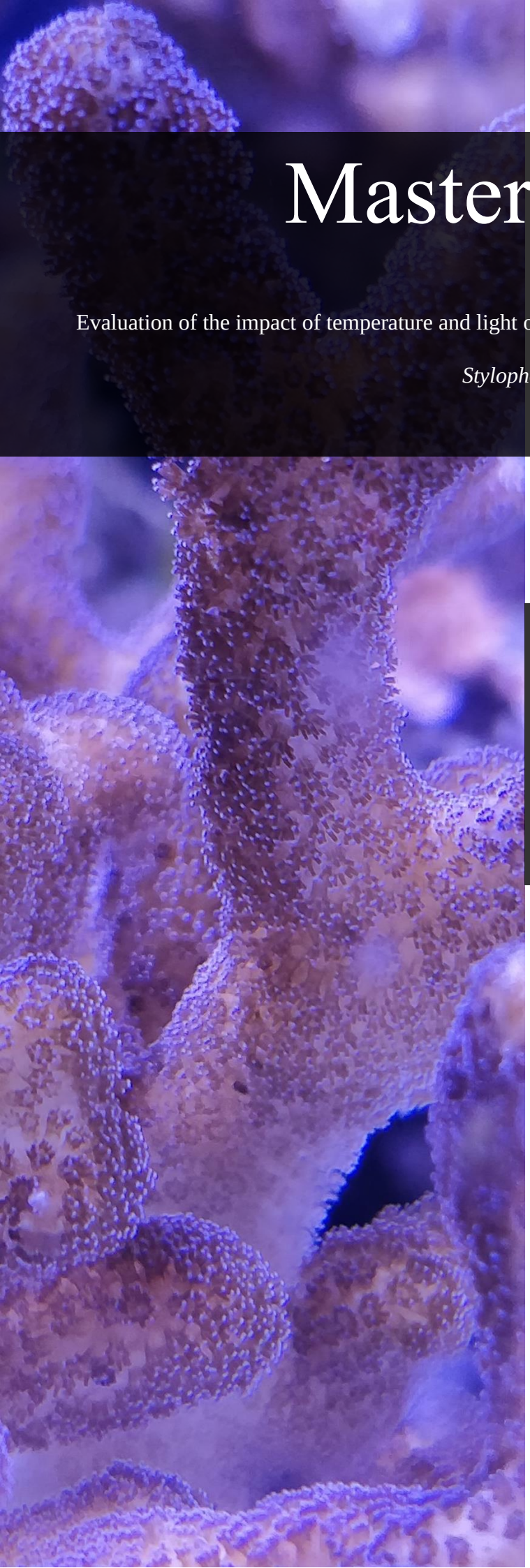


Master's thesis

Evaluation of the impact of temperature and light on bioenergetics (photosynthesis/respiration) in the coral *Stylophora pistillata*

Laboratory of Genetics and physiology of Microalgae

Supervised by Pr. Pierre Cardol

Tutor : Royen Edmée

Academic year 2021-2022

Collard Olivier. Master's student in Oceanography

LIÈGE université

1 TABLE DES MATIERES

2 LIST OF ABBREVIATIONS

acpPC = chlorophyll a – chlorophyll c2 – peridinin protein complex

AEP = alternative electron pathway

CEF = cyclic electron flow

CF = coral fragment

CN = coral nubbin

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

NPQ = non photochemical quenching

PPFD = photosynthetic photon flux density

PSU = practical salinity unit

ROS = reactive oxygen species

DD/DT = diadinoxanthin/diatoxanthin cycle

rETR = The relative electron transport rate

Evaluation de l'impact de la température et la lumière sur la bioénergétique (photosynthèse/respiration) chez le corail *Stylophora pistillata*

Étudient : Collard Olivier
Année: 2021 - 2022
Laboratoire: Génétique et Physiologique des microalgues Phytosystems (ULiège)
Promoteur: Pr. Pierre Cardol

La symbiose entre cnidaires et Symbiodiniaceae est une symbiose modèle qui prospère depuis des centaines de milliers d'années. Leurs associations ont donné naissance aux biotopes parmi les plus riches en biodiversité, les récifs coralliens. Chaque partie de l'association tire un bénéfice non négligeable. Les Symbiodiniaceae lèguent une partie des produits de leur photosynthèse tandis que l'hôte fournit des nutriments essentiels, une place stable dans la colonne d'eau et une protection contre les brouteurs. Cette symbiose est cependant très fragile et sensible aux différentes agressions extérieures. Les stress thermiques est lumineux sont les deux facteurs les plus connus car ils induisent de nombreux dommages au sein de l'appareil photosynthétique et sont déstiné a augmenter avec le réchauffement climatique. La rupture de cette symbiose conduit à terme à la perte des Symbiodinaceae de l'hôte corallien induisant à un phénomène appelés « blanchissement ». Ce mémoire porte sur l'évolution de la bioénergétique du corail *Stylophora pistillata* en réponse à un long stress thermique/lumineux. Nous avons d'abord soumis le corail à un court stress chaleur/lumière de 3 jours afin de déterminer la température adaptée pour la réalisation du stress long. Nous avons constaté que la lumière (obscurité et lumière ambiante) ainsi que la chaleur (28°C, 30°C, 32°C, 34°C) avaient un impact considérable sur la bioénergétique du phostosytème II de *Stylophora pistillata*. La photosynthèse a également été significativement réduite lors d'une exposition à un stress thermique et lumineux de courte durée. Pour le stress long, la présence de plusieurs défauts dans la mise en œuvre du montage a empêché l'obtention de résultat concluant. La configuration étant un système fermé, le facteur externe tel que le climat et la chaleur de lampe ont eu un grand impact sur la stabilité de la configuration de la température. De nombreux autres problèmes sont également survenus en dehors du principal. Le stress lumineux appliqué n'était pas homogène sur l'ensemble du bac. Ce travail a cependant permis

la mise en lumière des bonnes pratiques à mettre en place pour la réalisation d'un setup de stress élaboré. Setup qui n'a jamais été réalisé auparavant au sein du laboratoire de Génétique et physiologie des microalgues.

Evaluation of the impact of temperature and light on bioenergetics (photosynthesis/respiration) in the coral *Stylophora pistillata*

Student: Collard Olivier
year: 2021 - 2022
Laboratory: Laboratory of Genetics and Physiology of Microalgae (ULiège)
Promotor: Pr. Pierre Cardol

The symbiosis between cnidarians and Symbiodiniaceae is a model symbiosis that has been thriving for hundreds of thousands of years. Their association gave birth to the most diverse biotopes, the corals reefs. Each party of the association derives a non-negligible profit from this symbiosis. The Symbiodiniaceae gives a part of the products of their photosynthesis while the host provides nutrient, a stable place in water column and a protection from grazer. This symbiosis is however very fragile and sensitive to external stress. Heat and light stress are the most well-known factors inducing the rupture of the symbiosis as they induce causes a lot of damage to the photosynthetic apparatus and is destined to increase with global warming. The rupture of this symbiosis eventually conducts to the loss of the Symbiodinaceae from the coral host, leading to the phenomenon called "bleaching". This Master's thesis focuses on the evolution of the bioenergectics of the coral *Stylophora pistillata* in response to a long heat/light stress. We firstly subjected the coral to a 3-day short heat/light stress to determine the temperature adapted for the realisation of the long stress. We found that both light (darkness and ambient light) and heat (28°C, 30°C, 32°C, 34°C) impacted greatly the bioenergetic of phostosytem II of *Stylophora pistillata*. The photosynthesis was also significantly reduced when exposed to short heat and light stress. For the long stress, the presence of several flaws in the implementation of the setup prevented the acquisition of conclusive result. The setup being a closed system, the external factor such as climate and lamp heat had a great impact on the stability of the temperature of the setup. Many other problems also occurred apart from the main one. The light stress applied was not homogeneous over the whole beaker. This work has however allowed the highlighting of good practices to be put in place for the realization of an elaborate stress setup. Setup that has never been done before in the Laboratory of Genetics and Physiology of Microalgae's lab.

3 ACKNOWLEDGEMENTS

Well well well, it is little to say that this master's thesis has been a roller coaster of wonderful and not so wonderful emotion. Between the discovery of a job that I have always dreamed of and the massive death of over 400 hand cut corals fragments in a day, the last 6 month was far from dull. But in the end, this work was an amazing experience that I will always remember of, thanks to the amazing team who accompanied me during this journey.

I firstly want to thank Pr. Pierre Cardol without whom this amazing work that teach me many aspects of bioenergetics and coral biology would not have happened. I also want to thanks of course my favorite climbing and coral genocide acolyte Edmée Royen for her help and follow-up without which I would never be able to overcome this work. The time she took to teach me everything from R programming to Bioenergetics brought me a lot. Moreover, I would like to warmly thanks Stéphane Roberty for his good humor and his indispensable help when setting up the aquariums. Many thanks also to the laboratory of Genetics and physiology of Microalgeae's team who welcomed me with open arms. A special thanks to Gabriel Berger for the care and true love he brings every week to our corals but also for its good cocktail. And at last, I also want to thanks to my parents who have supported me through all my journey and during my little car speeding.

Oh, I almost forgot, thanks Lise Le Vern for parasitizing my room during the last week of writing.

4 INTRODUCTION

4.1 SYMBIOSIS CNIDARIANS/SYMBIODINIUM

Cnidarians are very diverse animals in terms of morphologies, ecological function, and life history, yet, they all have one characteristic in common, which is the presence of a specialized cell type called cnidocyte (Ghorai & Priyam, 2018). Cnidocyte is a type of cell that are used by cnidarians to catch preys or to defend themselves from predators. The cell is composed of a capsule called the cnida. This capsule contains an inverted tube that, with appropriate stimulation, will evaginate and entangle or penetrate the prey to finally release a venom (ANDERSON & MCKAY, 1987).

Symbiosis is defined to be an association where two or more different species live together for a prolonged period. One of the best well-known is the mutualism between the Cnidarian and the dinoflagellate algae of the family Symbiodiniaceae. This symbiosis exists since the Triassic and is today the main builder of the coral reefs ecosystem, which covers around 0.1% of the ocean (Côté & Reynolds, 2006) and hosts an enormous biodiversity as well as providing ecological services.

The algae, also known as the zooxanthellae, live inside the endodermis of its cnidarian host within a vacuolar compartment known as the symbiosome (Sheppard et al., 2018). The symbionts supply the cnidarian with photosynthetic products, providing up to 90% of the host's energy needs (Ghorai & Priyam, 2018). In return, the algae have access to certain nutrients from the waste of the host as well as a stable position in the water column and good protection from the grazers (Davy et al., 2012) (Côté & Reynolds, 2006). The cnidarians can host a great diversity of symbionts, however, each species is generally dominated by a symbiont type (Yellowlees et al., 2008)(Sproles et al., 2019).

This symbiosis between cnidarians and zooxanthellae can be formed in 2 ways. The first is the maternal or vertical transmission. In this case, the symbionts are passed from one generation to another through eggs. The other is the horizontal transmission where the cnidarians acquire their symbionts through their surrounding environment (Yellowlees et al., 2008). In this last case, the symbionts are ingested inside the organism in the same manner that a food particle would. Depending on the recognition of the microbes as good or bad, the vacuole will avoid or fuse with a lysosome (Yellowlees et al., 2008). This recognition

between host and symbiont depends mainly on the interaction between different membrane proteins present on the surface of the host and the microbe. The host expresses a large panel of membrane proteins called pattern recognition receptors (PRRs). These proteins are specialized in the recognition of signature microbial compounds present at the surface of the microbes and are called microbe-associated molecular patterns (MAMPs). Depending on the interaction of these compounds the host will trigger a metabolic cascade to either destroy the microbes or start a mutualistic relationship (Davy et al., 2012).

It is, however, far from being the only condition to establish the mutualism relationship between a microbe and a host. Some studies (Hohman et al., 1982) (O'Brien, 1982) have shown that the cnidarian *Hydra viridis* was able to differentiate heat-killed symbionts from healthy ones. The phagosome containing the heat-killed symbionts was then labeled with some specific markers while the one carrying the healthy one didn't show any label.

4.2 DIVERSITY IN CORALS

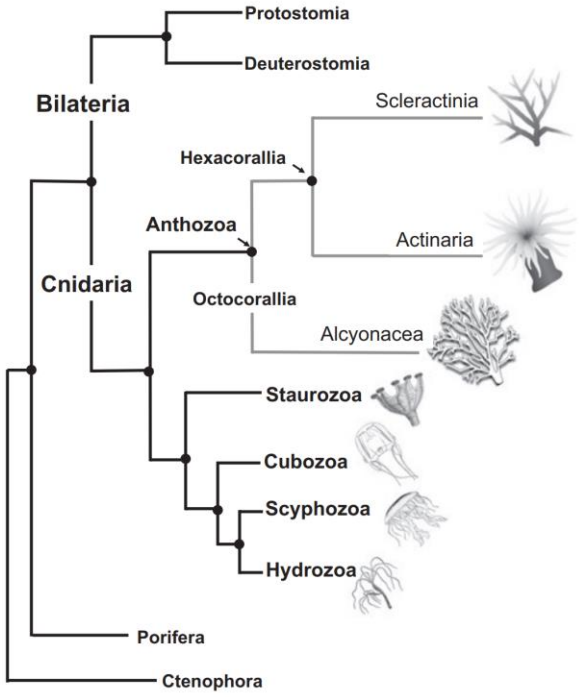


Figure 1 phylogenic tree of the Cnidarians. Retrieved from (Palmer & Traylor-Knowles, 2018)

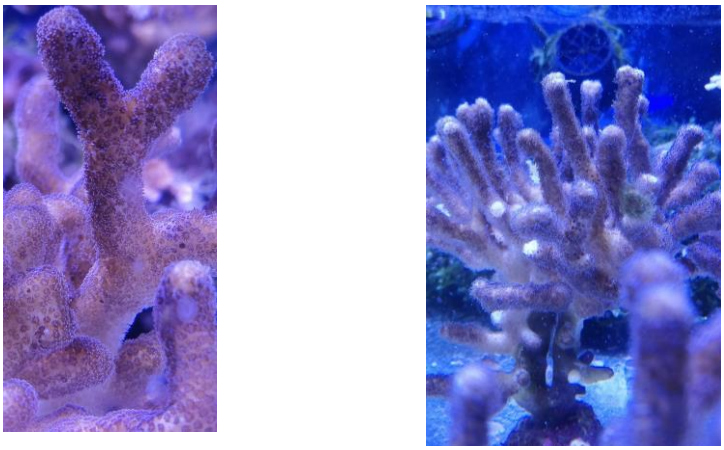


Figure 2 Pictures of the Coral *Stylophora pistillata* taken in the aquarium of the Ecology and Evolution Ecophysiology and animal physiology lab (University of Liège)

Cnidarians are divided into different groups (Ghorai & Priyam, 2018) (**Figure 1**). The class of Anthozoa is the most diverse in the Cnidarian phylum. This class brings together more than 7500 species separated into two different subclasses, Hexacorallia and Octocorallia. The difference between the two generally depends on the symmetry of the polyps. Hexacorallia unites species that have a 6-fold symmetry while Octocorallians have a symmetry of 8. Hexacorallia is then divided further into two groups, the Scleractinians and the Actinarians. Scleractinians, also called hard corals, are the ones that build a hard skeleton and are the founder of the coral reef ecosystem (Ghorai & Priyam, 2018).

This present thesis will focus on the bioenergetics of the Scleratinians Stylophora *pistillata* (Figure 2). It belongs to the family Pocilloporidaea and lives in the Red Sea (Kochman et al., 2021). They generally live on the reef plate until 25 m deep and are a model species when it comes to studying heat stress on corals (Kochman et al., 2021).

4.3 CNIDARIANS AND THE BIOENERGETICS OF PHOTOSYNTHESIS

Photosynthesis and its proper functioning are key to the maintenance and growth of corals.

In plants and algae such as symbiodiniaceae photosynthesis takes place in the chloroplast. The whole protein machinery (see **Figure 3**) involved in the capture of light energy is plugged into a membrane called the thylakoid membrane. To function, the photosynthetic apparatus is composed of three main proteins (photosytheme I, photosysthem II and b6f complex) that work together to exploit light energy (Figure 3) (Allen, 2002). The two reactive centers involved in photosynthesis (Photosystem I and II)are themselves composed of 2 large parts. The first part is made up of a hundred light-harvesting pigments (chlorophylls and carotenoids). The second part is the reaction center which initiates the electron transport from an energized specialized chlorophyll molecule (Allen, 2002).

The main process that drives carbon fixation is the linear electron flow (LEF). When the system receives light energy, electrons get extracted from water contained in the lumen and get carried to NADP+ localized in the stromal side of the membrane. This transfer is conducted by the two photosystems which are the bridge between the photosensitive chlorophyll and an acceptor molecule. The electrons get carried between photosystems II and I through different electron carriers such as plastoquinone (PQ), plastocyanin (PC), cytochrome c6 (cyt c6), or ferredoxin (Fd). This LEF induces the transport of protons from the stroma to the lumen. The energy contained in this proton gradient is then used by the ATP synthetase to produce ATP from ADP+Pi (Eberhard et al., 2008).

Many different electron flows exist within the photosystem apparatus. Unicellular algae in particular have a vast panel of electron pathways that help them adapt and optimize their photosynthesis in a constantly changing environment (Cardol et al., 2011). the ATP produced by the LEF process alone isn't sufficient for to fixation of CO2 (Cardol et al., 2011). Alternative electron pathways (AEP) exist in order to mitigate this ATP shortage. The main AEP is the cyclic electron flow (CEF) that occurs around the PSI. Instead of reducing NADP+ into NADPH, the electrons are brought back into the PQ pool to reach the donor side of PSI

(Eberhard et al., 2008). Hence, there is more transport of proton from the stroma to the lumen without producing NADPH.

Other AEP use oxygen as final electron acceptor through different processes such as chlororespiration and the Melher reaction (Cardol et al., 2011). In the Mehler reaction, O_2 is reduced in superoxide ion (O_2^-). O_2^- is a reactive oxygen species (ROS) that is then converted into water by two enzymes, the superoxide dismutase (SOD) and the ascorbate peroxidase (APX) (Roberty et al., 2014) (Eberhard et al., 2008). The Mehler reaction has two main functions. It has a photoprotective role by offering another electron sink and decreasing the excitation pressure on photosystems as well as an energetic function to increase the ATP/NADPH ratio (Eberhard et al., 2008) (Roberty et al., 2014).

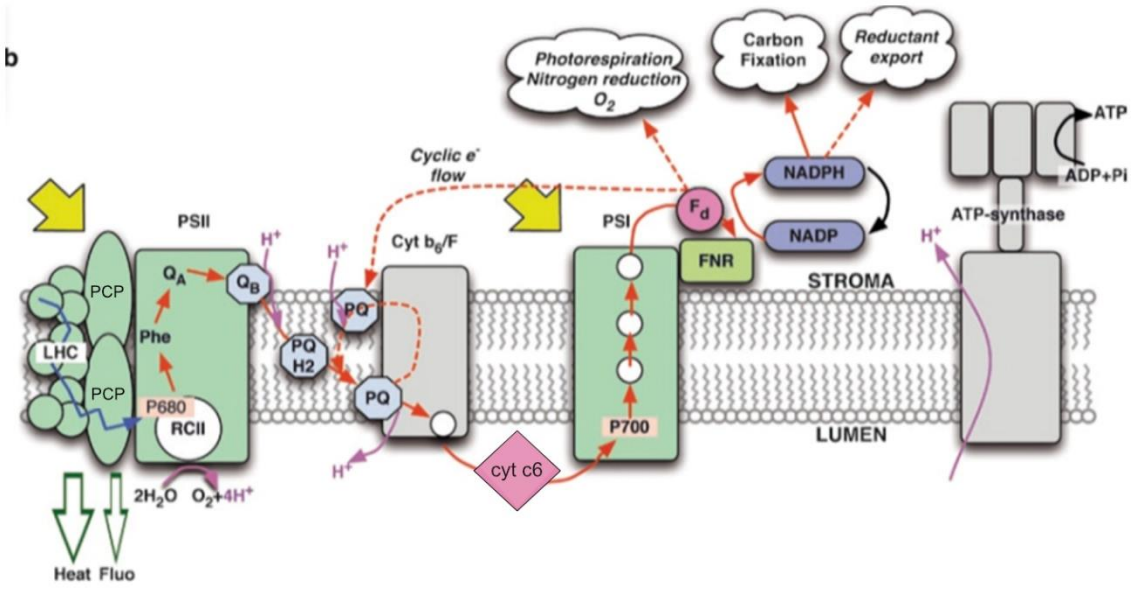


Figure 3 Schematic of the linear electric flow (LEF) and circular electric flow (CEF) as well as the alternatives pathway. Figure retrieved from (Huot & Babin, 2010) and adapted to dinoflagellates. Here the LEF is represented by a solid line and the alternative pathways (CEF amongst others) are represented by a dashed line. PCP stands for peridinin-chlorophyll a protein complex, PQ and PQH2 for oxidized and reduced plastoquinone, Cyt c6 for Cytochrome c6, Cyt b6/f for cytochrome b6/f complex, Qa and Qb stand for quinone a and b and Fd for ferredoxin.

4.4 RUPTURE OF THE SYMBIOSIS

4.4.1 Coral bleaching

Coral bleaching is among the most important process that affects coral reefs nowadays. This phenomenon involves the breakdown of the mutualistic partnership with symbiodiniaceae or at the very least the loss of photosynthetic pigments from the symbiodiniaceae. The final result is that the coral becomes free from pigments, giving the animals a translucent appearance and the sight of the white skeleton beneath the animal (Sheppard et al., 2018). Bleached coral undergoes a reduction in tissue growth, fecundity, and calcification (Sheppard et al., 2018). The survival of the bleached corals becomes extremely threatened but if the stress is short and/or mild, the coral can recover from it. In the current situation, anthropogenic changes, such as high-water temperatures and acidity, get more and more severe, resulting in longer and sharper stress events (Sheppard et al., 2018).

There are different ways in which the Symbiodinium are removed from the host. One way is by degradation of the symbiont under the effects of it's own ROS production. The second way is the withdrawal of the symbionts from its tissue. It can proceed according to different process, by exocytosis of symbionts, host cell detachment, apoptosis, or necrosis (Weis, 2008).

This breakdown can be caused by multiple stressors such as a change in salinity or temperature, an increased sedimentation, and an excess of nutrient or pollutants. The best known are the change in temperature and high solar radiation (Weis, 2008).

The temperature change is a stress of particular interest, as it is mostly linked to climate change (Davy et al., 2012) and corals are very sensitive to it. A small increase in temperature of 1-2°C can produce the bleaching of a whole reef, especially during the season with high luminous intensity (Weis, 2008) (Kochman et al., 2021).

The best known and studied mechanism that explains coral bleaching due to a change in temperature and a high radiance exposure is the **photoinhibition** of the Symbiodinium photosystems. It is, however, not the only phenomenon that is involved in the process of coral bleaching. The loss of symbionts can also occur in complete darkness (Tolleter et al., 2013). For example, the production of nitric oxide (NO), a cytotoxic molecule, by heat-stressed symbiodiniaceae can also alter the health of the host (Sheppard et al., 2018).

4.4.2 Photoinhibition

Photoinhibition is a phenomenon that principally involves an excess of electrons in the photosystems. This excess of electrons in the photosynthetic apparatus leads to the production of reactive oxygen species (ROS) such as singlet oxygen and superoxide (Yellowlees et al., 2008). It is believed that the main source of ROS comes from the Mehler reaction explained above (Roberty et al., 2014). The other alternative electron pathways (AEP) such as the CEF or chlororespiration has a very low contribution to photoinhibition (Roberty et al., 2014).

The two partners of the symbiosis possess multiples mechanisms to handle the production of ROS. For example, both can produce a large diversity of ROS handling enzymes such as ascorbate peroxidase to degrade those compounds (Weis, 2008) (Roberty et al., 2014). However, those mechanisms can only handle a certain amount of ROS, and the phenomenon of bleaching start to occur when this threshold is reached (Weis, 2008).

The ROS produced by the reaction leads to multiple cellular damages such as denaturing proteins and altering the structure of nucleic acids. In addition to the ROS produced by the symbionts, it can also be built within the mitochondrial cell of the host and the algae when there is a heat stress (Weis, 2008).

The excess energy can also be dissipated through the conversion of the xanthophyll diadinoxanthin to diatoxanthin (DD/DT cycle). The two pigments switch from one to another depending on the light intensity. The formation of diatoxanthin is brought by high light intensity while the conversion to diadinoxanthin is induced when the light is low (Eberhard et al., 2008).

4.4.3 Light/Heat stress

As explained above, heat stress coupled with high radiance is thought to be the main responsible party for mass bleaching that occurs at an increasing frequency (Sheppard et al., 2018). The impact of temperature on coral bleaching and thus on photoinhibition comes mainly from its different impacts on the photosystems. Those two stresses can impact photosynthesis in different ways. It can make the PSII protein D1 dysfunctional, destabilize the thylakoid membrane and induce a loss of Calvin cycle activity (Sheppard et al., 2018) (Venn et al., 2006). The D1 protein is a component of the water-splitting complex of the PSII. It is very easily destabilized and thus is considered the sensible point of the photosynthetic apparatus (Weis, 2008). RuBisCo is the most important enzyme in the dark phase. It is the enzyme responsible for the fixation of CO2. Like many other enzymes, its activity is

influenced by different external factors such as temperature. Its activity starts decreasing at 30°C and is almost reduced to zero above 36°C (Lilley et al., 2010). A slowdown of its activity would lead to a slump in the energy flux and an accumulation of electrons. This phenomenon also happens at low temperatures and could explain why corals suffer from bleaching at low temperatures (Sheppard et al., 2018) (Roberty et al., 2014). The degradation of different key proteins within the photosystems such as ATP synthase can also lead to a dysfunction of the electron transport. The photosystems apparatus, therefore, stops the production of NADH and ATP but remain reduced potentially inducing oxygen reduction (Weis, 2008).

All the Symbiodiniaceae species do not react equally to heat/light stress. Some of them are more tolerant to high temperatures giving the host a better resistance to heat/light stress (Sproles et al., 2019) (Sheppard et al., 2018). Corals may even be able to "switch" for Symbiodiniaceae more heat-resistant after a coral bleaching allowing the host to better resist future bleaching (Sproles et al., 2019).

4.5 Study of Photosynthesis

4.5.1 Fluorescence study

The chlorophyll fluorescence is phenomenon that allows the observation of the photophysiological status of algae (Huot & Babin, 2010) (Ralph et al., 2010a). It is a characteristic that has been originally used for plants and that was introduced in aquatic sciences during the 1980s (Huot & Babin, 2010).

Chlorophylls absorb wavelengths in the blue (centered at 430 nm) and the red (centered at 662 nm) regions of the spectrum of light. When absorbing light, chlorophylls can make an electronic transition from the ground to the first or second excited state. Each of these states is also characterized by different substates that correspond to various molecular vibrational energy giving the chlorophyll *a* large absorption band (Huot & Babin, 2010). Energy absorbed by the chlorophyll that are part of the PSI and PSII's reaction center can then be reemitted in 3 different ways. It can drive the photosynthesis, be re-emitted as heat or as light (fluorescence). The **yield of fluorescence** is the percentage of the photons absorbed by the chlorophyll that are re-emitted as fluorescence. It is worth noting that in waveband near 685 nm, the fluorescence comes exclusively from PSII. To measure the fluorescence of PSI, the fluorescence have to be measured at a larger waveband near 700 nm (Huot & Babin, 2010).

When the light hits the algae, the chlorophyll from PSII reaction center, called P680, can transfer one electron to an electron acceptor, a quinone called Qa. Until the transfer of the electron to the next carrier Qb, Qa cannot accept another electron from P680. The reaction center is then in its "closed state" (Murchie & Lawson, 2013). Depending on different conditions such as light intensity or temperature, a larger or a smaller portion of the reaction center will be closed. The more the reaction centers are closed the more the **quantum efficiency** (the part of the energy that is used for photosynthesis) of PSII declines. After the absorption of light energy, the fluorescence then declines gradually. This decline is so-called **quenching** (Murchie & Lawson, 2013)**.**

There are two types of quenching: **photochemical quenching** and **non-photochemical quenching (NPQ)**. The process of photosynthesis takes time to start up in dark-adapted algae. This boot time comes mainly from enzymes that are part of the Calvin cycle and that need to be activated to reach their full activity. As this process unfolds, the sink for electrons rises, the quantum efficiency of photosynthesis increases, and the fluorescence decreases. This quenching comes from the process of photosynthesis itself and is called **photochemical quenching** (Murchie & Lawson, 2013).

Non-photochemical quenching (NPQ) is the energy sink that comes from anything but the photosynthesis process and fluorescence. It is a photoprotective mechanism that takes place when the excitation energy contained in the chlorophyll is too high, engendering the production of free radicals such as ROS (Murchie & Lawson, 2013). One of the processes included under the terms NPQ occurs when the acidification of the lumen becomes too high due to a strong proton supply from the stroma. This NPQ response involves the diadinoxanthin (DD)/diatoxanthin (DT) cycle explained above (Ralph et al., 2010b).

When it comes to chlorophyll analysis, several values/ parameters need to be measured. To achieve that, a fluorescence imaging system is used. The device uses the concept of "dark pulse". It generates an actinic light that is cut just before the measurement and re-established right after the image has been captured. The fluorescence measured is triggered during this break of actinic light, by a weak blue light, that is cut right before the actinic light is back on. The sensor has a red filter that allows the filtering of the red fluorescent light from the blue excitation light.

On an unstressed dark-adapted sample (there is no NPQ and all the reaction centers are open), when the weak blue measuring light is applied, only a small part of the energy received

by the system is reemitted as fluorescence. This low fluorescence signal is called **F0** (Ralph et al., 2010b)**.**

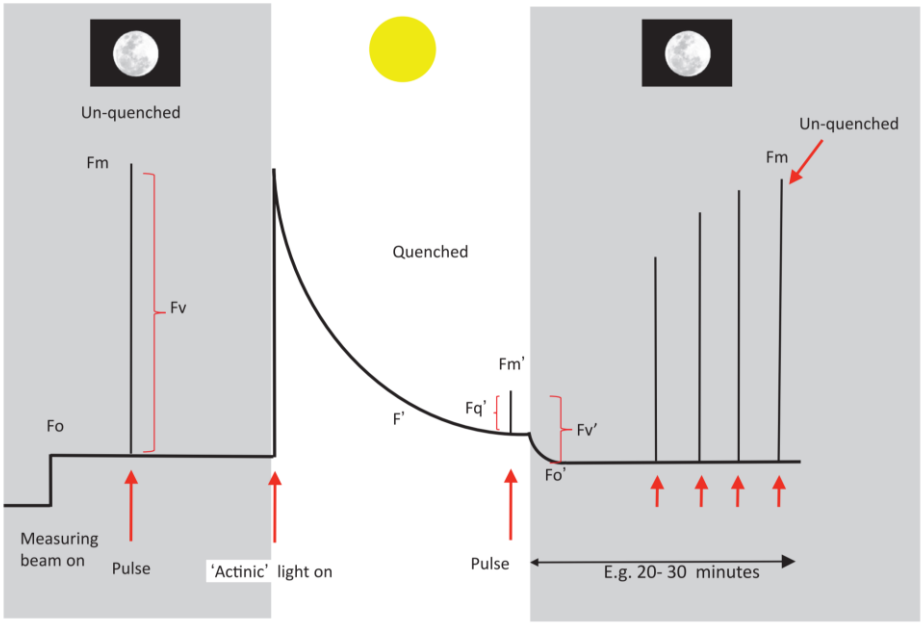


Figure 4 **Model fluorescence plot obtained by measurement on a fluorescence imaging system.** The figure retrieved from (Murchie & Lawson, 2013) shows all the different parameters that can be obtained through fluorescence analysis. Each red arrow represents a pulse of light.

After measuring the F0, a brief saturated flash (<1s) is applied to the sample. All the reaction centers of the PSII switch from their "open" states to their "close" state. Therefore, no electron can be transferred to Qb and all the energy received from the weak detecting light is re-emitted as fluorescence. Since the sample is not adapted to light, no NPQ has been set up, meaning that the fluorescence is at its peak intensity. This value is called **the maximal fluorescence (Fm)** (Ralph et al., 2010b).

Upon measurement of those two fluorescence points, the same process can be repeated multiple times. (Ralph et al., 2010b). When the sample is exposed to actinic light, part of the Qa electron acceptors is closed as photosynthesis has been activated. Fluorescence triggered by the weak blue detecting light is therefore increased compared to F0 and called F0'. When a saturating flash is applied, the fluorescence triggered reaches a peak in a similar way as in the dark-adapted state, but other processes influence its value. Indeed, if the actinic light step was long enough to trigger NPQ processes, the value of the peak F'm is reduced compared to Fm, as the maximum energy entering the system is reduced (Ralph et al., 2010b).

From these four values of fluorescence (Fo, Fo', Fm, Fm') other parameters can be calculated. The difference between Fm and F0 is the **variable fluorescence (Fv). Fv/Fm** is a good parameter used to determine the maximum quantum yield of the PSII. On unstressed samples, this parameter is quite consistent. However, when the sample undergoes stress-inducing PSII damage, the Fv/Fm decreases, making Fv/Fm a good indicator of stress (Ralph et al., 2010b). The difference between Fm' and F0' is called **Fv'**. **Fv'/Fm'**, also known as Y(II), allows the determination of the effective quantum yield of PSII. This parameter indicates the portion of the reaction center that is in an "open" state (Ralph et al., 2010b). The relative electron transport rate (**rETR)** can then be calculated by multiplying the Y(II) by the photosynthetic photon flux density (PPFD) deployed by the actinic light. The rETR indicates the rate of electron transport through the PSII depending on the intensity of the light (Ralph et al., 2010b).

(Fm-Fm')/Fm' is a parameter that allows estimating the intensity of NPQ. However, this parameter should not be compared between samples with different Fv/Fm. The result of (Fm-Fm')/Fm' varies depending on the PSII damage induced, meaning that the comparison between two different samples with different Fv/Fm will not be possible (Ralph et al., 2010b).

4.5.2 Study of the absorbance of P700

To measure the parameters related to PSI activity, other methods must be used. The reaction center of the specialized chlorophyll of PSI reaction center, P700, has an absorbance spectrum that changes depending on its redox state (Klughammer & Schreiber, 2008). Unlike PSII, the fluorescent yield of PSI is independent of the state of the reaction center of the reaction.

A Joliot-type spectrophotometer (JTS) is used for the measurement of PSI parameters. The device possesses 2 different modes: fluorescence and spectrophotometer.

The fluorescence mode works similarly to the one explained above.

The spectrophotometer mode works differently. First, a pulse of light is emitted by a weak light. The beam of light is then divided into two beams by a semi-transparent mirror. One of the beams will be measured by the reference detector while the other will go through the sample and be measured by the principal detector. The difference between the two will determine the absorbance of the sample. When the actinic light is off, the P700 is in its reduced state. At 703 nm, the absorbance of P700 is at its maximum and is called **P0** (Klughammer & Schreiber, 2008). When the actinic light is switched on, a part of the reaction centers of the P700 becomes oxidized. The absorbance measured at this state is the factor **P**

(Klughammer & Schreiber, 2008)**.** Then, a pulse of a saturating light is emitted to oxidize all the reaction centers that are active inducing a signal of absorption called **Pm'** (Klughammer & Schreiber, 2008). Since photosynthesis is active, the PSI keeps receiving an electron flow from PSII preventing them from being completely oxidized. To obtain the absorption signal of the oxidation from all the reaction centers of PSI 2 different methods exist. First, the PSII can be inhibited by using (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (DCMU) which blocks the electron flow of PSII (Klughammer & Schreiber, 2008). The second method consists in sending an far-red light to fully oxidize the PSI reaction centers. By applying one of the two methods the maxima signal **Pm** can be obtained (Klughammer & Schreiber, 2008). It is worth mentioning that the application of the DCMU is a destructive method. In this present work, the second method will be preferably used but it is worth noting that the far-red method is not as efficient as the DCMU method. Some algae possess antennae that can adapt to the far-red making it difficult to fully oxidize the PSI.

These various factors make it possible to determine different parameters. The effective quantum yield of PSI **Y(I)** can be determined as the fraction of open reaction centers (Pm'-P) over the amount of P700 (Pm-Po). The general formula gives Y(I)=(P'm-P)/(Pm-Po) (Klughammer & Schreiber, 2008). In the same way, as for PSII, the rate of electron transport through PSI ($rETR_{PSI}$) can be calculated by multiplying Y(I) by the PPFD (Klughammer & Schreiber, 2008).

5 RESEARCH OBJECTIVES

The main goal of this work was to measure different bioenergetic parameters on the reef-building coral *Stylophora pistillata* during light/heat stress. The main experiment was to subject the coral to heat/light stress for 4 weeks. We measured different bioenergetic parameters using various methods such as oxygen exchange, HPLC, and fluorescence/absorption measurements. The results of the work were, however, compromised by various incidents in the stress setup. The work will thus partially focus on the problems encountered throughout the experiments and the adjustments made to overcome those issues.

6 METHODOLOGY

6.1 BIOLOGICAL MATERIAL

Coral colonies of *Stylophora pistillata* variety Milka were obtained from Dejong Marinelife, an aquatic pet store located in the Netherlands. The corals were maintained in a 300 L tank

filled with artificial seawater with a salinity of 34 PSU (Coral Pro Salt, Red Sea Fish LTD) for the duration of 2 month. The temperature of the aquarium was set at 26°C and subjected to light of 100 µmol photon m^{-2} s^{-1} using a LED spotlight (A360W tuna Blue, Kessil, USA) and with a light-dark cycle of 12h:12h. the corals were also fed 3 times a week with freshly hatched artemia.

6.2 CORAL FRAGMENTS AND PIECES PREPARATION

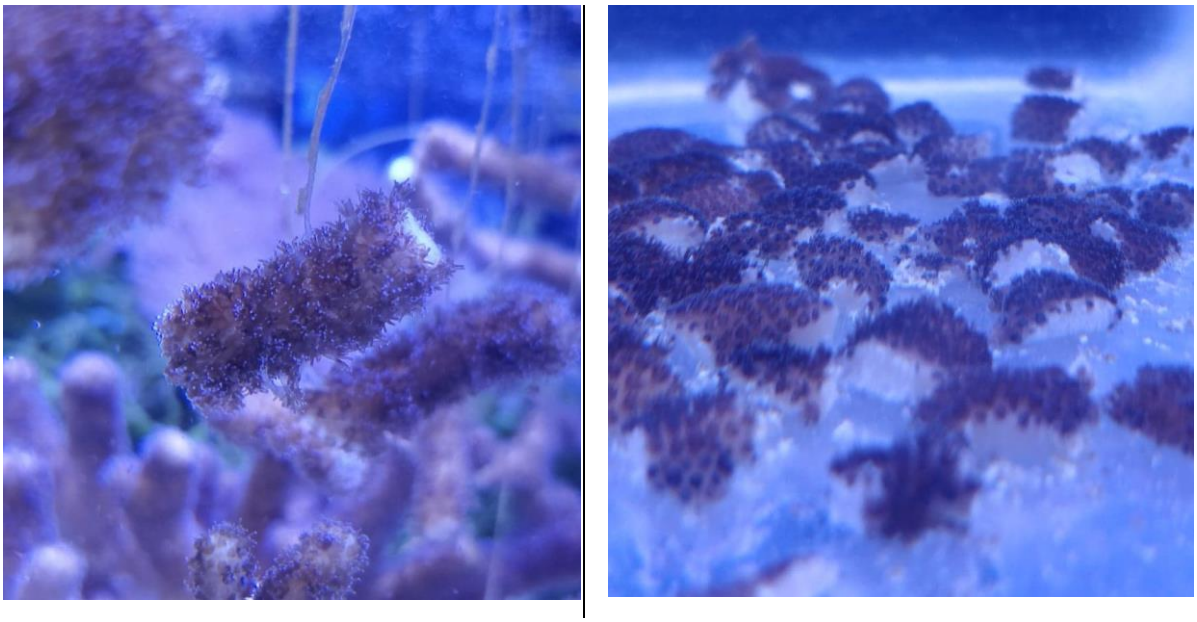


Figure 5 Pictures of the two different sample types used during this work. Left is a picture of a Coral nubbin hung with a nylon thread. Right is Coral Fragments placed on the skeleton side.

In this work, two types of sample preparation were used (Figure 5). Coral fragments (CF) fit for bioenergetics measurement were about 5 by 5 mm. The fragments were cut from the colonies using a Dremel 8220 (Dremel, USA) fitted with a diamond disk. For the extraction of the coral fragments from the colonies, a transversal cut was performed at the top of the coral branch to remove the growing part of the branch (if there was any). The second cut of about 5 mm was then performed longitudinally on the branch before cutting away the fragment from the coral. The fragment obtained was in the shape of a half cylinder, with a side exposing the polyps to the water and the side of the calcareous skeleton facing the bottom. This shape allowed the use of bioenergetic devices and turned out to be a convenient way to drop them off the bottom of the tank without crushing the polyps. This method of sampling has already been used by Félix Vega de Luna et al. (Vega de Luna et al., 2020) for the same purpose.

Coral nubbins (CN) were necessary for experiments requiring more biological matter such as HPLC. The pieces were about the length of 1 cm and were also cut using the same Dremel.

CNs were then hung in the aquarium tank using Nilon given that the sample did not have a polyp-free side where the sample could be laid.

6.3 General stress setup

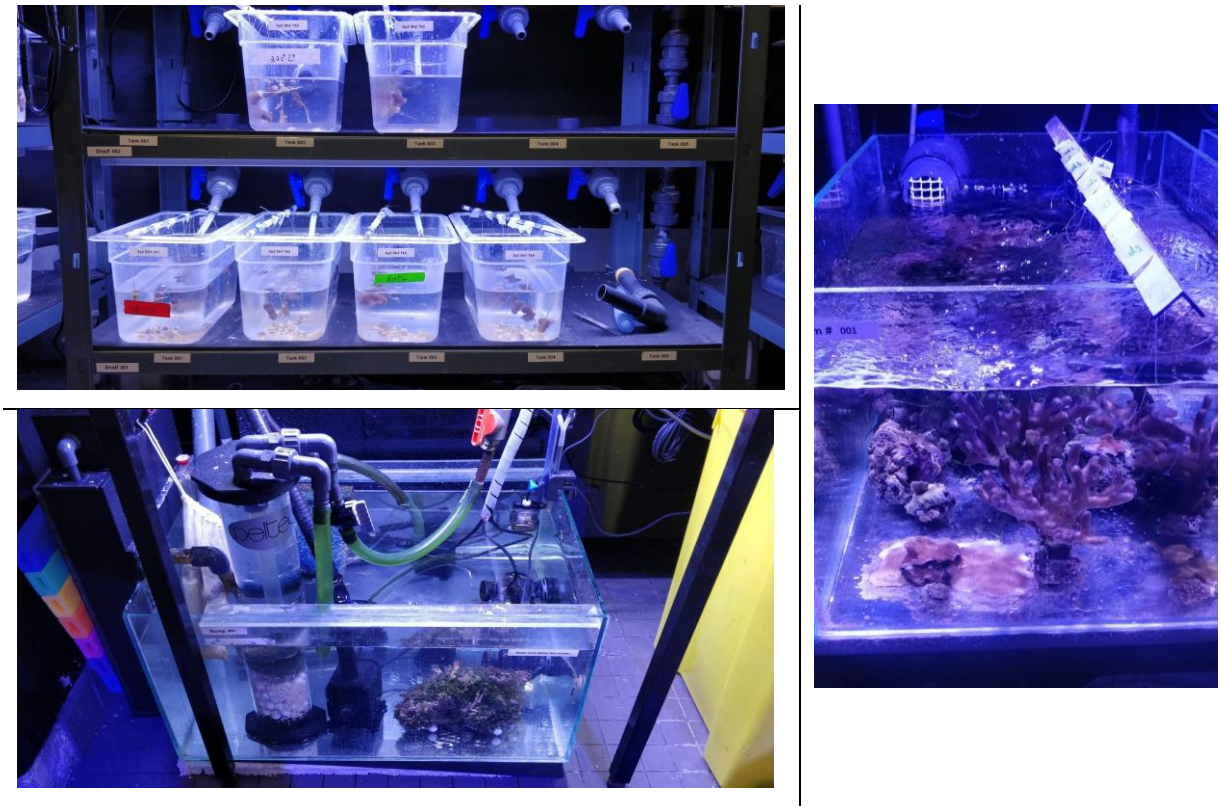


Figure 6 pictures of the different tanks that compose the stress setup overall. Upper left is the shelf used to place the different beakers supplied in water by the sump tank (bottom left). At the right is the reserve tank where the different colonies were placed before being sampled.

The stress setup (**Figure 6**) consisted of different beakers spread over 2 shelves and supplied with water through a pipe system. Each beaker could contain 5 liters of water and could be removed and adjustable according to the need. The supplied water is contained in two sump tanks in which the various instruments for controlling the conditions of the water are put in place. The sump tank was a 30 liters tank that contained a heater (Aquamedic, 200W), a thermometer, a protein skimmer, a stone retrieved from the reserve aquarium, a bacteria filter, and a water level adjuster (Aquastat) The stone has been retrieved from the reserve aquarium and placed in the sump tank to provide the water with healthy microbial flora. The reserve aquarium is a 300 L tank described above in section 6.1. The Aquastat is a device that supplies the sump tank with distilled water as the water evaporates. It allows us to maintain the same level of water and particularly the same salinity.

Each shelf has a sump tank where the temperature can be set for all the beakers above. The shelves were subject to a 12 hours light cycle using a spotlight (100 µmol photons m^{-2} s^{-1}) (solar stinger marine sun strip 70w) attached above the beakers.

6.4 GENERAL EVALUATION

6.4.1 cell count

To normalize the results of some experiments such as pigment measurement through HPLC, the concentration of Symbiodiniaceae within the sample was determined. 50 µl of the sample was mixed in 10 ml of Isoton (90g NaCl/50 ml formaldehyde/1L distilled water) diluted 10 times. The concentration of cells was then determined using a cell measuring device (Beckman coulter, Z2 particle count, and size Analyzer).

6.4.2 surface delimitation of fragments

The standardization of oxygen exchange measurement was conducted by measuring the CF surface. The multi-well plate containing the CF was placed over a square A4 sheet and a photo was taken using the camera of a smartphone. The surface of the CF in cm^2 was then determined using the FIJI software (ImageJ).

6.4.3 pigment extract

The raw pigment concentration of the CF was determined by placing the CF in 0.5 mL of 100 % methanol for 24 hours in a cold dark room (4-10°C). The extract was then centrifuged, and the absorbance was measured at 632, 665, and 750 nm using a spectrophotometer (Perkin Elmer, PDA UV/Vis Lambda 265).

The absorbance at 632 and 665 nm allow the determination of the concentration of chlorophyll *a* and c2 by the 2 following formula.

$$chll\ a = (abs\ 630\ nm \times 3{,}451) + (abs\ 665\ nm \times 13{,}6849)$$

$$chll\ c2 = (abs\ 630\ nm \times 32{,}9371) + (abs\ 665\ nm \times -7{,}0140)$$

$$chll\ tot = chll\ a + chll\ c2$$

The absorbance at 750 nm allows the detection of impurity and should be close to 0.

6.5 PHOTOSYNTHETIC PARAMETERS MEASUREMENT

6.5.1 PSII variable fluorescence measurement

For this work, the fluorescence of PSII was determined using a “pump-and-probe” fluorescence imaging system (Speedzen, BeamBio/API, France) or with a Joliot-type spectrophotometer (JTS-10 (BioLogic, France). The pump-and-probe method consists of the alternation of probe flashes to determine the fluorescence without inducing photosynthesis and short pump flashes to saturate the PSII reaction centers (Huot & Babin, 2010). The probe flashes were emitted by blue LEDs (light-emitting diodes 450-470nm) and the pump flashes as well as the continuous light steps were performed by LEDs at 660nm. The fluorescence was measured by a CCD camera (UI-3240CP-NIR-GL Rev.2, IDS, Obersulm, Germany) equipped with a long-pass red filter (>685 nm).

Different parameters can be retrieved from this equipment such as the short photosynthesis irradiance (PI) curves from rETR of PSII as well as the Fv/Fm using the different formulas explained in section 4.5. The light sequences used for this method can be found in the appendices.

6.5.2 oxygen exchange measurement

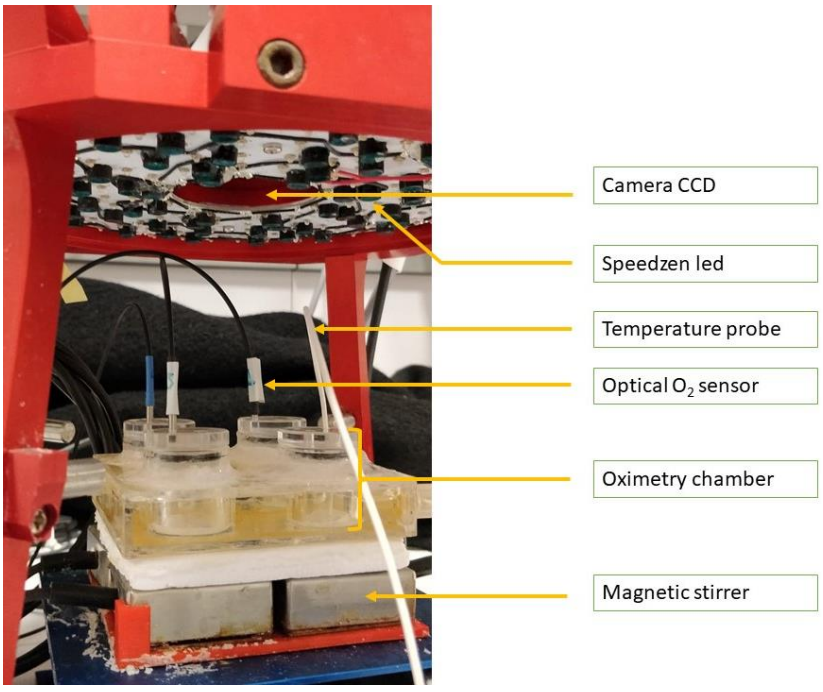


Figure 7 Picture of the setup used for the oxygen exchange measurement. The setup was placed in a dark room to minimize the influence of external light. To reduce the heat transfer between the magnetic stirrer and the oximetry chamber, a plate of styrofoam was placed between the two.

The oxygen exchange measurement was performed using homemade cylindrical chambers (8 mL, 2.54 cm in diameter) with translucent acrylic walls (**Figure 7**). The temperature of the chamber was adjusted through a compartment that surrounded the chamber. The compartment was filled with running water set at the desired temperature. The oxygen concentration inside the chamber was measured with oxygen electrodes (Firesting O_2, Pyroscience oxygen probe) that were placed in the hermetically closed chambers. The saltwater inside the chambers was mixed with a magnetic stirrer separated from the animal using a small plastic table.

The setup was placed under the Speedzen to measure the light-dependent oxygen exchange using a light sequence that can be found in Appendix 10.2. The sequence used in this method is similar to the one used for the PSII fluorescent measurement except that the light steps last for 3 minutes. This change in time sequence allows the determination of the long PI curve from $rETR_{PSII}$ as well as the contribution of NPQ. The light sequence of oxygen exchange starts 10 minutes before the beginning of the data logging to measure the contribution of respiration. This respiration rate was then removed from the result of the oxygen exchange to obtain the net O_2 production.

The result from the oxygen measurement was then normalized to the surface of the coral fragments as well as the content of the total chlorophyll.

6.5.3 P700

To compute several indicators of the state of PSI, the change in absorbance in P700 was measured as explained in section 4.5. Those measurements were performed with a JTS-10 spectrophotometer (BioLogic, France). The detection light that was used to monitor the redox change in P700 was emitted by LEDs in front of which a 705 nm-filter was placed. The actinic light was emitted by LEDs at 660 nm. 2 receiving photodiodes are used for the measurement. One is placed above the sample and the other measures the incident light.

The sequence of light used is presented in Appendix 10.3.

6.5.4 77K

At low temperatures (77 Kelvin), the fluorescence of both the PSI and the PSII become more distinguishable from each other. The fluorescence is also stronger, the fluorescence yield of PSII is 2 times higher and the one from PSI is 10 times higher than at room temperature. Indeed, this temperature, most of the electron transport reaction is inhibited and the electrons accumulate on the acceptor side of the PSI and PSII (Lamb et al., 2018).

For this method, the fluorescence is measured at 77K and between 600 and 800 nm. Two peaks are particularly interesting: The signal at 675 nm matches with the fluorescence of peridinin-chlorophyll protein (PCP), which are soluble light-harvesting complexes (LHC) present in the luminal side of the thylakoid membrane., and at 685 nm, the fluorescence peak is linked to the chlorophyll a-chlorophyll c2-peridinin protein complex (acpPC) and the remaining part of PSII. The acpPC is an insoluble LHC present within the thylakoid membrane. In this work, the ratio between the two peaks 675/685 is used to indicate the state of PSII stress (Lamb et al., 2018).

6.6 HPLC

Symbiodinium was extracted from CN by air-brushing. The method consists in placing the CN into liquid nitrogen to snap freeze the animal tissue and avoid stress in the following step. The frozen CN is then inserted into a small plastique bag containing 1 ml of artificial seawater and the animal tissue is taken off the skeleton using a thin airflow. The Symbiodiniaceae are then separated from the animal tissue through centrifugations. The pigments were then extracted from the algae using dichloromethane-methanol and 500 µm diameter silica balls.

The solution was placed in a bead beater (Tissue Lyser II, QIAGEN) for 2 × 15 minutes at 30 Hz at 4°C. The cell lysate was centrifugated again at max speed for 15 minutes to separate the cellular debris from the pigments. Around 250 µl of pigment extract was filtered on Acrodisc 13 mm with 0.2 µm PTFE (PALL Corporation, USA), transferred into 2 ml glass vials, and diluted with approximately 500 µl of methanol to match the minimum volume of the HPLC sample.

40 µl of the solution was loaded on the Nova Pak column (Waters, USA), which is a C18 on 4µm silicate beads with a porosity of 60 A. The size of the column was 150mm × 3.9 mm (L. x I.D), used on a Shimadzu HPLC (Japan, Kyoto).

6.7 Pre-experiment measurement

6.7.1 survivability of coral fragment

To check that coral fragments survive in our experimental conditions without stress, the PSII fluorescence of multiple CF in the beaker was monitored for 19 days. The beaker was set according to the same parameters of the reserve aquarium (26 °C and at a salinity of 34 PSU and with the same light conditions).

24 CF were cut and placed in two 12-well plates to make their transportation more convenient. One plate was placed in a beaker and the other in the reserve aquarium. The fluorescence of PSII was measured almost every day using the method explained in section 6.5.1. The maximum quantum yield of PSII (Fv/Fm), as well as the $rETR_{PSII}$, were measured to determine if the beaker itself could induce stress compared to the reserve aquarium.

6.7.2 survivability of coral fragments depending on the heat stress

To determine the heat stress that will be used for the 3 weeks experiment, four 1-week heat-stress experiments (28°C, 30°C, 32°C, and 34°C) were made to determine the optimal temperature.

34 CF were extracted from various colonies and placed uniformly in eight different multi-well plates. For each 1-week heat stress experiment, one control plate was placed in a beaker set at 26°C and another in the beaker subject to the higher temperature. The CF PSII activity was monitored using the method described in section 6.5.1 as well as in section 6.5.2. The measurement was taken the days 0, 1, and 3 of the week with day 0 corresponding to Monday.

6.8 LONG STRESS EXPERIMENTS

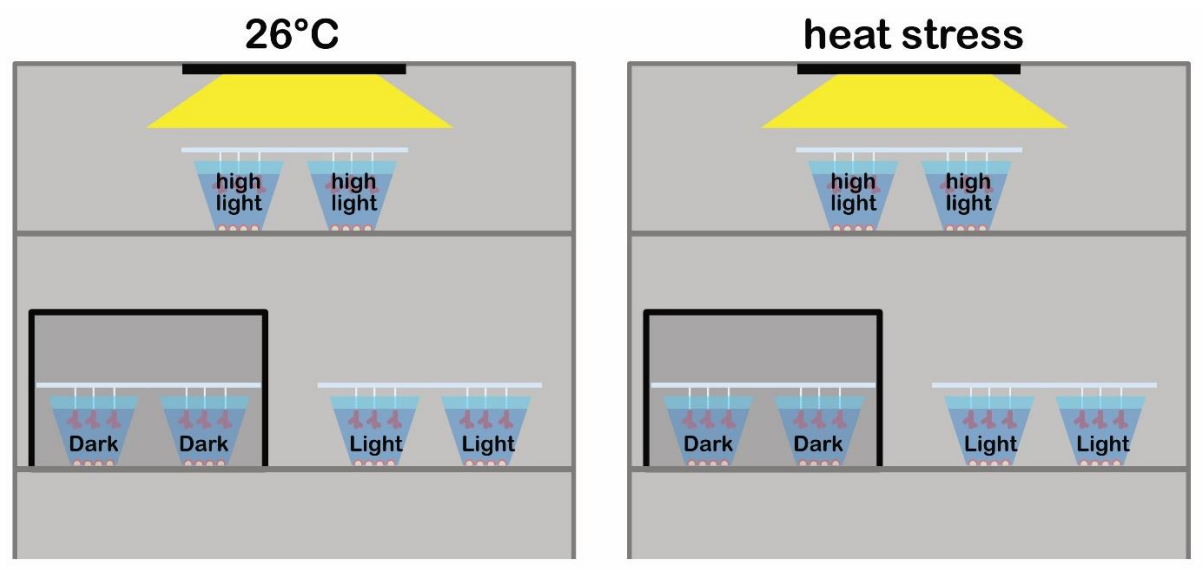


Figure 8 Plan of the setup used for the long-stress experiment. The CF was deposited skeleton facing the bottom of the beakers. The CNs were hanging from a metal bar using a Nylon thread. The dark room was built using 2 cardboard boxes. The high light condition was created using a 2 spotlight (Aquamedic). The conditions were also monitored using data loggers.

For the long 3-week stress experiment, two temperature shelves were put in place, one shelf at 26°C to serve as a control, and one shelf at the heat-stress temperature (**Figure 8**). Each setup of temperature included three separate conditions (Dark, Light, and High light) for a total of

six conditions. Each condition was composed of two beakers where the different samples were placed.

390 CF and 72 CN were extracted from various S. *pistillata* colonies. For each condition, 65 CF and 12 CN were distributed between the 2 beakers. All the samples were used throughout the 4 week's experiment as follows.

Measure	Day	Necessary CF and CN
P700 (PSI activity)	D0, D1, D8, D15, D22, D29	4 CF/cond/day
Oxygen exchange measurement		
PSII activity	D0, D1, D8, D15, D22, D29	5 CF/cond
ECS*	D0, D1, D7, D14, D2, D28	3 CF/cond/day
77K	D0, D1, D8, D15, D22, D29	3 CF/cond/day
HPLC	D0, D1, D8, D15, D22, D29	1 CN /cond/day
FAMES*	D0, D1, D8, D15, D22, D29	1 CN /cond/day
Total		72 CN/ 390CF

Figure 9 Table of the use of samples depending on the method used as well as the measurement day.
* due to complications during the main experiment, the Electrochromic shift (ECS) and the analysis of Fatty acid methyl ester (FAMES) through Gas chromatograph could not be realized and therefore have not been described in the introduction and methodology section.

The same coral fragments were used for the measurement of oxygen exchange and P700 redox change, as these techniques are not destructive. For the 77K fluorescence analysis, the CF was directly placed in liquid nitrogen to snap freeze the CF to then be placed in a freezer maintained at –80°C until the measurement. The same process was carried out for the CN used for the HPLC and FAMES.

7 RESULTS

7.1 SURVIVABILITY OF CORAL FRAGMENTS

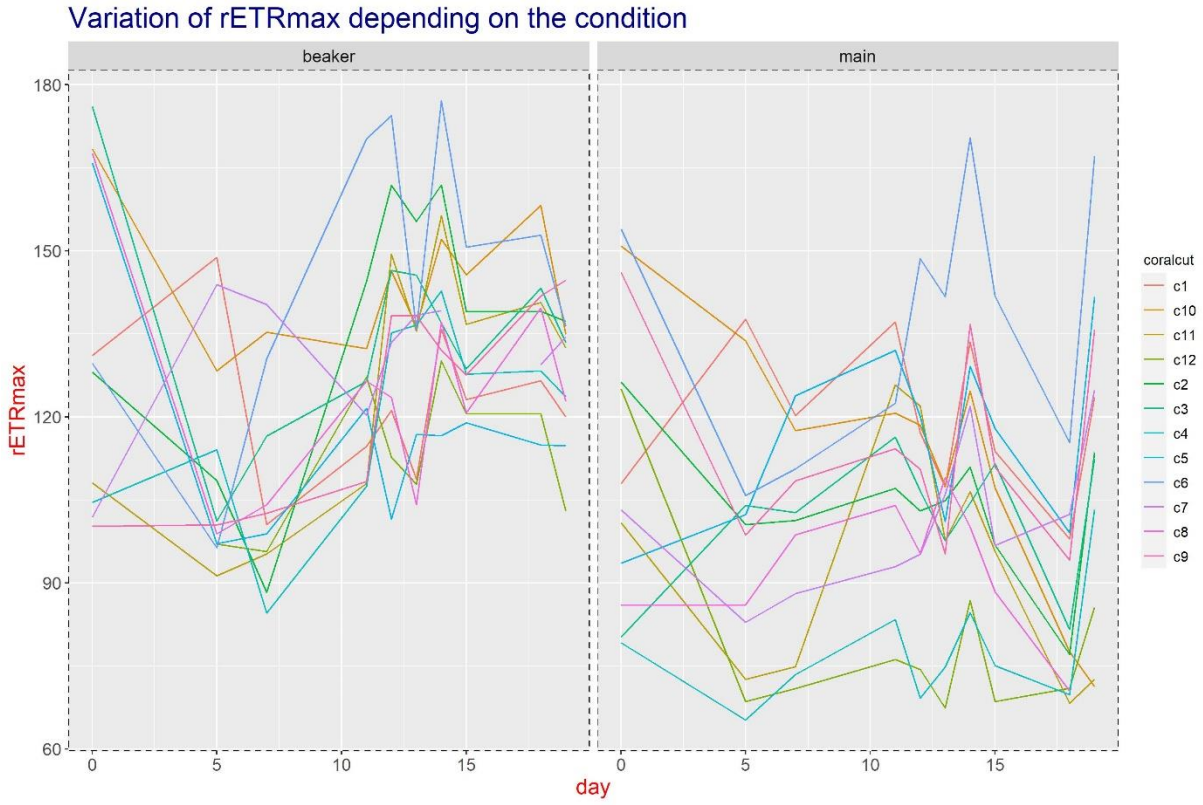


Figure 10 graphic showing the variations of the maximum $rETR_{PSII}$ (in µmol é.m^{-2}.s^{-1}) at PPFD = 700 µE m^2 s^{-1} during 18 days of incubation in an experimental beaker and the reserve tank.

The hypothesis that the incubation in the beaker had an impact on the overall health of the CF compared to the one incubated in the reserve tank was tested by measuring the relative maximal electron transport rate of PSII over days (Figure 10). The statistical test comparing the different setups (reserve tank and beaker) gives a p-value of 0.502 on day 0 and 0.976 on day 19. Both p-values were above the 0.05 threshold indicating that the beaker had no influence on the health of CF compared to the reserve tank. Most of the variation observed on the graph comes from the fluorescence measuring device as some of them are the same for all the CFs.

From these results, we concluded that the CF could be used in the beaker for a long period without a significant impact on their physiological state of health.

7.2 SURVIVABILITY OF CORAL FRAGMENTS DEPENDING ON THE HEAT STRESS

7.2.1.1 PSII fluorescence measurement

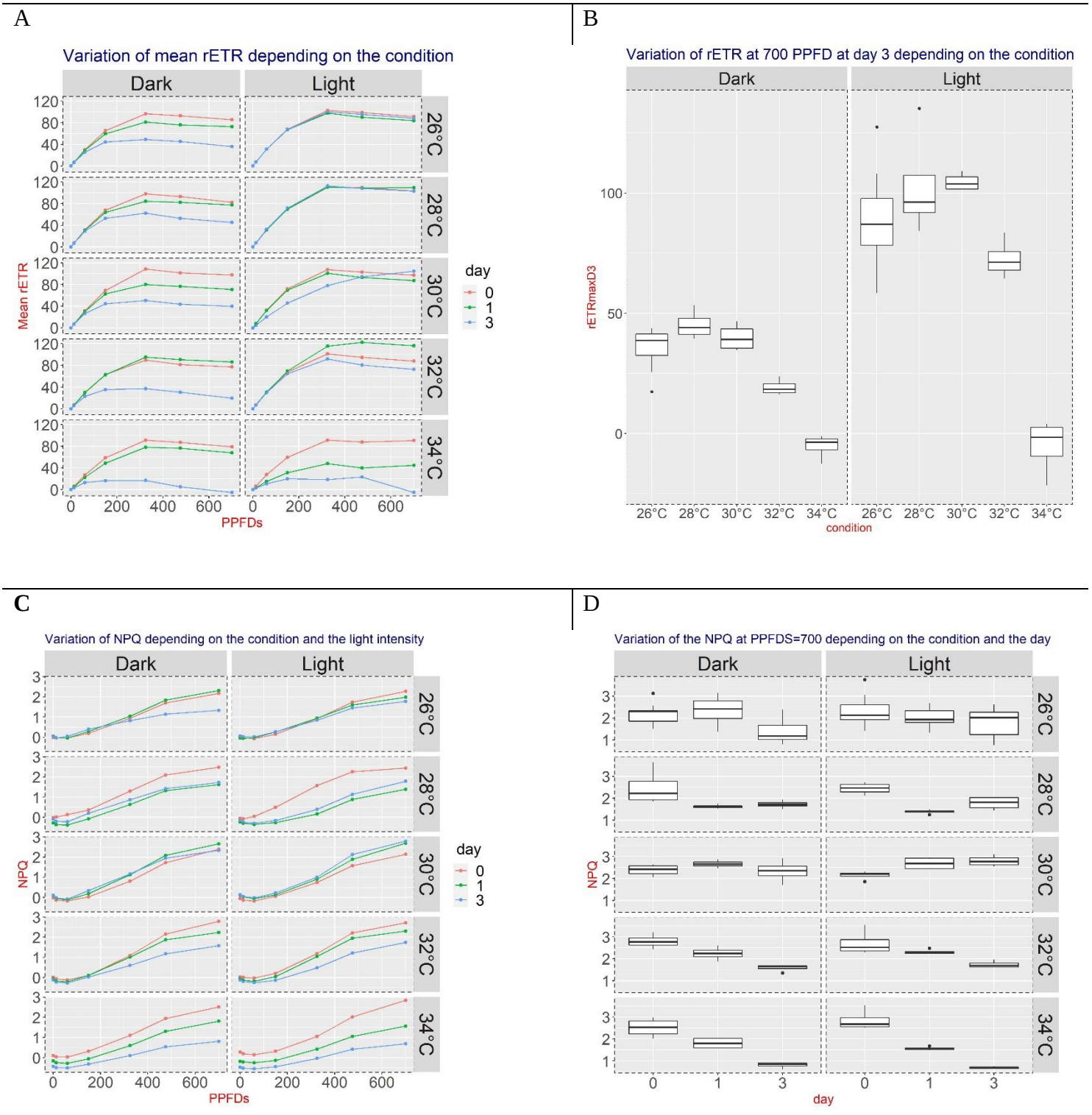


Figure 11 **Graph obtained through PSII fluorescence measurement.** Graph A is the mean $rETR_{PSII}$ variation depending on the light intensity, light condition, and temperature. Graph B is the $rETR_{PSII}$ max (obtained at 700 µE m^{-2} s^{-1}) depending on the temperature and the light condition. Graph C is the variation of the NPQ depending on the light intensity, light condition, and temperature. Graph C is the variation of NPQ at 700 µE m^{-2} s^{-1} depending on the day and the condition.

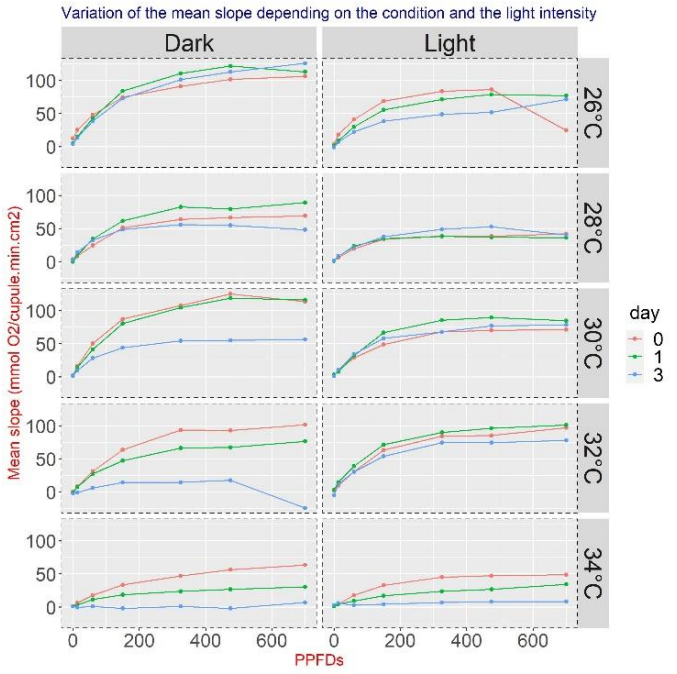
We then evaluated the impact of heat stress on the photosynthetic activity of coral fragments. The results of the short PI curves measured throughout the five 1-week heat stress experiments are displayed in Figure 11 A. Both temperature and light seem to have an impact on the colonies. The light condition has a greater impact than temperature, with a p-value of $4.5.10^{-12}$ when comparing the maximum return-PSII at 26°C between both darkness ($rETR_{PSII}$ = 36 µmol é.m^{-2}.s^{-1}) and light ($rETR_{PSII}$ = 88 µmol é.m^{-2}.s^{-1}). As can be seen in Figure 11 A, the absence of light throughout the week has a clear impact on rETR-PSII, even at 26°C. The temperature does not show a significant impact until 34°C. In Figure 11 B, the values obtained at 28°C, 30°C, 32°C, and 34 °C show a p-value of 0.49, 0.34, 0.37, and $4{,}4.10^{-12}$ respectively at light when we compare it to the control (26°C at ambient light) and 0.93, 0.99, 0.28 and $3.6.10^{-6}$ in darkness. Given that the impact of the treatment at 34°C was far too stressful, a temperature of 32°C was therefore chosen for the long-stress experiment.

From the data collected through the measurement of chlorophyll fluorescence yields during 3 min light step (Appendix 10.2), the NPQ developed in each condition and at different PPFDs could be computed. The NPQ shows a general trend that increases with the light intensity with a maximum of 700 µE $m^{-2}s^{-1}$ (Figure 11 C). At this maximum PPFDs, the NPQ does not show a significant difference whether the sa

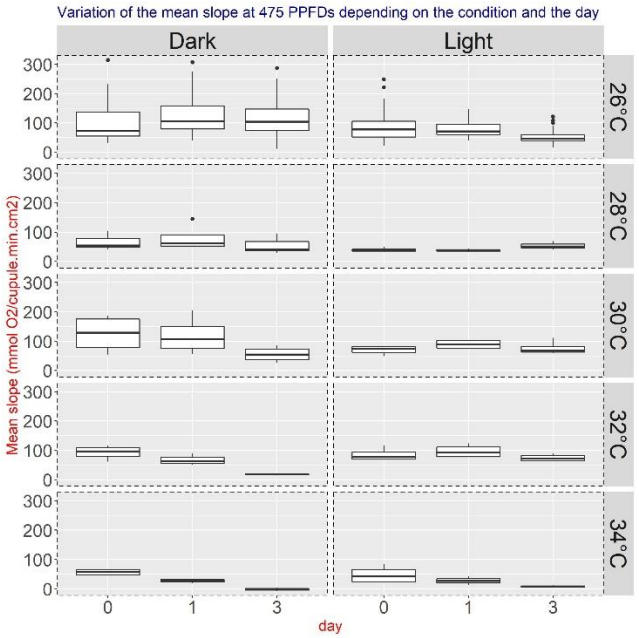
mple is in darkness or normal light in all the temperature conditions. When we compare day 0 and day 3, the main difference occurs at 34°C, in light (NPQ D0=2.85, NPQ D3=0.694) (p-value = 2.10^{-8}) and in darkness (NPQ D0=2.51, NPQ D3=0.8) (p-value = 4.10^{-8}). The NPQ decreases with time at a high temperature which could indicate the impairment of the mechanisms involved in NPQ under the effect of heat, or less likely to reflect an acclimation to high temperature.

7.2.1.2 Oxygen exchange measurement

A



B



C

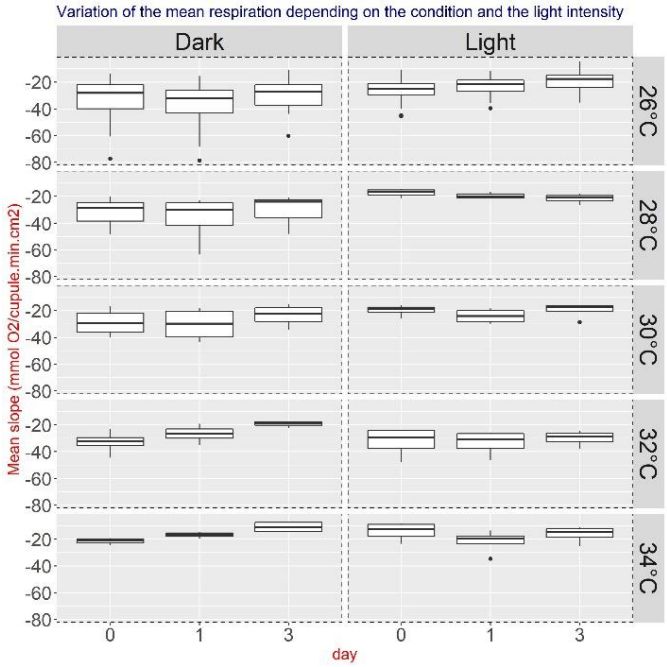


Figure 12 **Graphic obtained through the Oxygen exchange measurement**. Graph A is the mean net photosynthesis at different light intensities depending on the day and the condition. Graph B is the mean net photosynthesis at 475 µE $m^{-2}s^{-1}$ depending on the day and the condition. Graph C is the mean respiration depending on the day and the condition.

The results of oxygen exchange measurements are shown in Figure 12 A. At day 3 and 475 µE $m^{-2}s^{-1}$ (Figure 12 B), the 34°C (net photosynthesis = -1.78 mmol $O2.min^{-1}.cm^{-2}$) and 32°C (net photosynthesis = 17.9 mmol $O2.min^{-1}.cm^{-2}$) Dark conditions show a significant decrease

compare to the 26°C Dark condition (net photosynthesis = 113 mmol O2.min^{-1}.cm^{-2}) (P-value=3.37.10^{-6} and 2.61.10^{-4} respectively) whereas at normal light condition, the two temperature conditions do not show any difference (P>0.05).

After three days of incubation, the respiration rate (Figure 12 C) is significantly increased at 34°C in the dark (respiration = -11 mmol O2.min^{-1}.cm^{-2}) compared to 26°C in the dark (respiration = -29.3 mmol O2.min^{-1}.cm^{-2}) (p-value = 7,25.10^{-4}). The light alone also shows a significant impact on the respiration rate (respiration dark = -29.3 mmol O2.min^{-1}.cm^{-2}) (respiration light = -19.2 mmol O2.min^{-1}.cm^{-2}) (p-value = 3,87.10^{-9}).

7.3 SETUP 1

7.3.1 Setup difficulties

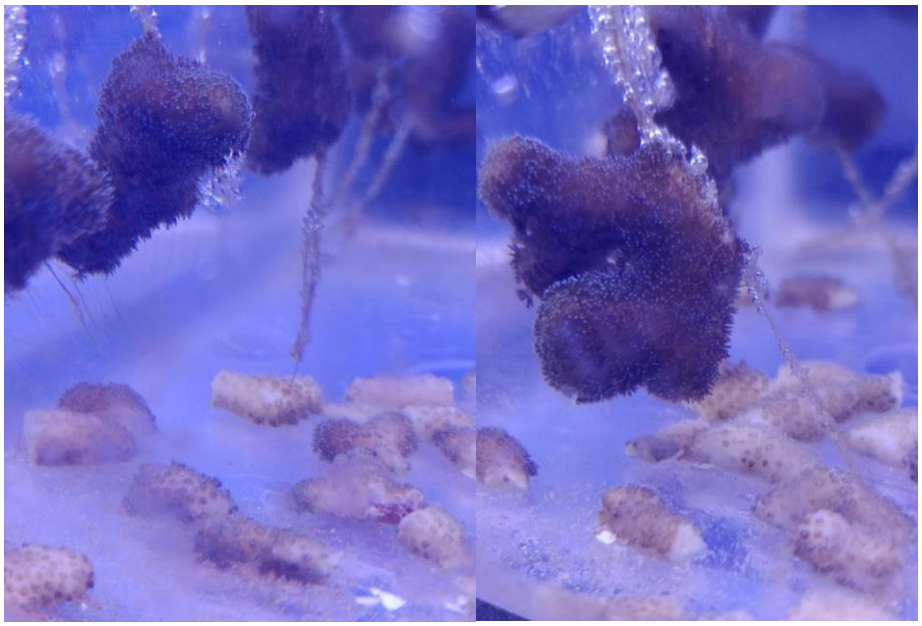


Figure 13 pictures of the CF and CN in the beaker after 5 days of acclimatization at 28°C and subjected to the light of 100 µmol photon m^{-2} s^{-1}.

After 5 days of acclimatization in the beakers before starting the long stress experiment, all the CF had bleached. It is worth noting that only the CF had bleached, the CN remained visually in good health after the acclimatization (Figure 13). This outcome was relatively unexpected considering that the previous experiment explained in section 7.1 gave positive results.

This result may originate from different factors. Firstly, the experiment of section 7.1 was performed in March and the long stress experiment took place in June. The aquariums being stored in an old cold room and the aquarium having no cooling system, the heat had great

difficulty escaping from the system. Unfortunately, the temperature of the beaker was not monitored at that time, since the stress was not triggered yet.

Secondly, this experiment shows that the sampling preparation method does have an impact on the sample's health. As shown in the photos (Figure 13), the CN does not show any bleaching compared to the CF.

7.4 SETUP 2

7.4.1 Setup rearrangement

For the second setup, different adjustments have been made to answer the problem encountered in the first setup. Since the sample preparation method has an impact on the survivability of the sample, all the samples were prepared as CN. Some of them were used entirely at once, while others were partially cut to obtain CF. The temperature of the 26°C beakers was also set 1°C lower since the temperature obtained previously was closer to 27 ° C.

The chlorophyll fluorescence measurements (short PI curve) with the Speedzen were conducted with CNs instead of CFs. The CNs were marked at the extremity of the nylon thread to monitor the same CN throughout the stress. 3 CNs per condition were monitored throughout the experiment.

7.4.2 Setup difficulties

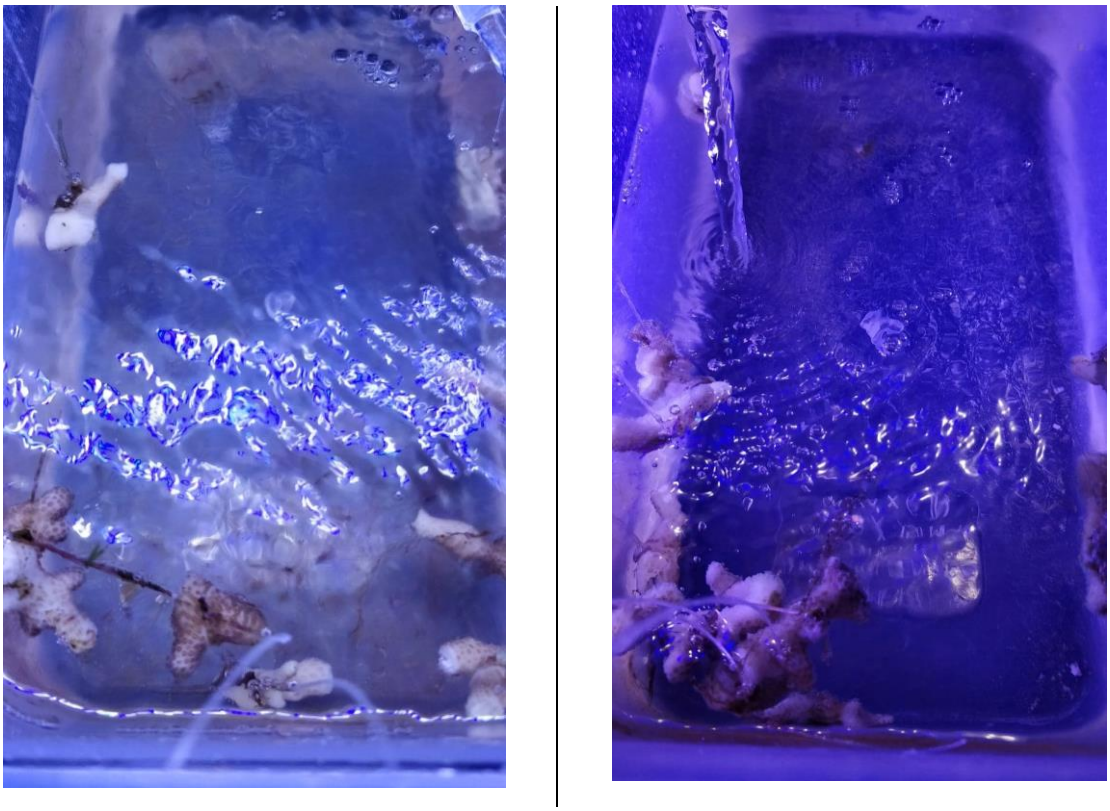


Figure 14 pictures of the CN in the beaker after 4 days of stress. Left is high light at 26°C and right is normal light at 26°C.

Several problems occurred during the first seven days of incubation (**Figure 14**). On June 18 (the fifth day of stress), a strong heat wave occurred in Belgium, raising the temperature in the beakers by 3 °C above the set temperature. Furthermore, On June 19 (the sixth day of stress), a failure occurred at the level of the 32°C beakers water supply pump, preventing water supply in the beakers and causing the temperature to drop drastically.

Given that the heat stress had not been constant throughout the week, the experiment was stopped after 7 days of incubation.

Another minor inconvenience from this setup was the extraction of CF from CN. The CN being around 2 cm long, the extraction of CF with the Dremel was complicated, dangerous, and time-consuming.

7.4.3 Results obtained through the experiment

7.4.3.1 PSII fluorescence

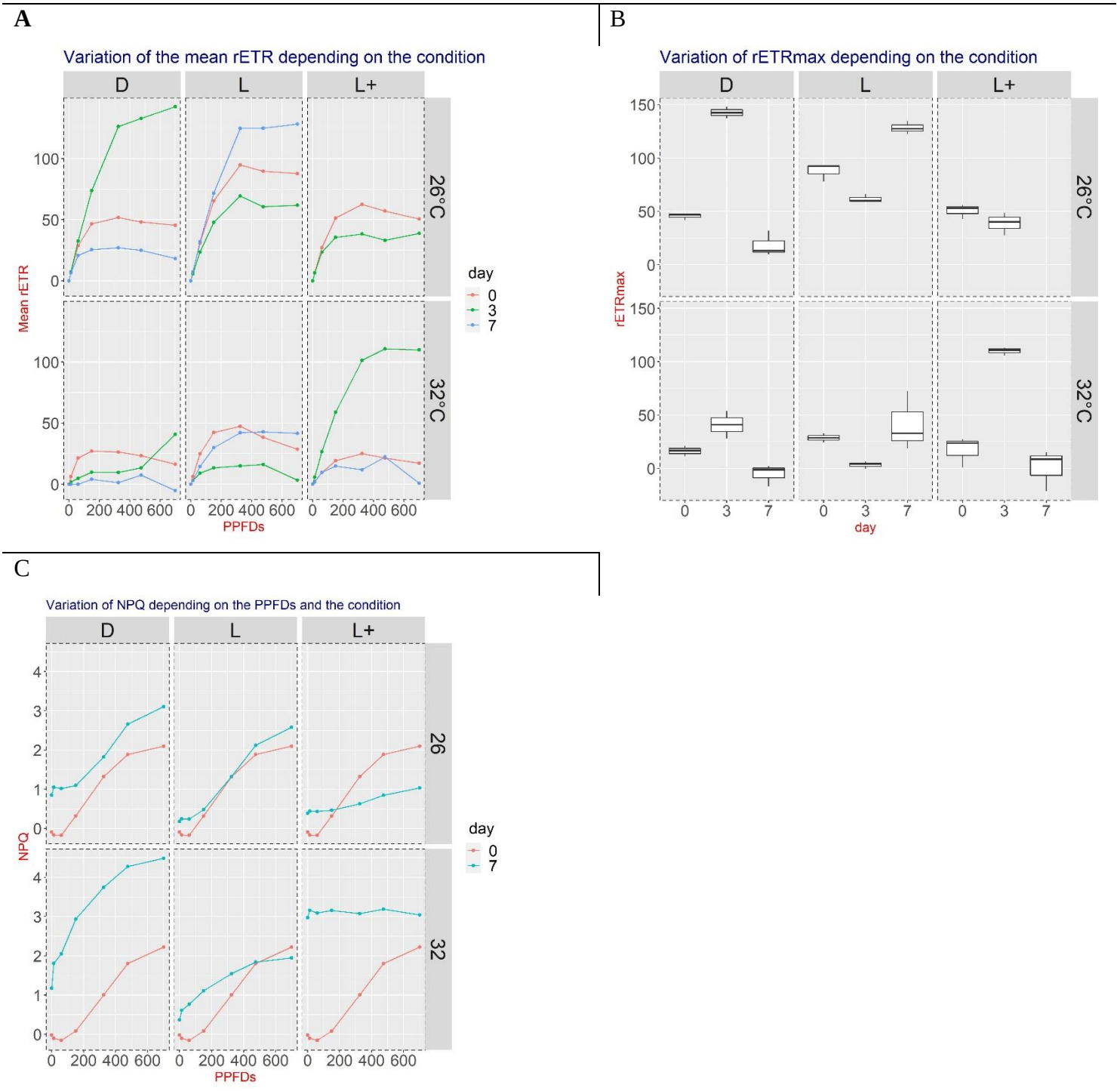


Figure 15 **Graphs obtained from the measurement of the long PI curve of PSII**. Graphic A shows the mean $rETR_{PSII}$ as a function of PPFD depending on the light and temperature conditions. Graph B shows the mean $rETR_{PSII}$ as a function of time depending on the light and temperature conditions. Graph C shows the mean NPQ as a function of PPFD depending on the light and temperature conditions. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

Figure 15 shows the result obtained through the long PI curve produced using the Speedzen with the use of CNs. The condition 26°C Dark shows a stronger $rETR_{PSII}$ on day 3 than on day 0. This result is different than the one obtained in section 7.2 for the same condition. This difference also occurs for the condition 32°C L+ and 26 L. The main explanation is the orientation of the CNs when measuring the fluorescence of PSII as well as a high temperature variability. Since the CNs have a cylindric form and the bleaching phenomena do not occur uniformly throughout the CNs, depending on the orientation of the sample during the measurement, the result may vary significantly. It is also important to mention that the light used for the L+ condition was not perfectly uniform over the entire beaker. Some of the CNs were more exposed to light than others and therefore showed stronger bleaching. This phenomenon was not as severe when using CFs given that the samples were placed at the bottom of the beakers and not hung by a nylon thread.

7.4.3.2 *Oxygen exchange measurements*

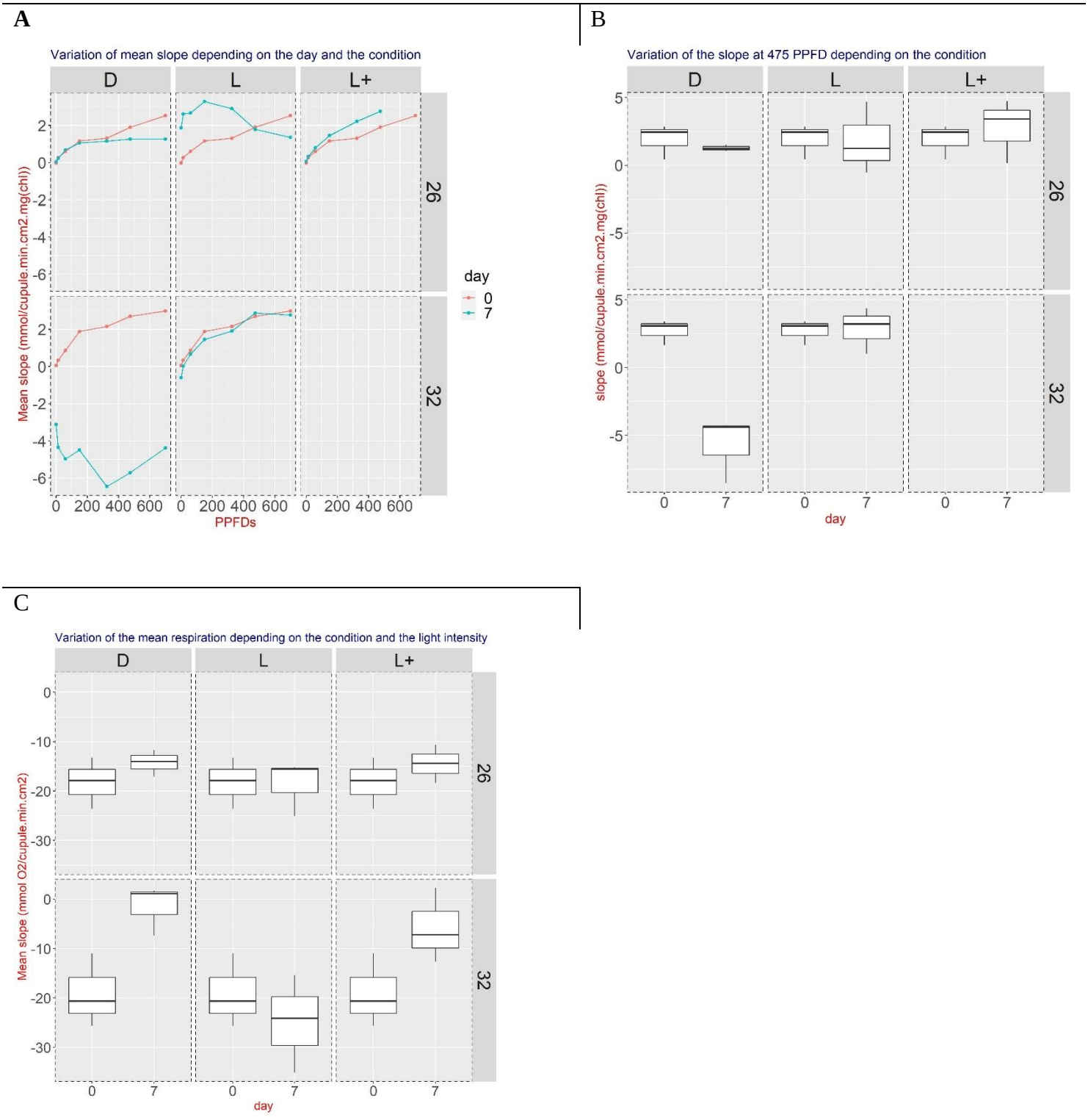


Figure 16 **Graphic obtained through the Oxygen exchange measurement.** Graph A is the mean net photosynthesis at different light intensities depending on the day and the condition. D stands for dark, L stands for light, and L+ stands for high light. Graph B is the mean slope at 475 µE $m^{-2}s^{-1}$. The data from 32°C L+ were removed because they showed an aberrant trend.

After 7 days of stress, the results obtained through the Oxygen exchange method for the different light conditions at 26°C do not show any significant difference (Figure 16 A).

However, at 32 °C, conditions D show a significant reduction of the net oxygen production after 7 days of incubation (net photosynthesis D0= 2.70 mmol $O_2.min^{-1}.cm^{-2}$.mg chl, net photosynthesis D7 = -5,72 mmol $O_2.min^{-1}.cm^{-2}$.mg chl), with a P value of $6{,}801.10^{-3}$.

For the respiration rate, a significant decrease of respiration is observed in 32°C (Figure 16) in dark condition (respiration dark = -1.5 mmol $O_2.min^{-1}.cm^{-2}$) and light condition (respiration dark = -5.9 mmol $O_2.min^{-1}.cm^{-2}$) compared to normal light condition (respiration light = -24.8 mmol $O_2.min^{-1}.cm^{-2}$) (p-value = 0.006 and 0.03 respectively).

7.4.3.3 PSI reaction center absorbance measurements

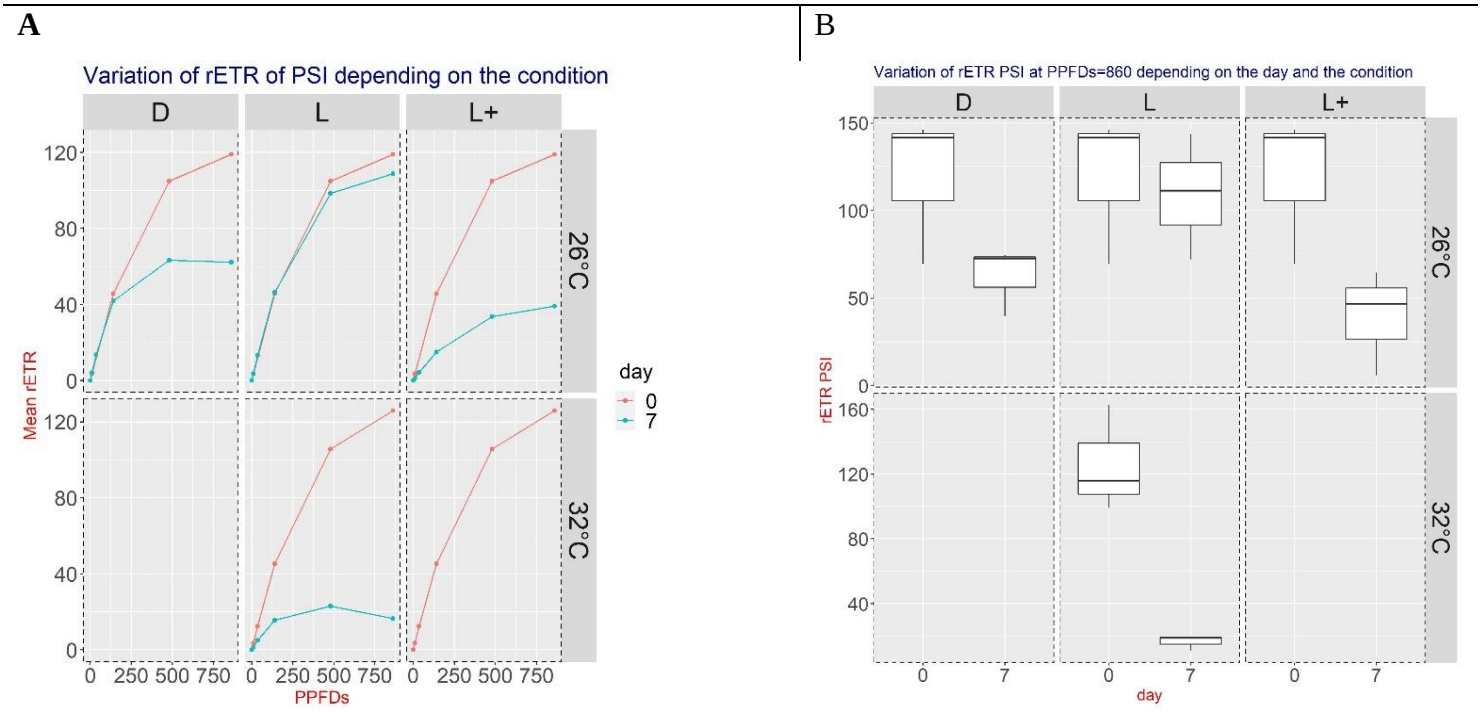


Figure 17 **Graph obtained through the P700 redox change analysis method**. Graph A shows the variation of $rETR_{PSI}$ depending on the light intensity, day, and condition. Graph B shows the variation of $rETR_{PSI}$ depending on the say and the conditions. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

The main parameter measured using the JTS device was the $rETR_{PSI}$ (Figure 17) at different light intensities (short PI curve). The 26°C conditions show the most significant variation both in Dark ($rETR_{PSI}$ D0 = 119 µmol é.$m^{-2}.s^{-1}$, $rETR_{PSI}$ D7 = 62 µmol é.$m^{-2}.s^{-1}$) and high light ($rETR_{PSI}$ D0 = 119 µmol é.$m^{-2}.s^{-1}$, $rETR_{PSI}$ D7 = 39 µmol é.$m^{-2}.s^{-1}$) conditions after 7 days of incubation with a P-value of 0,45 and 0,15 respectively. The result also shows a significant decrease in the $rETR_{PSI}$ after 7 days of incubation at 32°C ($rETR_{PSI}$ D0 = 126 µmol é.$m^{-2}.s^{-1}$, $rETR_{PSI}$ D7 = 16 µmol é.$m^{-2}.s^{-1}$) (P-value=0.0003).

7.4.3.4 *Fluorescence at 77 K*

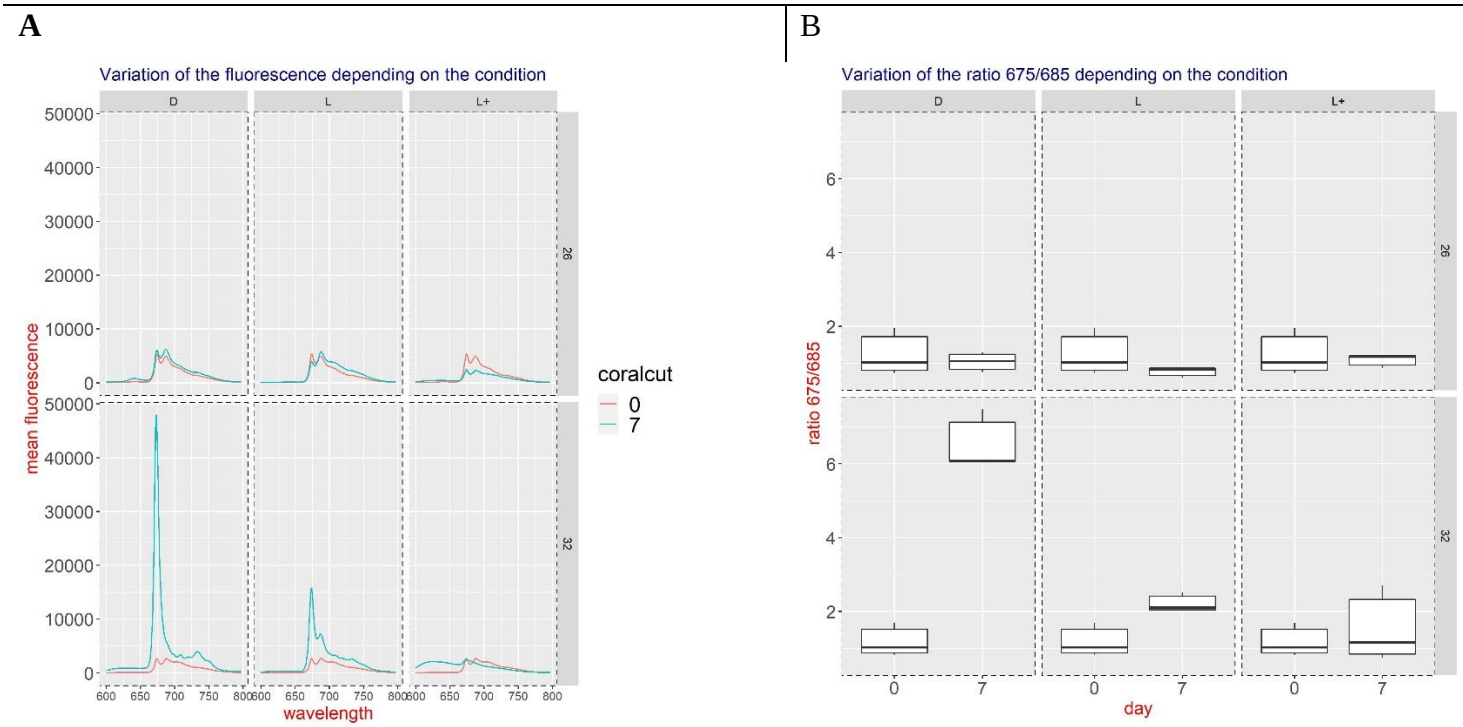


Figure 18 Graph obtained through the measurement of fluorescence at 77 K. Graph A shows the variation of the fluorescent signal depending on the wavelength. Graph B shows the ratio between the fluorescence at 675 and 685 nm depending on the condition. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

The result obtained through 77K fluorescence analysis are shown in (**Figure 18**). The result does not show any significant difference between conditions except at 32°C in the dark on day 7 ($P<0.005$). This could indicate the presence of NPQ or damage to the acpPC.

7.5 SETUP 3

7.5.1 Setup rearrangement

Figure 19 **Picture of the alternative coral sampling methods facilitating the extraction of CF.**

For the third setup, an air conditioner device set at 22°C was placed at the entrance of the aquarium room to maintain a constant temperature throughout the stress incubation. This allowed for a more stable temperature in the beakers despite the very variable summer temperatures in Belgium. Another type of coral sampling was also put in place to facilitate CF extraction (Figure 19). For this, larger CFs were cut from the coral colonies and fixed on a test tube cap using suitable glue.

7.5.2 Setup difficulties

Figure 20 **Picture of the beaker set at 32°C in normal light after 3 days of stress incubation**.

The difficulties that occurred during the third setup were mainly linked to the condition of the equipment used for the beakers. The heater used for the 32°C conditions had a malfunction

causing the temperature to rise to 34°C on day 2 and fall to 24°C on day 5. The data for the 32°C conditions could then not be used as the stress endured by the corals was too uncertain to be reliable.

For this setup, two different colonies were used. The CN situated in the upper left of the pictures (Figure 20) was cut from a colony bought a couple of weeks earlier at the same location described in section 6.1 to replenish the biological material stock. It had arrived approximately one week before the beginning of the experiment and was probably not used to the environmental conditions of the aquariums. The other CN (bottom right) was extracted from the colony that had already been used for the other experiments and described in section 6.1. It is worth noting that the new colonies (upper left) were in a much worst state of bleaching that the old colonies (bottom right) after undergoing the same stress. Both of the colonies were from the same species and were in the same visible state of health when they were placed in the beakers.

7.5.3 Results obtained through the experiment

7.5.3.1 JTS analysis of PSI reaction center redox change and PSII fluorescence

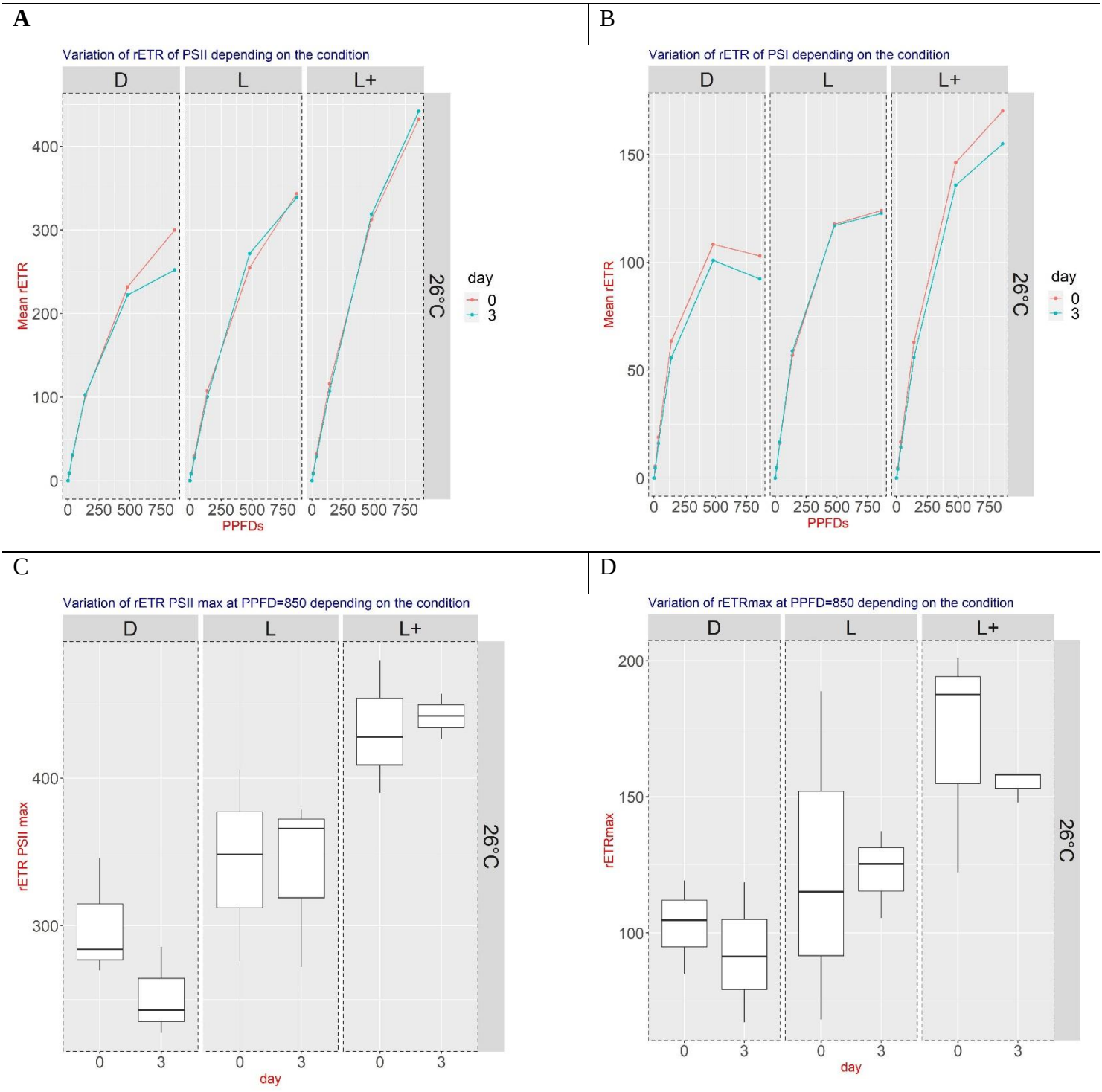


Figure 21 Graph obtained through the analysis of P700 redox change and PSII fluorescence variation with the JTS. Graph A and Graph B are the variations of the $rETR_{PSII}$ et $rETR_{PSI}$ respectively depending on the light intensity and light condition. Graphs C and D show the variation of $rETR_{PSII}$ and $rETR_{PSI}$ at 850 µE $m^{-2}s^{-1}$ depending on the day and the condition. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

For the results of $rETR_{PSII}$ and $rETR_{PSI}$ shown in Figure 21, none of the conditions shows a significant difference compared to day 0. The 32°C conditions are absent (due to the strong temperature instability) and the stress lasts only 3 days. The $rETR_{PSI}$ and $rETR_{PSII}$ values did not indicate any sign of stress.

7.5.3.2 77K fluorescence

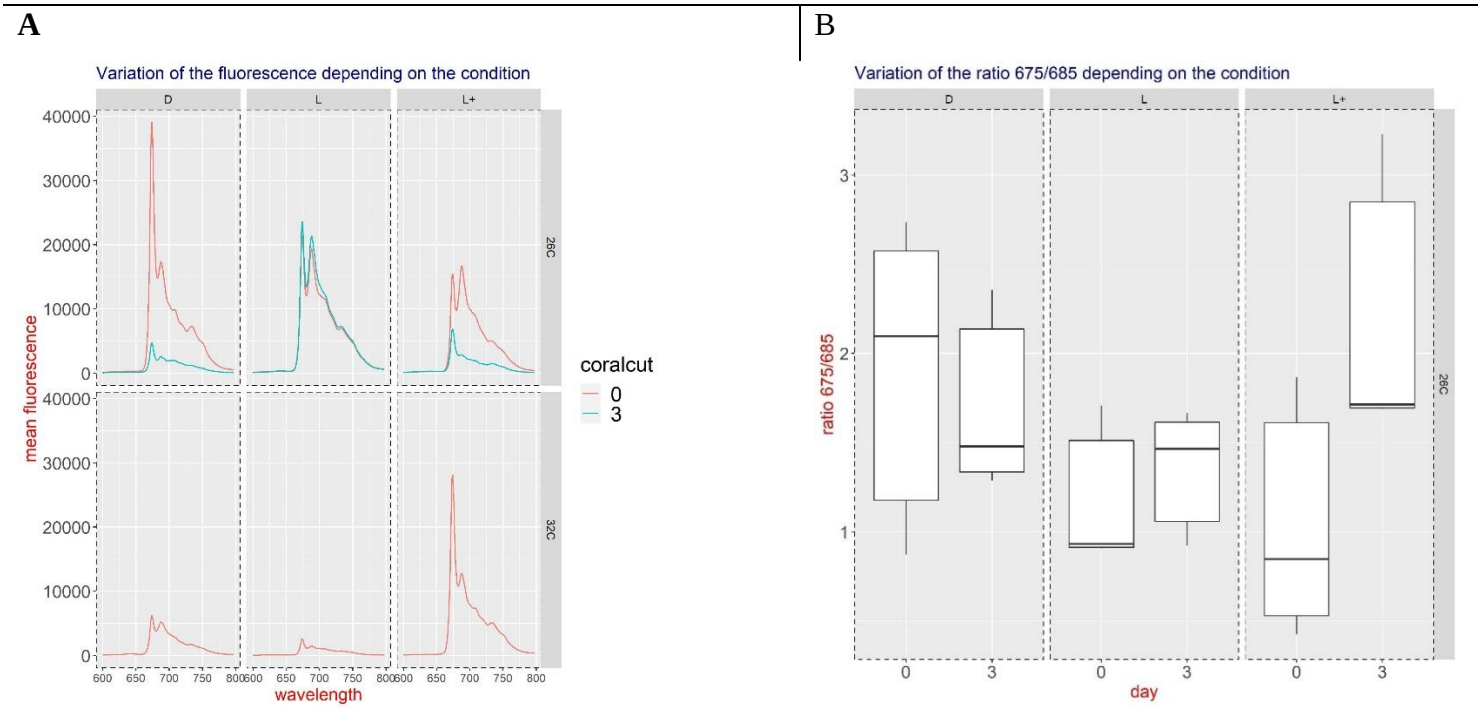


Figure 22 Graph obtained through the analysis of fluorescence at 77K. The graph shows the ratio between the fluorescence at 675 and 685 nm depending on the condition. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

The analysis of the fluorescence spectra recorded at 77K (**Figure 22**) indicate a significant change when the sample is exposed to high light after 3 days ($P < 0.005$). The darkness condition and the control do not show any significant variation in the ratio 675/685. The increase in this ratio indicates the presence of NPQ or damage to the acpPC.

7.5.3.3 *Oxygen exchange*

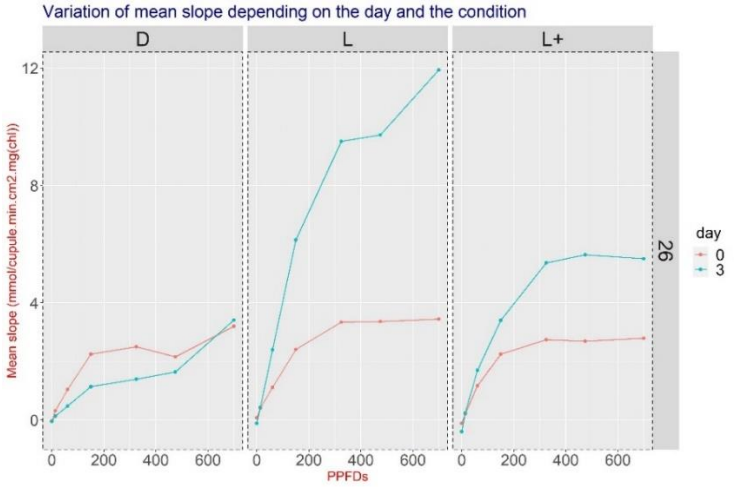


Figure 23 Graph obtained through the measurement of oxygen exchanges. The condition at 32°C could not be measured. The stress caused by the different problems encountered was too important. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

For setup 3, the result obtained for the oxygen exchange measurement show no logical trend (**Figure 23**). The control condition (26°C L) shows a strong positive variation after 3 days of incubation whole no variation is assumed to be observed.

7.5.3.4 *HPLC*

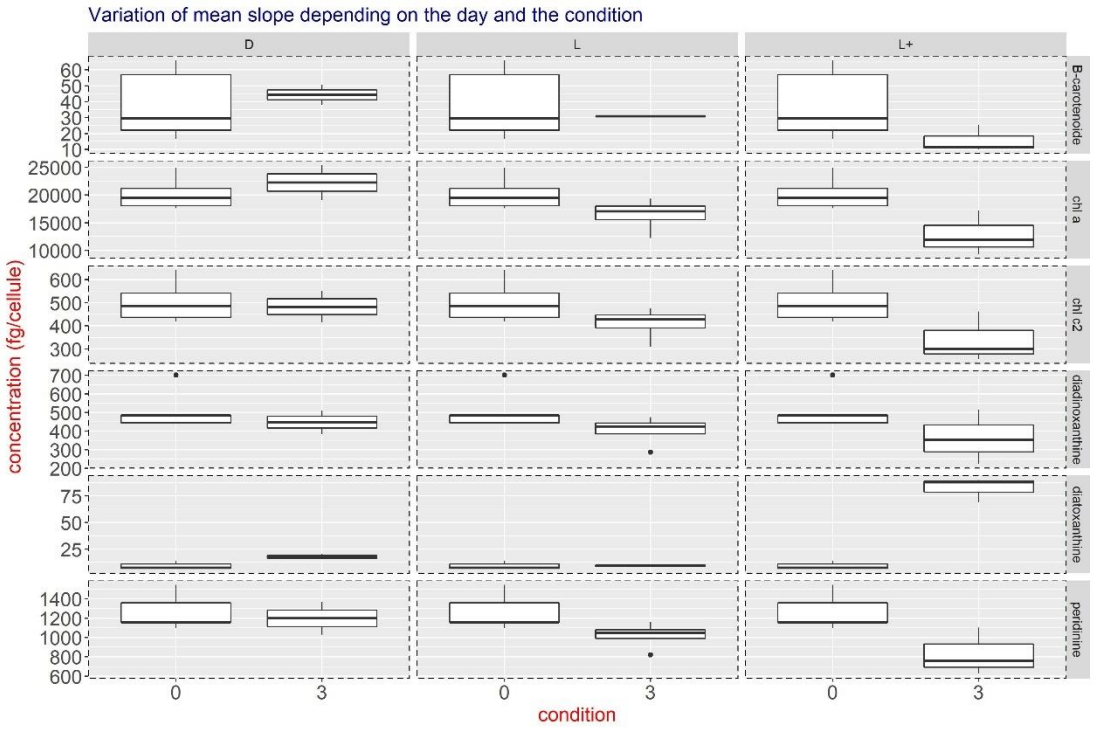


Figure 24 Graph obtained through the HPLC analysis of setup 3 after 3 days of incubation for the different conditions. D stands for Dark condition, L for normal light condition, and L+ for high light condition.

As for setup 2, the 26 L condition shows a slight decrease in all pigments on day 3 (**Figure 24**). As for the diatoxanthin, the only condition that shows a significant variation is 26 L+ (p-value=$2.68.10^{-8}$) when we compare day 0 (concentration = 9.928 fg.cellule^{-1}) and day 3 (concentration = 82.4 fg.cellule^{-10}). The other condition does not show any strong variation (P>0.005). Chlorophyll *a* also shows a significant variation in the condition 26L+ between days 0 (concentration = 20100 fg.cellule^{-1}) and 3 (concentration = 12823 fg.cellule^{-1}) (P-value=0.029). the variation of the other pigment over the different conditions does not express a significant change.

8 DISCUSSION

8.1 BIOENERGETICS OF PHOTOSYNTHESIS.

Exposing corals to temperature/light stress, showed a great impact on the chlorophyll fluorescence of PSII and the redox /absorbance variation of the reaction center of PSI. After a 3-day heat/light stress (Figure 11), S.*pistillata* tends to have a stable short $rETR_{PSII}$ PI curve up to 30°C. Above this temperature, the short $rETR_{PSII}$ PI curve starts to decline until it collapses at 34°C. The light has also shown a strong influence on the $rETR_{PSII}$. CFs incubated in complete darkness expressed a strong decrease in $rETR_{PSII}$ compared to the ones placed in normal light. The influence of light on PSII fluorescence has already been widely observed in other studies (Bhagooli & Hidaka, 2004; Hoogenboom et al., 2012).

When it comes to the NPQ, the result of Figure 11 shows a significant decrease as the temperature rise but no significant difference was visible between the darkness and normal light condition (Figure 11). However, when comparing the NPQ values between the different light conditions at 26°C for setup 2 (Figure 15), it shows a significant decrease when exposed to high light. This type of trend concerning NPQ is however the opposite of that measured in other studies (Hoogenboom et al., 2012). This difference from the studies is probably due to the unstable temperature as well as the light stress heterogeneity in the beaker.

Overall, the NPQ value seems to be influenced by high light and high temperatures but not by low light conditions. This trend is mainly related to the increase of photoprotective mechanisms, which is expressed under high light conditions and is manifested by an increase in the NPQ values (Hoogenboom et al., 2012).

The $rETR_{PSI}$ result obtained through the JTS analysis of setup 2, shows a similar trend to the $rETR_{PSII}$ of the 3-day short heat/light stress. Both low light and temperature have a significant

effect on the $rETR_{PSI}$ strongly reducing its values after 7 days of stress. Setup 3 shows similar results, however, the difference in values is much less significant since the stress only lasted for 3 days. Those results, nevertheless, do not completely match the ones obtained by Hoogenboom et al., 2012. Their research show, contrary to their expectation, a higher $rETR_{PSI}$ when the corals are exposed to a higher temperature such as 32°C. However, as mentioned for setup 2, the temperature varied enormously due to the absence of a cooler. The decrease in $rETR_{PSI}$ during this experiment could have been caused by the rise of the temperature due to the heat wave.

8.2 Oxygen exchange

When exposed to a short heat/light stress (Figure 12), the coral's net oxygen production tends to be more impacted by heat stress when exposed to low light conditions. (Figure 12 B). Short heat stress alone does not seem to influence significantly oxygen production ($P>0.005$). For setup 2 (Figure 16), the same trend is observed. At normal light and after seven days of 32°C heat stress, the oxygen production does not show any significant difference compared to day 0 at 32°C. However, when combined with low light, the net oxygen production gets significantly reduced. Those results do not match with the research carried out by Hoogenboom et al., 2012. They found no significant difference in photosynthesis after 11-14 days of 34°C low light stress. On the other hand, the research conducted by Kochman et al., 2021 show a decrease in net photosynthesis after 9 days of heat stress at 33°C. Nevertheless, as said above, the heat stress of setup 2 has varied during the week due to the heat wave. The corals were therefore subjected to greater stress. The decrease in net oxygen production could come from the damage suffered by the PSII or the rubisco. As said earlier enzymes are very sensitive to temperature, and the slowdown of the rubisco enzyme can lead to a slowdown of the Calvin cycle.

It is also important to mention that the CFs used for the short 3-day stress were all cut at once and stored in a control beaker at 26°C. The CFs used for the 34°C short heat stress were, therefore, stocked in a beaker for a longer period than the one used for the 26°C short heat stress. As shown by the setup 1 experiment, the survivability of CFs is very hazardous and can undergo great stress even at 26°C when placed in a beaker. The CFs used for the 34°C or even 32°C were possibly already in a state of stress before the start of the experiment.

The respiration rate shows a significant variation depending on light conditions. for the short stress experiment, respiration rate shows a lower result when incubated in the dark compared

to normal light conditions at 26°C and 34°C. for setup 2, the same trend is observed at 32°C. High light and dark conditions show a significantly lower value than the normal light 32°C conditions. This suggests that the holobiont has reduced the consumption of energy during both light conditions.

Respiration is known to be a sensitive element to temperature (Kochman et al., 2021). A small increase in temperature for a short time increases the respiration rate. However, when the stress is longer (days) and of greater intensity, the respiration rate gets reduced. This observation correlates with the one obtained during the 2 experiments given that the respiration gets significantly reduced at 32°C and 34°C.

8.3 PIGMENTS CONCENTRATIONS (HPLC)

With the result obtained by setup 3 through the HPLC analysis, multiple trends can be observed (**Figure 24**). The first thing to be observed is the stability of chlorophyll *a* through the 3 days of incubation under the control condition. This result allows us to conclude that the corals at ambient light and 26°C did not experience any bleaching phenomenon because of the setup (Rosic et al., 2020). For diatoxanthin and diadinoxanthin, there is a strong increase of diatoxanthin for the L+ condition ($P<0.005$) and a slight decrease in diadinoxanthin. These results correspond to the results expected by the DD/DT cycle explained in section 4.4.2. As the light increase, the diadinoxanthin is converted to diatoxanthin to evacuate the excess energy stored by the photosynthetic apparatus. At high light conditions, the chlorophyll and the other pigments also decrease significantly, highlighting the onset of the bleaching process.

In dark conditions, the concentration of the pigments studied does not express any significant variation. According to Rosic et al., 2020 , the absence of light does not have a significant impact in terms of the pigment content of the cell. The two main factors influencing the pigment concentration are the temperature and the feeding of the corals (Hoogenboom et al., 2012a).

8.4 PROSPECT FOR IMPROVEMENT

Although the different setups put in place during this work did not achieve their goal, they shed light on the complexity of setting up a long-stress experiment on corals. Despite the fact that different improvements have already been made during this work, several aspects of the heat stress setup still need to be corrected. The main problematic aspect of the setup was the use of CFs in the setup device. As shown in the problem of setup 1 (section 7.3.1), the CFs

have a much worse bleaching response than the CNs. The use of CFs as a base sample for the setup must preferably not be considered but rather be extracted from a small colony placed in the setup. However, for the monitoring of coral health through fluorescence measurement (short PI curve, Speedzen) it is preferable to use the same CFs to have an optimal result. Several studies (Bhagooli & Hidaka, 2004; Dobson et al., 2021; Hoogenboom et al., 2012b) do not extract CFs from the colonies but rather use a portable Mini-Pam device (Walz, Germany) allowing the measurement of fluorescence directly on the corals. Multiple spatially separated measurements of the fluorescence can thus be performed on a CN giving good monitoring of the coral's health state.

The review written by McLachlan et al., 2020 also identifies the different sensitive points when it comes to coral heat stress experiments. First of all, long heat stress is mainly performed using open flow-through-seawater systems. Close systems are on the contrary usually used for short-term experiments, many of which use natural seawater rather than artificial seawater. These different factors are important as they can influence many aspects of the physiochemical parameter that can affect coral health. Therefore, it is preferable to limit the times of the experiment carried out using the facilities presented in this work. When it comes to long-medium heat stress, many studies also use a “ramping method” to introduce the corals to the stress (Dobson et al., 2021; Hoogenboom et al., 2012a; Kochman et al., 2021; Rosic et al., 2020). Usually, both the control tanks and the high-temperature tanks are set at normal temperature (25-26 °C). the temperature of the heat-stress tanks is then gradually increased to the desired temperature at a rate of 0.5 to 1 degree a day. In this work, the corals were put directly in the set temperature with no adaptation time.

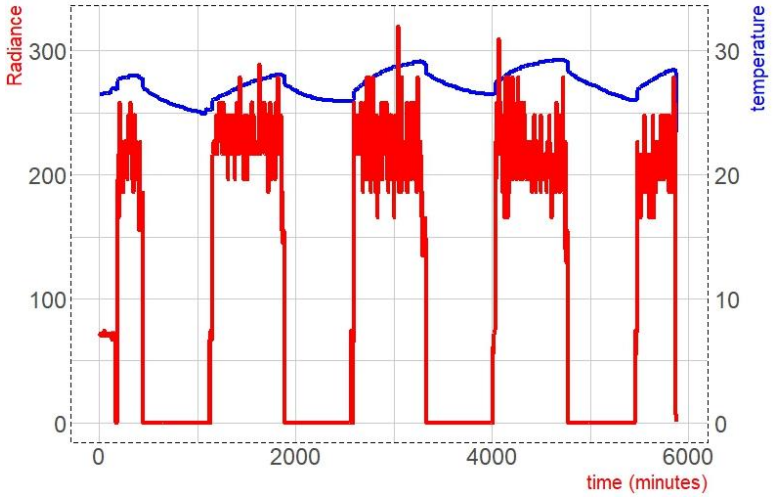


Figure 25 Graph obtained through the monitoring of the 26°C high light condition for 4 days. Radiance is expressed in µmol photon.m^{-2}.s^{-1} and the temperature in °C.

For the heat/light stress establishment, several aspects must be considered. First of all, since the system used is a closed system, the temperature of the water gets relatively easily influenced by external factors such as light and room temperature. One of the main problems was the high light condition (Figure 25). With the beakers exposed to increased light intensity and with the absence of a cooling system, the temperature would rise to a maximum of 30°C at day time and fall back to 26°C at night time. The room temperature was also an important factor in temperature variability. As said for setup 2, the weather can also influence the temperature of the setup. This problem however was partially solved by the installation of an air conditioner. The installation of a cooling system, especially in a closed system, is essential and must be taken into consideration.

The high light condition was also a very complicated condition to homogenize. The spotlight used was placed on the right side and the left side of the beaker. Since the CNs used for setup 2 and setup 3 were 3D objects and the light was not homogeneous, the light would affect only a side of the CN. This would lead to the bleaching of one side of the CN and not the other. The bleaching was also stronger on the CNs that were placed closer to the spotlight than the ones placed further. All these variabilities have induced heterogeneous stress, making the measurements very difficult.

Feeding frequency and amount must also be considered. Under stress conditions, this factor has a strong impact on the physiology of the CNs (Dobson et al., 2021; Hoogenboom et al., 2012b; Levy et al., 2016). The quantities of Artemia used must be similar for each of the beakers.

9 CONCLUSION

Short 3-day temperature stress impacted negatively the fluorescence of PSII of S. *pistilllata.* The stress was stronger when put in darkness and induced complete bleaching at 34 °C. NPQ also got significantly reduced by heat stress but did not show any different results whether the coral was placed in the darkness or ambient light (100 µmol photons.m^2.s^{-1}). For the different setups used during this work, several trends could be observed. NPQ is significantly reduced after seven days of incubation at high light (300 µmol photons. m^{-2}.s^{-1}). The production of oxygen is also getting significantly influenced by the temperature. However, the influence is stronger when incubated in darkness rather than in light.

For the different setups used during this work, several trends have been observed. However, multiple of these trends were not reliable due to the numerous problems of stability of the conditions carried out in the different setups. After seven days of incubation, the oxygen production was negatively influenced when the corals were placed at 32°C in darkness but remained constant in ambient light. At 26°C, the oxygen production did not show any significant variation whether the coral was placed in darkness, ambient light, or high light. The P700 redox change analysis showed no change of the $rETR_{PSI}$ at PPFD = 860 µmol photon.m^{-2}.s^{-1} after the 3-day incubation at 26°C at low light and high light (setup 3). However, after seven days of incubation, the $rETR_{PSI}$ showed a significant decrease at 32°C ambient light and at 26°C high light (300 µmol photons.m^2.s^{-1}) and in darkness. The measurement of fluorescence at 77k did not show any significant variation of the ratio 675/685 nm after 3 and 7 days of incubation at 26°C but does at 32° in low light conditions. Light also has an impact on the pigment content of Symbiodinaceae when increased. Chlorophyll *a* tended to be reduced when exposed to high light in ambient temperature, expressing the onset of bleaching. On the other hand, the initiation of the DD/DT cycle was reflected by the increase of diatoxanthin after three days in high light.

Overall, the main objectives of this work, namely, understanding the influence of a long heat/light stress on the bioenergetics on the reef-building coral *Stylophora pistillata*, could not be achieved. This conclusion is mainly due to the numerous flaws in the stress setup carried out during this thesis. To quote some, the use of a closed system for long stress experiment was not adopted, the temperature of the system was not well stabilized during the stress, the light stress was not well homogenized on the entire beaker, and the stressed corals should have been acclimatized to stress through a gradual increase in temperature, the feeding frequency and the quantity of Artemia used should have been more precisely monitored

This work has however allowed the highlighting of good practices to be put in place for the realization of an elaborate stress setup. Setup that has never been done before in the Laboratory of Genetics and Physiology of Microalgae's lab.

10 APPENDICES

10.1 SEQUENCE USED FOR THE MEASUREMENT OF SHORT RETR PI CURVE

30msE0!3sE0!30µsD30msE15!150msE0!30µsD
30msE3!3sE0!30µsD30msE15!150msE0!30µsD
30msE5!3sE0!30µsD30msE15!150msE0!30µsD
30msE7!3sE0!30µsD30msE15!150msE0!30µsD
30msE8!3sE0!30µsD30msE15!150msE0!30µsD
30msE9!3sE0!30µsD30msE15!150msE0!30µsD
30msE10!3sE0!30µsD30msE15!150msE0!30µsD100msD

10.2 SEQUENCE USED FOR THE MEASUREMENT OF OXYGEN EXCHANGE AND LONG RETR PI CURVE USING THE SPEEDZEN FLUOROMETER

10(60sD)
3(30msE0!20sE0!90µsD30msE0!20sE0!90µsD30msE0!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE3!20sE0!90µsD30msE3!20sE0!90µsD30msE3!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE5!20sE0!90µsD30msE5!20sE0!90µsD30msE5!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE7!20sE0!90µsD30msE7!20sE0!90µsD30msE7!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE8!20sE0!90µsD30msE8!20sE0!90µsD30msE8!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE9!20sE0!90µsD30msE9!20sE0!90µsD30msE9!20sE0!90µsD30msE15!150msE0!90µsD)
3(30msE10!20sE0!90µsD30msE10!20sE0!90µsD30msE10!20sE0!90µsD30msE15!150msE0!90µsD)100msD

10.3 SEQUENCE USED FOR THE MEASUREMENT OF ABSORBANCE CHANGE IN P700 WITH THE JTS SPECTROPHOTOMETER

2(10msD)5msE0!300µs/E0!300µsD30µs/E0!2msZE0!5msZE0!10msZE0!20msZE0!50msZE0!100msZE0!300msZE0!500msZE0!1sZE0!1sZE14!150msE0!300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1sD

2(10msD)5msE3!300µsZE3!2msZE3!5msZE3!10msZE3!20msZE3!50msZE3!100msZE3!300msZE3!500msZE3!1sZE3!1sZE14!150msE0!
300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1sD

2(10msD)5msE5!300µsZE5!2msZE5!5msZE5!10msZE5!20msZE5!50msZE5!100msZE5!300msZE5!500msZE5!1sZE5!1sZE14!150msE0!300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1s

2(10msD)5msE7!300µsZE7!2msZE7!5msZE7!10msZE7!20msZE7!50msZE7!100msZE7!300msZE7!500msZE7!1sZE7!1sZE14!150msE0!300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1s

2(10msD)5msE9!300µsZE9!2msZE9!5msZE9!10msZE9!20msZE9!50msZE9!100msZE9!300msZE9!500msZE9!1sZE9!1sZE14!150msE0!
300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1sD

2(10msD)5msE10!300µsZE10!2msZE10!5msZE10!10msZE10!20msZE10!50msZE10!100msZE10!300msZE10!500msZE10!1sZE10!1sZE14!150msE0!300µsD300µsD2msD5msD10msD18msD30msD75msD200msD500msD1sD

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