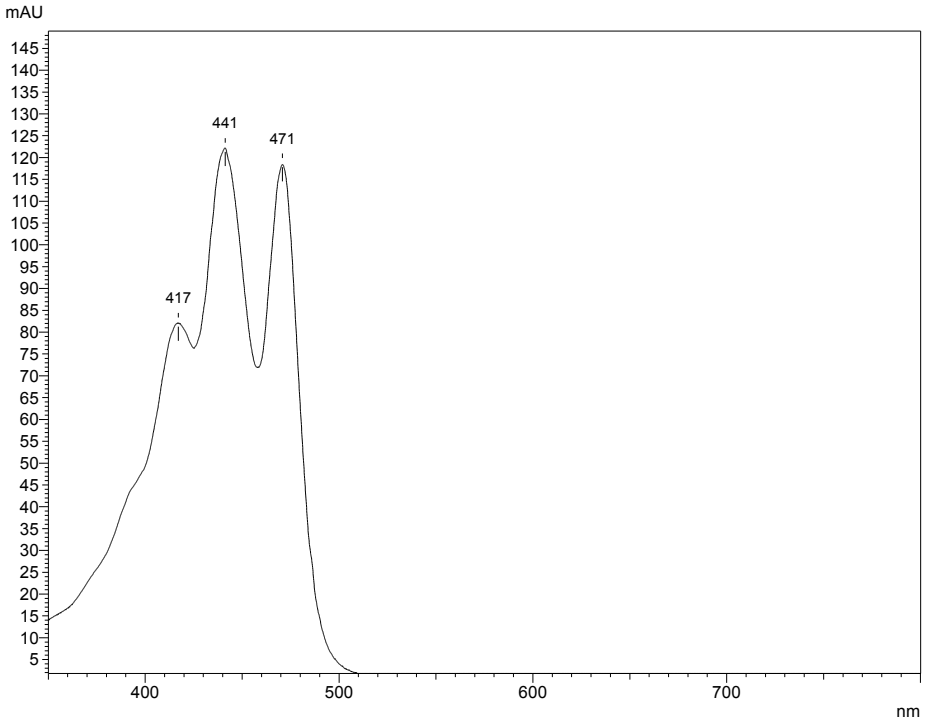
Siphonaxanthin-dodecenoate (Siph-do)



trans-Neoxanthin (t-Neo)



Violaxanthin (Viola)



Zeaxanthin (Zea)