MARINA TONETTI BOTANA

The role of *Symbiodinium* membrane lipids in response to heat shock: implications for coral bleaching

Thesis presented to the Instituto Oceanográfico of the Universidade de São Paulo, in partial fulfillment of the requirements for obtaining the degree of Master in Sciences, Oceanography Program, concentration area of Biological Oceanography.

Supervisor: Prof. Dr. Paulo Y. G. Sumida

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Evaluated in:

Prof(a). Dr.(a)	Grade
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“Look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. Science is not only a discipline of reason, but also, one of romance and passion”

Steve Hawking

“Commit yourself to the process. Not the goal. The final score take care of itself”

James Clear

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RESUMO

Recifes de coral do mundo inteiro vêm sendo devastados pelo fenômeno de branqueamento, o qual as evidências indicam que seja causado pelo stress oxidativo promovido pelo aquecimento global e eventos catastróficos de El Niño. A grande variabilidade genética da Família Symbiodiniacea também é sugerida como determinante da susceptibilidade do coral hospedeiro porque cada espécie possui limites fisiológicos específicos, tanto no modo de vida livre como em simbiose. Neste estudo apresentamos pela primeira vez o sucesso da utilização da de técnicas de lipidômica (i.e, caracterização dos lipídeos globais em um determinado organismo) oferecendo suporte para as investigações moleculares de investigação dos mecanismos relacionados ao stress térmico em espécies de endosimbiontes de coral. *Symbiodinium minutum* foi sensível às temperaturas elevadas, enquanto *S. microadriaticum* e *S. goreaui* apresentaram distintos níveis de termo tolerância. Os fenótipos lipídicos das espécies após o stress, incluindo o transportador de elétrons do fotossistema II – plastoquinona – sugerem que cada um apresentou uma estratégia diferente para sobreviver. Além disso, os lipídeos específicos do cloroplasto com ácidos graxos poliisaturados (PUFA) formado, principalmente, por espécies com ômega 3 (n-3) foram essenciais para manter a bioenergética celular à longo prazo (10 dias após stress) em todos os *Symbiodinium* spp. A capacidade de manter altas concentrações de n-3 na membrana dos cloroplastos determinou a sobrevivência dos *S. microadriaticum* e *S. goreaui.* Os dados apresentados nesta dissertação revelam, pela primeira vez, o aumento de ácidos graxos oxidados na membrana do cloroplasto e também na forma livre (FFA) em resposta aos dados de stress oxidativo causados pelo calor. O estudo das membranas lipídicas é fundamental para melhor compreensão da bioenergética dos simbiontes e para determinar a vulnerabilidade da relação de simbiose com o coral aos estressores climáticos em um futuro com temperaturas mais elevadas.

Palavras-chave: *Symbiodinium*, recifes de coral, lipidômica, stress térmico, stress oxidativo.

ABSTRACT

Coral reefs around the world have been largely devastated by the phenomenon of “coral bleaching”, which causes have been reported to be strongly related to oxidative stress promoted by climate change drivers, including mainly global warming and catastrophic El Niño events. Genetic variability in coral endosymbionts from the Family Symbiodiniacea was also suggested as determinant of host susceptibility to stress because they present distinct physiological boundaries when in free living or in symbiosis. Here we present for the first time the successful use of lipidomics (*i.e*., the global characterization of lipids in a given organism) supporting molecular investigation in the oxidative mechanisms related to thermal stress in coral endosymbionts phylotypes. *Symbiodinium minutum* was thermal sensitive, whereas *S. microadriaticum* and *S. goreaui* presented different levels of thermal tolerance. Their lipid phenotypes after stress, including the photosystem electron transporter - plastoquinone - suggested they had different survival strategies. In addition, chloroplast specific lipids with polyunsaturated fatty acids (PUFAs) mainly formed by omega 3 (n-3) seemed to be essential to sustain *Symbiodinium* cells bioenergetics in the long term (10 days after stress). *S. microadriaticum* and *S. goreaui* capability of keeping high n-3 concentrations in the chloroplast membranes determined their survival. The present thesis reports, for the first-time, upregulation of oxidized lipids derived from precursor chloroplast membranes and free fatty acids (FFA) in response to oxidative stress damage caused by heat. The study of lipid membranes is of paramount importance to better understand the bioenergetics of symbionts and to determine the host/endosymbiont vulnerability to climate change stressors in a warmer future.

Key words: *Symbiodinium*, coral reefs, lipidomics, thermal stress, oxidative stress

LIST OF ACRONYMS AND ABBREVIATIONS

ARA - arachidonic acid

AC - *Symbiodinium* phylotype A1 control sample

AT – *Symbiodinium* phylotype A1 temperature stressed sample

ATP – adenosine triphosphate

BC - *Symbiodinium* phylotype B1 control sample

BT - *Symbiodinium* phylotype B1 temperature stressed sample

CC - *Symbiodinium* phylotype C1 control sample

CE – cholesterol ester

Cer - ceramide

Chl-a -chlorophyl-a

CL - cardiolipin

CT - *Symbiodinium* phylotype C1 temperature stressed sample

DAG – diacylglycerol

DGCC - 1,2-diacylglyceryl-3-(O-carboxyhydroxymethylcholine)

DGDG – digalactosyldiacylglycerol

DGTS - diacylglyceroltrimethylhomoserine

DHA - docosahexaenoic acid

EPA - eicosapentaenoic acid

ER - endoplasmic reticulum

ESI-TOFMS – electron spray ionization time of flight mass spectrometer

FFA - free fatty acids

Gluc Acid – glucoronic acid

HUFA – High unsaturated fatty acids

LC - liquid chromatography

MGDG - monogalactosyldiacylglycerol

MUFA - monounsaturated fatty acid

MS – mass spectrometer

PC – phosphatidylcholine

PE - phosphatidylethanolamine

PG – phosphatidylglycerol

PI - phosphatidylinositol

PL – polar lipids

PUFA - polyunsaturated fatty acid

RPLC – reverse phase liquid chromatography

ROS - reactive oxygen species

SFA - saturated fatty acid

SQDG – sulfoquinovosyldiacylglycerol

SM - sphingomyelin

TAG - triacylglycerol

UHPLC - ultra-high-performance liquid chromatography

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CHAPTER 3: Concentration of omega 3 fatty acids in membrane lipids determine *Symbiodinium* phylotypes (A1, B1 & C1) fate after temperature stress

CHAPTER 1: GENERAL INTRODUCTION

1.1 Coral reefs and their symbionts in the context of global warming

Coral reefs are one of the most productive ecosystems on Earth, representing one of the most diverse marine environments (Grigg *et al*., 1984). They hold high biodiversity and ecosystem services important for sustaining higher trophic level organisms and providing goods for large numbers of people (Martinez *et al*., 2007; Alves de Lima *et al*., 2010; Knowlton *et al*., 2010). Corals are defined as ecosystem engineering organisms responsible for controlling the availability of resources to other organisms through physical changes in biotic and abiotic materials (Jones *et al*., 1994). Therefore, studying them is pivotal to understand marine community structure and production of resources to humankind.

Tropical calcium carbonate reefs are built by stony corals (Scleractinia) associated with dinoflagellates of the family *Symbiodinidaceae* perceived as a mutualistic symbiosis. Photosynthetically produced endosymbiont metabolites are exchanged with coral hosts guaranteeing their survival and growth even in nutrient-poor waters (Muscatine & Porter, 1977; Leggat *et al*., 2003). In addition, the family *Symbiodinidaceae* is divided into nine clades (A-I) (Pochon & Gates, 2010) and multiple phylotypes within each clade (Thornhill *et al*., 2014), which shows distinct degrees of host-specificity and different tolerances to environmental conditions (Toller *et al*., 2001; Baker, 2003; Chen *et al*., 2003; Coffroth & Santos, 2005; LaJeunesse, 2005; Berkelmans & van Oppen, 2006; Goulet, 2006). Recent research has focused on the strength of this mutualistic relationship in response to predicted climate alteration (Davy & Cook, 2001; Cervino *et al*., 2004; Barneah *et al*.,2007; Hoegh-Guldberg *et al*.,2007), which is the main culprit causing extensive coral reef degradation (Hoegh-Guldberg & Smith, 1989; Graham *et al.* 2008; Wilkinson, 2008).

1.2 Coral bleaching and the oxidative stress theory

Coral bleaching is defined by visible whitening of the coral as a result of decreasing densities of *Symbiodinium* spp. cells and/or declines in their photosynthetic pigments (Fig.1.1). Since corals are extremely dependent on endosymbiont metabolites, their lack leads to coral degradation, eventually followed by coral death (Glynn, 1996; Brown, 1997). A recognized biochemical explanation for the phenomenon was first proposed by Lesser (1997) and coined by Downs *et al.* (2002) as “the oxidative stress theory of coral bleaching”. They proposed that excessive light and temperature cause physiological stress in the symbiont in combination with increased production of reactive oxygen species (ROS). ROS (i.e., hydrogen peroxide, superoxide, hydroxyl and singlet oxygen - Valko *et al*. 2007) can trigger the oxidation of essential biomolecules such as proteins and lipids in both coral and *Symbiodinium* spp. cells, thereby leading to disruption of symbiotic association.

Figure 1.1: Bleached corals of the genus *Mussismilia hispida*, endemic from the tropical waters of Brazil, observed in Ubatuba (São Paulo State north shore) in the summer of 2019.

The concept of ROS playing a key role in bleaching events has been an important subject of research in the last decades. The increased ROS production in symbiont cells may be caused by combination of temperature, light and even other stressors (e.g., alterations in

carbonate chemistry) (Tchernov et al., 2004; Smith *et al.*, 2005; Ragni *et al.*, 2010; Roberty *et al.*, 2015; Goyen, 2017). Today, despite the evidences, the specific causes triggering excessive ROS production leading to bleaching and harmful impacts on host physiology and impairment of symbiosis are still a matter of debate and further investigation.

1.3 The effect of high temperature in the viscosity of membranes leading to free radical and ROS formation in the chloroplast

The membranes of both cells and organelles are universally formed by lipids. Lipid composition directly affects membrane motion and fluidity, which are determined by the size and saturation levels of fatty acids chains of polar lipids. Polyunsaturated fatty acids (PUFA) tend to increase membrane's fluidity, whereas saturated fatty acids (SFA) have the opposite effect. Besides, membrane characteristics can also be altered by abiotic factors, such as temperature. Higher temperatures tend to increase spaces between fatty acids chains increasing membrane fluidity (Fig. 1.2). Therefore, organisms must adjust their membrane composition with variations in abiotic factors in order to maintain cellular functions, a process known as homeoviscous stability control (Sinensky *et al*., 1974; Cossins *et al*., 1978; Kellerman *et al.,* 2016).

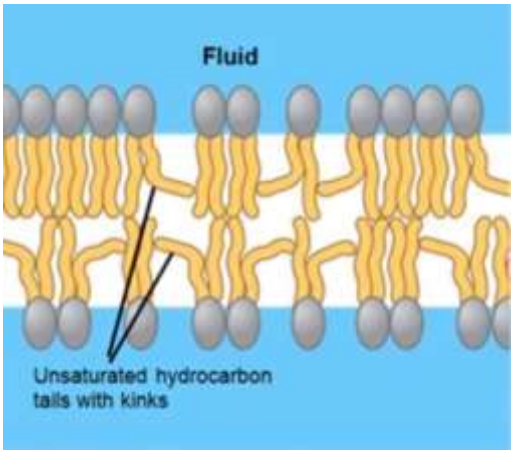

Figure 1.2: **Exemplification of how membrane lipids are affected by fatty acid composition and temperature**. Figure shows how polar lipids with unsaturated fatty acids attribute high motion and low viscosity to the membrane. Plus, elevated temperatures also promote higher membrane's motion and fluidity. Combination of elevated temperatures and high polyunsaturated fatty acids can compromise membrane's electron transport chain viability. Source: adapted from bio.libretexts.org.

Controlling membrane composition is pivotal for survival of all life forms, from bacteria to plants to mammals. The lipid composition of energy transducing membranes (i.e., cytoplasmic membranes of bacteria, thylakoid membranes of chloroplasts and mitochondrial inner membranes) is extremely specialized and must perform two essential functions: 1) control permeability of ions such as protons and sodium; and 2) tighten the electron transport at the membrane level. In chloroplasts (Fig. 1.3), the high-energy electrons coming from the water split at photosystem II (PS II) and must be safely transported within membranes by a plastoquinone to energize protein complexes or proton pumps (cytochrome b6f and PSI), a process known as electron transport chain. These pumps generate a proton gradient (high in the lumen and low in the cytoplasm) needed to promote adenosine triphosphate (ATP) synthesis (Fig.1.3). In retrospect, this ion gradient can only be established by controlling permeability of energy transducing membranes. All living forms must always adapt and keep membrane motion and fluidity stable in order to make ATP or energy, thus, lipids and bioenergetics are inseparable.

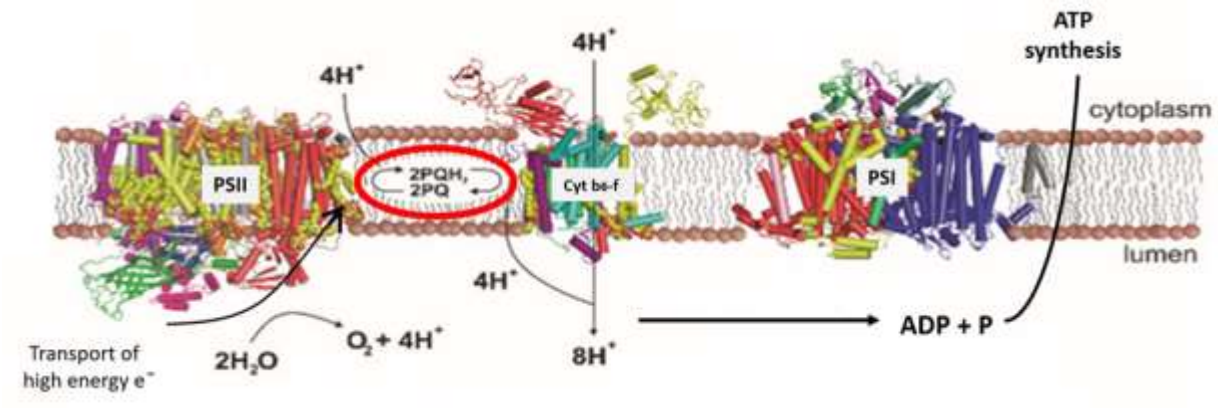

Figure 1.3: Electron transport chain resulting in ATP synthesis in the thylakoid membranes of chloroplasts. Protein complexes photosystem II (PSII), cytochrome b_6-f (cyt b_6-f) and photosystem I (PSI) are highlighted. Plastoquinone pool responsible for electron transport from PSII to cyt b_6-f is circled in red. Source: Adapted from Wada & Murata, 2009.

Thylakoid membranes of chloroplasts are very unique in that they are composed of glycolipids and specially at protein complexes such as the PS II. They are also decorated with several pigments including carotenoids and chlorophylls (Wada & Murata *et al.*, 2009). The arrangement or conformation of lipids and pigments has been honed by Darwinian evolution to prevent the leakage of both protons and electrons at the membrane level. Any leakage of protons through the membrane may prevent the generation of proton gradients needed for ATP synthesis (e.g., Kellerman *et al*., 2016; Yoshinaga *et al*., 2016). On the other hand, leakage of high-energy electrons may lead to free radical generation, which combined with oxygen leads to the formation of ROS (Polle, 1996). Both free radicals and ROS, if not contained by the cell's antioxidant machinery (including carotenoids and antioxidant enzymes such as catalases), can cause oxidation of biomolecules: proteins, pigments and lipids (Augusto & Miyamoto, 2011; Yin *et al*. 2011). Lipid peroxidation is the substitution of a bis-allylic hydrogen in the lipid structure by a free radical, yielding the formation of a lipid radical (L•). This lipid radical can easily react with oxygen, generating a lipid peroxyl radical (LOO•) and undergo complex cyclic reactions that might propagate through the membrane through a process best known as chain reaction (Niki, 2009; Yin *et al*., 2011). In this context, therefore, fatty acids containing high unsaturation levels, such as polyunsaturated fatty acids (PUFAs),

are the main targets of radical and ROS attack in their double bounds and propagate lipid oxidation even further unless stopped by an antioxidant agent.

The combination of high temperatures and high concentration of PUFAs in the chloroplasts of *Symbiodinium* is likely a strong trigger for coral bleaching, and the associated "oxidative theory of coral bleaching" (Lesser, 1997; Downs *et al,* 2002). Drastic changes in membrane permeability and fluidity are expected to occur as a consequence of high temperatures. Altering the thylakoid membrane conformation most likely promotes leakage of protons and electrons, thereby leading to decreased energy production and increased oxidative stress, respectively. It is, however, unknown whether the disruption of symbiosis occurs by a decreased supply of metabolites to the coral host, death of symbionts or simply symbionts themselves representing a potential threat to the host due to high ROS production.

1.4 Lipid membrane profiles of symbionts and their fate after thermal stress

Strong evidence suggests that *Symbiodinium* spp. can modulate the lipid composition of cells and organelles membranes in order to keep homeoviscous stability and adapt to environmental alterations (D'amico *et al*., 2006). Higher abundance of SFAs relative to PUFAs has been reported to enhance physical stability of thylakoid membranes of *Symbiodinium* sp. in response to thermal stress (Tchernov *et al*., 2004; Bachok *et al*., 2006; Tolosa *et al*., 2011). The rationale is that *Symbiodinium* spp. thylakoid membranes are enriched in PUFA, which are highly susceptible to oxidative damage by free radicals and ROS (Wada, 1994; Lesser, 2006; Catalá, 2009). However, *Symbiodinium* spp. may protect the photosynthetic membranes against ROS and thus acquire thermal tolerance altering the ratio saturated/unsaturated fatty acids. Tchernov *et al.* (2004) have even suggested that thermal tolerance is not associated with a single monophyletic phylotype, but rather with the level of saturation of their membrane lipids.

The above-mentioned studies marked the initial investigations of the role of lipids in coral bleaching. They were essential to establish that bulk fatty acid composition is crucial for survival and supports the "oxidative theory of coral bleaching" (Lesser, 1997; Downs *et*

al, 2002). In this dissertation, we generated data based on the global lipidome of some *Symbiodinium* phylotypes (e.g., glycolipids, phospholipids, aminolipids and storage lipids), including their pigments, in response to thermal stress. Thus, we not only report data on fatty acids, but also the lipid molecular species containing these fatty acids. That is, we are able to pinpoint whether thermal stress affects thylakoid membranes by characterizing their specific glycolipids rather than bulk fatty acids derived from other pool of lipids such as the triglycerides or phospholipids. Such detailed and comprehensive lipid analysis could only be achieved by recent analytical developments in mass spectrometry and the advance of lipidomics (Jones *et al*., 2012; Yao *et al*., 2015; Nygren *et al*., 2017).

1.4.1 Lipidomics as tool to better characterize microalgae lipids

Lipidomics is a fairly recent technique that evolved from metabolomics in its own research field (Tomita & Nishioka, 2006; German *et al*., 2007). Previous lipid analytical techniques allowed qualitative information about polar lipids such as acquired by thin-layer chromatography or quantitative analysis of bulk fatty acids by gas chromatography. Contrasting with past lipid analytical techniques, lipid characterization by liquid chromatography coupled to mass spectrometry (LC-MS) enabled precise characterization and quantification of every lipid molecular species present in a given sample (German *et al*., 2007; Oresic *et al*., 2008), including molecules that are specific markers of chloroplast, such as glycolipids and plastoquinones. For example, a great diversity of glyco and amino membrane lipids in *Symbiodinium* spp. and other dinoflagellates has been described by LC-MS analysis, including few alterations when growing in distinct temperatures (Leblond *et al.,* 2000, 2006, 2010, 2015; Gray *et al.,* 2009; Dahmen *et al.,* 2013; Flaim *et al.,* 2014; Anesi *et al*., 2015, 2016). These studies were mostly focused on a specific class of polar lipid such as glycolipids or aminolipids, and not aimed at characterizing the global lipidome together with specialized lipids, such as plastoquinone and pigments using LC-MS. Besides the culture-based investigations, important data have been generated in environmental studies reported by Van Mooy *et al.* (2006, 2009 and 2010), Moutin *et al.* (2007), Schubotz *et al.* (2009), Xie *et al.* (2014) and Becker *et al.* (2018). These include not only data from phospho, glyco and

aminolipids, but also storage lipids and quinone molecules (e.g., ubiquinone in Becker *et al*., 2018). These studies describe the lipidome of the water column of the oceans, where a diverse variety of phytoplankton occurs, in response to diel temperature oscillations and distinct nutrient conditions. The present study is, to the best of our knowledge, the first attempt to characterize the global lipidome of a microalga, describing not only membrane lipids, but also storage lipids, pigments and plastoquinone from *Symbiodinium* phylotypes. This information will be linked to cell physiology data to further knowledge on their thermal sensitivity.

2. GOALS AND SCOPE OF THIS THESIS

The overall goal of this thesis is to investigate how the lipidomes of *S. microadriaticum* (phylotype A1), *S. minutum* (phylotype B1) and *S. goreaui* (phylotype C1) most prevalently associated with scleractinian corals are related to their thermal sensitivity. Description of *Symbiodinium* spp. lipids and pigments is very scarcely found in the literature, let alone their lipid alterations with stress events. For best comprehension all studied *Symbiodinium* spp. will be referred as their phylotypes A1, B1 and C1. Therefore, this thesis is divided into two further chapters:

Chapter 2:

Description and quantification of global lipidome and pigment profiles of *Symbiodinium* phylotypes A1, B1 and C1 growing under optimum conditions of temperature, light and nutrients. Here, the goals are to examine whether differences in lipidome and pigments profiles between *Symbiodinium* phylotypes can predict their thermal tolerance to heat stress events based on previous studies (e.g., Tchernov *et al*., 2004).

Chapter 3:

Monitor *Symbiodinium* phylotypes (A1, B1 and C1) growth rates and lipidome/pigment alterations after a heat shock event (4h under 34˚C) describing short-term (4 and 24 h) and long-term phenotypical alterations (10 days).

Main assumption: “High temperatures known to change thylakoid membrane stability and enabling scape of high energy electrons generate higher concentration of ROS leading to lipid peroxidation accompanied by reduction of ATP production. Both facts affect *Symbiodinium* spp. growth rates and reflect in drastic changes in their lipidomes and pigments profiles”.

Specific hypotheses:

1) *Symbiodinium* spp. growth rates are negatively affected by heat shock;

2) Changes in lipids and pigments are different between *Symbiodinium* phylotypes after heat shock;

3) Lipidome and pigments profiles of *Symbiodinium* phylotypes after stress are good indicators of oxidative stress caused by heat shock experiment;

4) Lipid peroxidation preferably occurs in polyunsaturated fatty acids (PUFA) from chloroplast membranes.

CHAPTER 2

Lipidomics as a throughput method for profiling lipids and pigments in *Symbiodinium* phylotypes (A1, B1 & C1): physiological and ecological applications

1. INTRODUCTION

Lipids are major subcellular components in *Symbiodinium* spp. and all microalgae cells as they present distinct physiological roles in processes essential for survival, growth and reproduction (e.g., membrane structure, cell division, photosynthesis) (Guschina & Harwood, 2006; Wada & Murata, 2009). Individual classes of lipids have specific roles and are located in distinct cell compartments (Mizusawa & Wada, 2012). Polar lipids and sterols are mostly found in cell and organelle membranes forming their structure and acting as selective permeable barriers. On the other hand, storage lipids are nonpolar molecules, mostly found in internal monolayer vesicles (i.e., lipid droplets), important for supplying the cell's energetic demands (Gurr *et al*., 2002). In order to precisely characterize this large diversity of compounds, the usual fatty acid characterization (Mansour *et al*., 1999; Dìaz-Almeyda *et al*., 2011, 2017; Kneeland *et al*., 2013) is not enough and more sophisticated techniques of mass spectrometry coupled with high performance liquid chromatography are pivotal for identification and quantification of intact polar lipids (i.e., whole molecule characterization).

The description of such robust phenotypical data permits a better comprehension of microalgae physiological processes regarding their lipid metabolism (Wada & Murata, 2009). In addition, it is important for ecological studies of marine food webs, considering that their lipids are major dietary components for primary consumers, representing a source of energy and essential nutrients (Guschina & Harwood, 2009). Microalgae lipids, especially polyunsaturated fatty acids (PUFAs) (e.g., docosahexaenoic acid - DHA and eicosapentaenoic acid - EPA), substantially guarantee neurological and sensorial

development of higher trophic-level organisms (Müller-Navarra *et al*., 2000). PUFAs are also essential for humans and terrestrial animals because they constitute their cell membranes and are precursors of important hormones, bioactive compounds and signaling molecules (da Costa *et al*., 2016). Therefore, the characterization of intact lipids has important applications on both physiological and ecological studies.

In the case of the dinoflagellate microalgae *Symbiodinium* sp., lipids are important in the composition and architecture of their cells and organelles membranes. Lipids are also part of the main metabolites transferred to coral hosts during their symbiotic state (Grottoli *et al*., 2004; Matthews *et al*., 2018). The genus *Symbiodinium* is currently divided in nine phylotypes (A-I) (Pochon & Gates, 2010), and recent studies suggest these phylotypes are, in fact, different species (Lajeunesse *et al*., 2018). Considering that they are different species we expect different total lipids and pigments profiles in each studied phylotype. A significant amount of data has been already reported for *Symbiodinium* spp. partial lipidomes, containing few groups of polar lipids (e.g., glycolipids and aminolipids) both *in vitro* and *in vivo* research (Leblond *et al*., 2000, 2010; Becker *et al*., 2018, respectively). However, culture studies did not mention which phylotype was involved and environmental studies analyzed *Symbiodinium* spp. together with the total phytoplankton biomass. Therefore, none has ever precisely investigated lipidome distinctions among distinct isolated *Symbiodinium* phylotypes. Aiming to fill this gap, the complete lipid and pigment profiles of three *Symbiodinium* phylotypes A1, B1 and C1 were analyzed for the first time. These particular phylotypes are highly associated with reef-building scleractinian corals (LaJeunesse, 2005; Berkelmans & van Oppen, 2006; Pochon & Gates, 2010).

A large diversity of molecular compounds and concentrations in each phylotype are reported in the present work, precisely determining what lipids are significantly different between them. Our data mechanistically suggests physiological differences in their lipid metabolism and, therefore, might also indicate distinctions in the fatty acids transference when they are associated with coral hosts. This study represents a first step into a more robust lipidome characterization of all *Symbiodinium* phylotypes and it can be used as a guideline for future lipid studies with both physiological and ecological applications.

2. MATERIALS AND METHODS

2.1. Experiment design

Symbiodinium sp. cultures of phylotypes A1-C1 were brought from University at Buffalo (NY-USA) and kept inside BMAK microalgae facility in the Oceanographic Institute of Universidade de São Paulo (IO-USP). They were grown in triplicates with autoclaved natural sea water in f/2 nutrient conditions (Guillard & Ryther, 1962). Room temperature was kept at 22 ºC; light conditions of 80 µmol photons m^{-2} s^{-1} into a 12L:12D cycle. Culture growth rates were monitored overtime and cells were counted in a Neubauer chamber under light microscope. Lipids and pigments samples were collected only during the exponential phase of growth. Volumes sampled were calculated in order to obtain a cell abundance greater than 1 million cells per filter. Culture media was filtered and used as blanks and exclude the possibility of any contamination. Sterile pre-combusted GF/F filters (5 minutes under 300 ˚C) were used for all filtrations.

2.2. Internal standards

Internal standards for sphingolipids, phospholipids, storage lipids and plastoquinone were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL. USA). They were added to each sample as a mix allowing for further identification and correction in the quantification. Description and concentration of each component is described in table S2.1.

2.3 Additional standards

Pigments standards were obtained from DHI Labs (Denmark). Amino and glyco lipids standards were kindly donated by Dr. Florence Schubotz (MARUM – University of Bremen, Germany). External calibration was made through dilution curves for the establishment of a correction index and further quantification. Description and concentration of each standard component and correction index (α) are described in table S2.2.

2.4. Lipid extraction

Lipid extraction was performed according to the method established by Bligh & Dyer (1959). Each GFF filter containing *Symbiodinium* sp. cells was macerated and homogenized in 1 mL of 10 mM phosphate buffer (pH 7.4) containing deferoxamine mesylate 100 µM. Then, 800 µL of ice-cold methanol and 200 µL of internal standard mix (10 µg/mL) were added. Next, 4 mL of chloroform/ethyl acetate (4:1) were added to each mixture, which was thoroughly vortexed for 1 minute. Samples were also sonicated for 20 minutes to enhance breakage of glycomembranes. After centrifugation at 2000 *g* for 6 min at 4 °C, the lower phase containing the total lipid extract (TLE) was transferred to a new tube and dried under N_2 gas. Dried TLE were again dissolved in 100 µL of isopropanol and the injection volume was set at 1 µL.

2.5. Lipidomics analysis

The TLE was analyzed by ESI-TOFMS (Triple TOF 6600, Sciex, Concord, US) interfaced with a high-performance LC (UHPLC Nexera, Shimadzu, Kyoto, Japan). The samples were loaded into a CORTECS® (UPLC® C18 column, 1.6 µm, 2.1 mm i.d. x 100 mm) column with a flow rate of 0.2 mL min^{-1} and oven temperature at 35°C. For RPLC, mobile phase A consisted of water/acetonitrile (60:40), while mobile phase B composed of isopropanol/acetonitrile/water (88:10:2). Mobile phases A and B contained ammonium acetate or formic acid (at a final concentration of 10 mM) for experiments performed in negative or positive ionization modes, respectively. The linear gradient during RPLC were as follows: from 40 to 100% B over the first 10 min, hold at 100% B from 10-12 min, decreased from 100 to 40% B from 12-13 min, and hold at 40% B from 13-20 min.

The MS was operated in both positive and negative ionization modes, and the scan range set at a mass-to-charge ratio of 200-2000 Da. Data for lipid molecular species identification and quantification was obtained by Information Dependent Acquisition 78 (IDA®). Data acquisition was performed with a period cycle time of 1.05 s with 100 ms acquisition time for MS1 scan and 25 ms acquisition time to obtain the top 36 precursor ions.

Data acquisition was performed using Analyst® 1.7.1 with an ion spray voltage of -4.5 kV and 5.5 kV (for negative and positive modes, respectively) and the cone voltage at +/- 80 V. The curtain gas was set at 25 psi, nebulizer and heater gases at 45 psi and interface heater of 450 °C.

2.6. Data processing

The MS/MS data was analyzed with PeakView®, and lipid molecular species were identified by an in-house manufactured Excel-based macro. Spectrums showing fragment breaks including exact masses and retention times used for identification are exemplified in the end of supporting information. Area of each lipid species were obtained by MS data from MultiQuant®. For quantification, the area ratio of each lipid species was calculated by the peak area of each lipid species divided by the peak area of the corresponding internal standard (shown in detail in table S1.1). Pigments, amino and glycolipids standards had external calibration curves relative to the internal standards lyso PC, TAG (17:0/17:0/17:0) and Cer (d18:1/17:0), respectively. They were injected separately following dilution curves with eight points each, following the ranges described in table S1.2. Through dilution curves each point had half the concentration of the previous point and lower limits were defined based on MS inferior limit of detection. Specific correction factors (α) were obtained from resulting curves and calculated by the ratio of slope of pigments, amino and glycolipids against their respective above-mentioned internal standards. Final concentrations were calculated by the peak area ratio divided by respective internal standard area ratio and multiplied by their respective correction factors. Quantification of DAG, PI and FFA followed the same procedure but had no internal standards. PI and FFA areas ratios were divided by area ratios of PC (17:0/17:0) and then multiplied by correction coefficients 0.65 and 2.294, respectively. DAG had its area ratio divided by TAG (17:0/17:0/17:0) area ratio and then multiplied by a coefficient of 0.576. Total amounts of each lipid and pigment were expressed in ng/cell of *Symbiodinium*. Correction coefficients of PI and DAG were obtained in the same way explained for glyco- and aminolipids considering PC (17:0/17:0) as internal standard.

2.7. Statistical analysis

All lipids statistical analyses were performed with Metaboanalyst (website: www.metaboanalyst.ca). Data were log transformed, and the statistical significance ($p < 0.05$) evaluated by t-test analysis of stressed cells x control cells in each sampling time. Differences were evaluated through a Volcano-plot adjusted for false discovery rate (FDR) also considering p values lower than 0.05.

For comparison of lipids and pigments profiles of control samples (AC, BC and CC), data were also log transformed, and the statistical significance ($p<0.05$) was evaluated by one-way ANOVA followed by Tukey's post-hoc test.

3. RESULTS

3.1. Description of total lipids and pigments profiles of *Symbiodinium* phylotypes A1, B1 and C1

A total of 241 molecules, including lipids and pigments, were identified (Fig. 2.1) and compounds were separated into main classes to facilitate comprehension regarding distinct cell compartment composition. Detailed description of molecules belonging to each mentioned lipid class can be found in table S2.3 together with their respective molecular weight and lipid classes fragment break spectrum utilized for their identification.

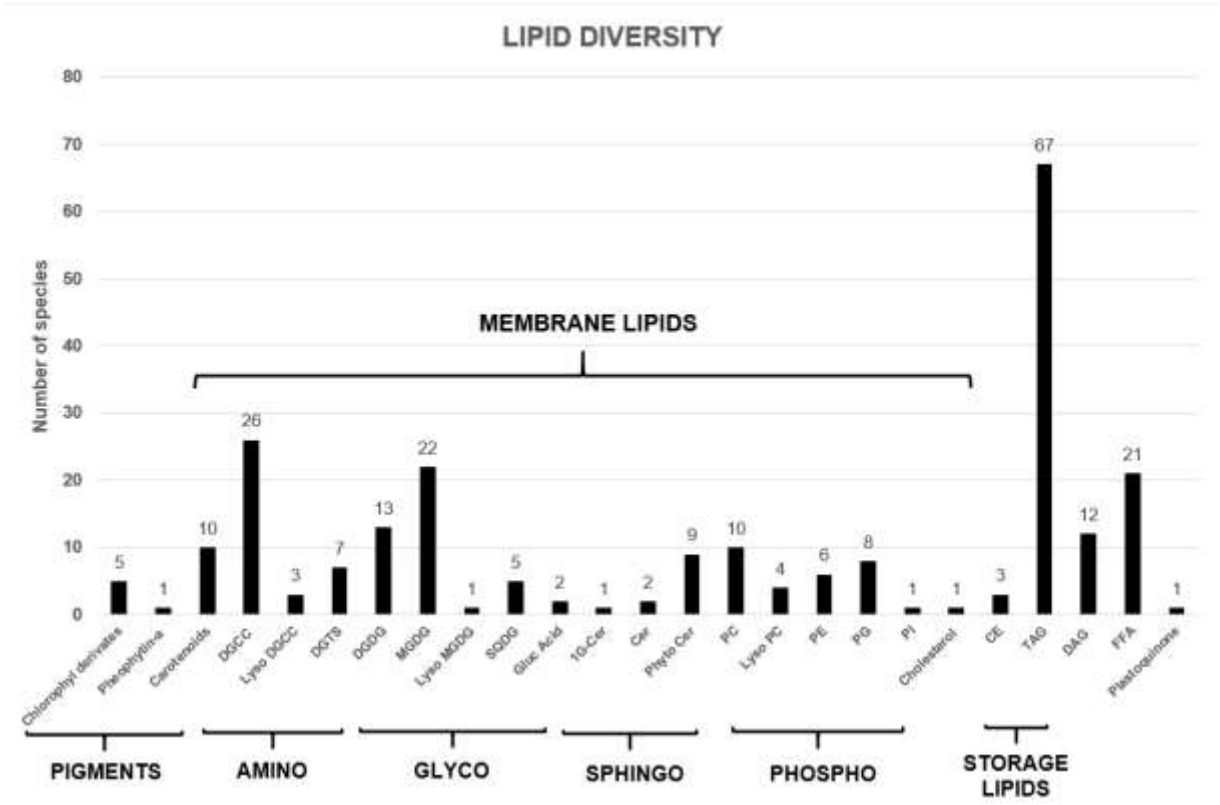

Figure 2.1: Diversity of lipid species identified in *Symbiodinium* phylotypes A1, B1 and C1 sorted into their respective lipid classes. Abbreviations: AMINO = aminolipids, GLYCO = glycolipids, SPHINGO = sphingolipids, PHOSPHO = phospholipids. Detailed description of lipid subclasses abbreviations are shown in Table S2.3.

The main lipid classes shown in Fig. 2.1 were monitored and quantified in *Symbiodinium* phylotypes and detailed data is presented in Fig. 2.2. Their total lipids content was not significantly different ($p > 0.05$) and the same fact was noticed for total pigments quantities, as well as diacylglycerol (DAG) and free fatty acids (FFA). However, B1 presented the lowest concentration of total membrane lipids and storage lipids because aminolipids and phospholipids were more abundant in phylotypes A1 and C1 (Fig. S2.1). All phylotypes showed differences in the cholesterol ester (CE) amounts, but their triacylglycerol (TAG) quantities were equivalent (Fig.S2.1). In the next following items, we show particular distinctions noticed in each specific lipid group and how did they behave in each studied phylotype.

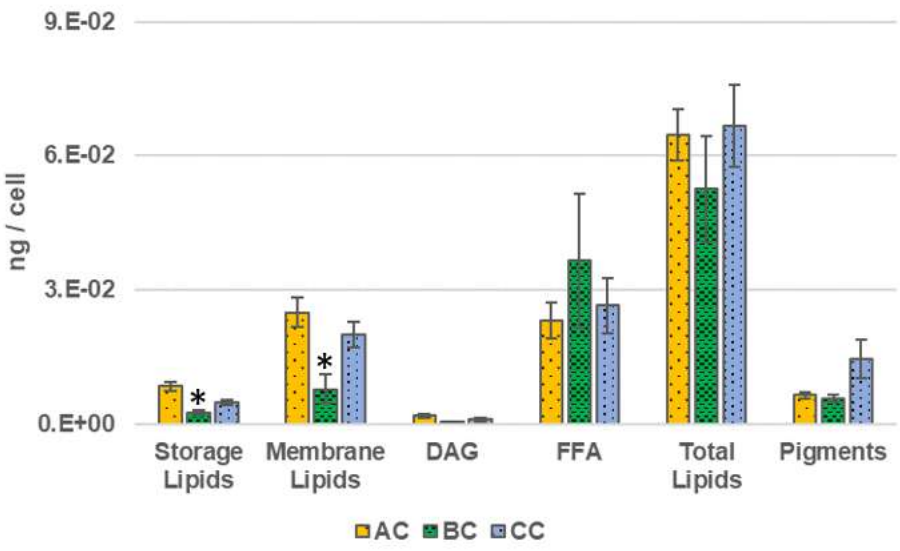

Figure 2.2: Lipid molecular species profiles of *Symbiodinium* phylotypes A1, B1 and C1 control samples. Cultures grew at 22 ºC; 80 µmols photons m^{-2} s^{-1}; 12L:12D cycle. Bars show the mean abundance of each lipid class per cells and their respective standard errors. Storage lipids encompass cholesterol esters and triacylglycerol; membrane lipids group sphingolipids, phospholipids, glycolipids and aminolipids; Diacylglycerol (DAG) and free fatty acids (FFA) are presented alone. * represents statistically significantly different ($p < 0.05$) mean values inside their respective lipid groups. Fig.S2.1 exhibits separately details about storage lipids and membrane lipids. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

3.1.1 Pigments

Chlorophyll-a, chlorophyll-c2 and peridinin related to the main photosynthetic apparatus of marine dinoflagellate protein complexes were the most abundant pigments in all analyzed samples. Carotenoids related to the xanthophyll cycles (Violaxanthin-Zeaxanthin and Diadinoxanthin-Diatoxanthin) and others were also present in lower quantities. Detailed data is shown on figure S2.2 a) and b). No statistically significant variations ($p > 0.05$) were noticed between phylotypes.

3.1.2 Membrane lipids

The most abundant membrane lipids were monogalactosyldiacylglycerol (MGDG), 1,2-diacylglyceryl-3-(O-carboxyhydroxymethylcholine) (DGCC), phosphatidylcholine (PC) and digalactosyldiacylglycerol (DGDG) (Fig S2.3 a, c, d, b, respectively). Other membrane lipid subclasses were also present in lower quantities and they are also described in figures S2.3, S2.4 and S2.5 All distinct membrane lipid subclasses are further described below.

Nomenclature of membrane lipids (glycolipids, phospholipids and amino lipids) is given as their headgroups (e.g., MGDG, PC, DGCC) followed by their respective fatty acid side chains (e.g. 14:0/16:0, 18:1/22:6). For all three groups, lyso species were characterized and nomenclature follows the same structure, except that lysolipids are composed of only one fatty acid chain. For sphingolipids, their nomenclature is given by their sphingosine base (e.g., d: 18:1, t: 18:0) and their n-acyl side chain (e.g., 24:0, 30:0).

3.1.2.1 Glycolipids

Glycolipids are exclusive from chloroplast membrane structures and they all present a sugar head connected to glycerol and fatty acid groups. Monogalactosyldiacylglycerol (MGDG) was the most abundant class for all phylotypes, followed by digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and glucoronic acid (Gluc Acid). The neutral lipids MGDG and DGDG were highly associated with the (n-3) polyunsaturated fatty acids (PUFA) octadecatetraenoic acid (18:4) and octadecapentaenoic acid (18:5) (Fig. S2.3 a, b, respectively), whereas SQDG was mostly associated with the saturated fatty acids (SFA) myristic acid (14:0) and palmitic acid (16:0). The most abundant glucoronic fatty acids were the palmitic acid (16:0) and DHA (22:6 – n (3); Fig. S2.5 c, d). There was no statistically significant ($p > 0.05$) variation in the abundance of different MGDG fatty acids among analyzed phylotypes. However, for DGDG, DHA was more abundant in C1 and 18:4 was more abundant in phylotype A. Other fatty acids present in lower quantities also varied significantly ($p < 0.05$).

3.1.2.2 Aminolipids

Two classes of amino lipids were identified: 1, 2 - diacylglycery l - 3 - (O - carboxyhydroxymethylcholine) (DGCC) and diacylglyceroltrimethylhomoserine (DGTS). DGCC was one of the most abundant lipids in all analyzed samples. It was the membrane lipid most associated with the (n-3) docosahexaenoic acid (DHA – 22:6). It was significantly more abundant ($p < 0.05$) in phylotypes A1 and C1 and occurred in lower concentrations in B1 (Fig. S2.3 c). On the other hand, DGTS was exclusively associated with SFA and the monounsaturated fatty acid (MUFA) oleic acid (18:1). A1 had the highest concentration and it did vary significantly across phylotypes (Fig. S2.4 a).

3.1.2.3 Phospholipids

Phosphatidylcholine (PC) was among the most abundant membrane lipids (Fig. S2.3 d). PC was highly associated with the MUFA oleic acid (18:1) and nonadecaenoic (19:1) fatty acids. All phylotypes had statistically significant ($p < 0.05$) distinct concentrations of the most abundant PC compounds. On the other hand, all other phospholipids did not vary significantly between phylotypes (F3S). Phosphatidylglycerol (PG) and phosphatidyletanolamina (PE) were highly associated with oleic acid (18:1). Palmitoleic acid (16:1) was the second more abundant in the PE lipids; whereas palmitic acid (16:0) was the second more abundant in PG. The only phosphatidylinositol (PI) characterized was a combination of palmitic acid (16:0) and DHA (22:6).

3.1.2.4 Sphingolipids

We described 3 distinct sphingolipid classes: ceramides (Cer), Glyco-ceramides (1G-Cer) and tri-hidroxy ceramides, also known as phytoceramides (Phyto-Cer). Phytoceramides were the most abundant sphingolipid in all phylotypes and polar sphingo heads were highly associated with very long chain SFA. Phylotype A1 had the highest statistically significant ($p < 0.05$) concentration of sphingolipids and particular variations are shown on detail in figure S2.4b).

3.1.2.5 Cholesterol

Cholesterol was the only sterol present among the 250 most abundant lipids found. Its concentration was not significantly different ($p > 0.05$) between phylotypes (Fig. S2.5 b).

3.1.3 Storage lipids

Triacylglycerol (TAG) was the most abundant and diverse analyzed group presenting 67 distinct molecules highly associated with SFA. No significative variation ($p > 0.05$) was found comparing total TAG abundance and relative TAG fatty acids concentration in *Symbiodinium* phylotypes (Figures S2.1 b); S2.3 e), respectively). Nomenclature of triacylglycerol compounds was given by the glycerol backbone followed by three fatty acid side chains (e.g., 16:0/18:1/DHA).

On the other hand, (n-3) PUFA was more abundant in cholesterol esters (CE) molecules (Fig. S2.4 c). Eicosatrienoic acid (ETE - n-3) was significantly ($p < 0.05$) less concentrated in B1; whereas phylotype A1 had highest concentrations of docosahexaenoic acid (DHA) and oleic acid (18:1). Cholesterol esters nomenclature were given by cholesterol linked to a single fatty acid side chain (e.g., 20:3). Although storage lipids were very diverse, they were not very abundant compared to other lipid classes.

3.1.4 Other compounds

We also found molecules that could not be fitted into the main described groups above. The characterized diacylglycerol (DAG) molecules were highly associated with palmitic (16:0) and palmitoleic (16:1) acids, but they did not show any statistically significant variation among *Symbiodinium* phylotypes (Fig. S2.5 a). Diacylglycerol nomenclature is given by its glycerol backbone followed by two fatty acid side chains (e.g., 16:0/18:0). Free fatty acids (FFA) were diverse and they were among the most abundant lipids in all phylotypes, but their concentration was also not significantly different between them (Fig. S2.3 f). On the other hand, plastoquinone, responsible for electron transport chain inside

chloroplast membrane, was significantly ($p < 0.05$) more concentrated in phylotypes A1 and C1, exclusively (Fig. 2.3).

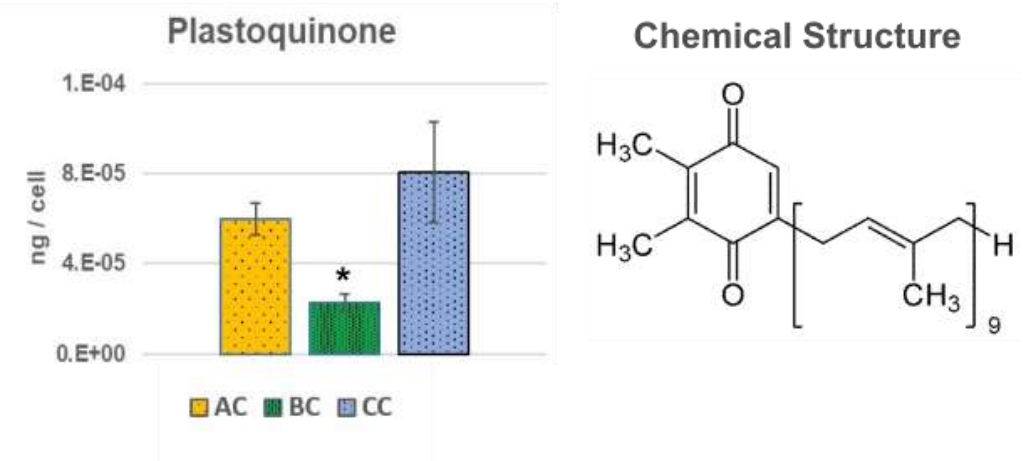

Figure 2.3: Plastoquinone concentration in *Symbiodinium* phylotypes A1, B1 and C1; and its respective molecular structure. * indicates that only BC is significantly different ($p < 0.05$) from other mean values in AC and CC. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

3.2. Main distinctions in *Symbiodinium* phylotypes are revealed in membrane lipids composition

One-way ANOVA followed by Turkey's post-hoc test ($p < 0.05$) was performed considering all lipid and pigment compounds presented above to better understand distinctions between *Symbiodinium* phylotypes. Only statistically significant compounds are shown in the heatmap and described in the right column (Fig. 2.4). Lipids were plotted with their normalized concentrations and triplicates position was stablished according to cluster analysis based on Pearson distance and Ward's algorithm. Compounds showed in red were upregulated, whereas blue compounds were downregulated. Nearly all significantly distinct lipids were represented by membrane lipids (>90%), consequently, the main differences in *Symbiodinium* spp. lipids were due to the composition of their cell's and organelle's membranes.

On heatmap's top we see grouped triplicates from phylotypes A1 and B1 (red and green) indicating that based on their lipid profile they are more similar when compared to C1(in

blue). Left cluster grouped significant lipid compounds that behaved alike. The upper part gathered mainly diacylglyceroltrimethylhomoserine (DGTS) and sphingolipids that represented 17.5% and 12.5%, respectively, of the total significantly different lipid compounds on heatmap. DGTS were high concentrated in A1, exclusively. All types of ceramides were target in sphingolipids and they were also more concentrated in A1. Although these two lipid classes had many significantly different compounds in the three studied phylotypes, we must keep in mind that their concentrations were very low compared to other more abundant membrane lipids (i.e. MGDG, DGCC and PC) – FS1.

In addition, the lower left cluster grouped other compounds that were mainly represented by membrane lipids with n-(3) fatty acids side chains that stood for 52.5% of total significantly distinct compounds. Their polar heads were constituted by phosphatidylcholine (PC), 1, 2 -Diacylglycery l - 3 - (O - carboxyhydroxymethylcholine) DGCC and glycolipids (mostly DGDG). Lower part on the left cluster showed that they were more concentrated in phylotypes A1 and, specially, in C1, which revealed more points target in red.

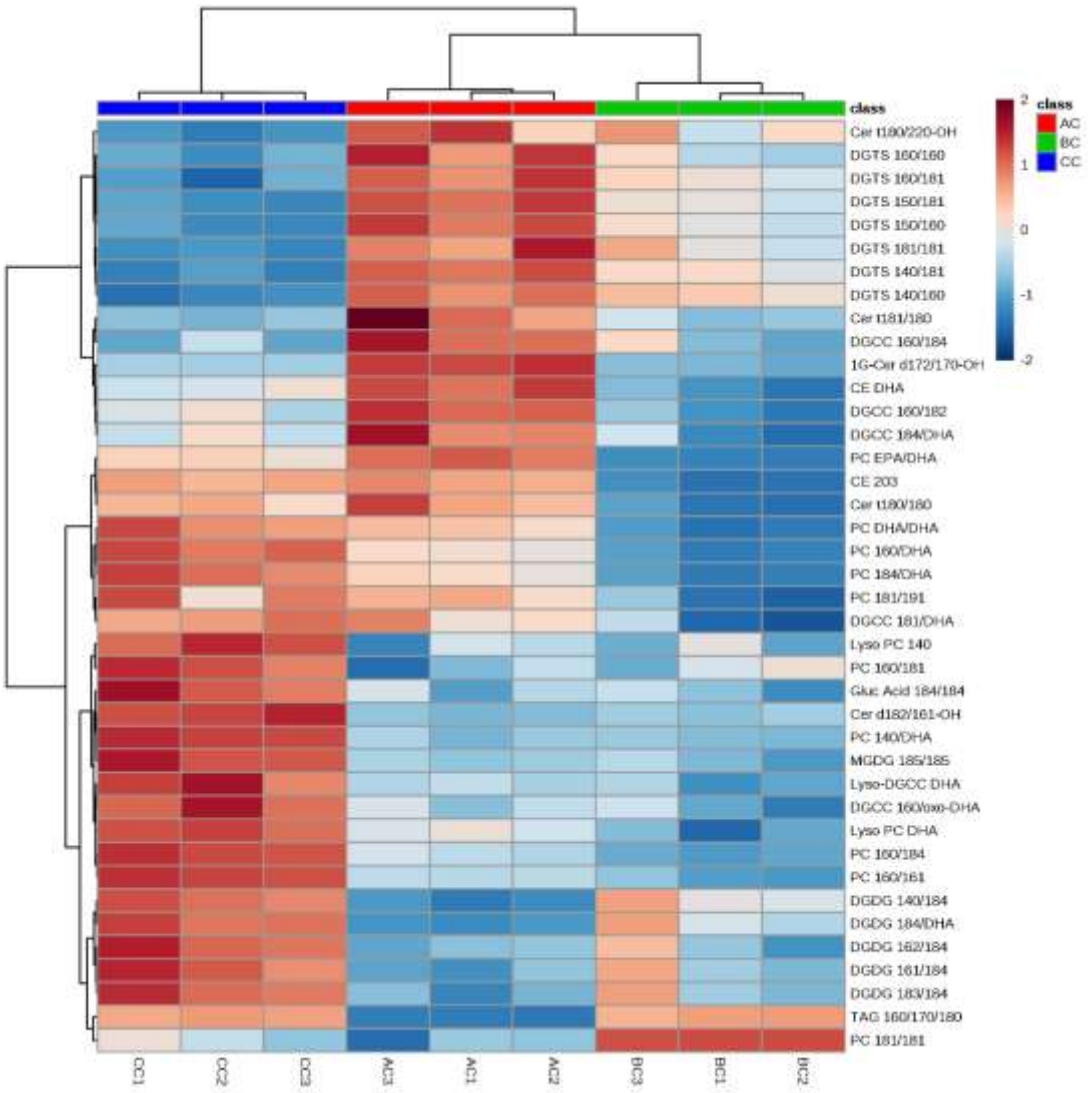

Figure 2.4: Heatmap of the most significantly different lipid molecular species between *Symbiodinium* phylotypes A1, B1 and C1. In total, 40 lipid molecular species are shown in rows and samples in column. Statistical significance was evaluated by one-way ANOVA followed by Turkey's post-hoc test ($p < 0.05$) using Metaboanalyst. Distance was measured in Pearson, and Ward´s clustering algorithm. Each colored cell on heatmap corresponds to normalized concentrations. Log transformation was used for data normalization value. Red color indicates upregulation, whereas blue means downregulation. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

Special attention was given to the most abundant omega-3 fatty acids. Figure 2.4 shows their total abundance and in which specific membrane polar head they were connected. Octadecatetraenoic acid (18:4) (Fig. 2.5 a) and octadecapentaenoic acid (18:5) (Fig. 2.5 b) exhibited low variation of relative percentage among *Symbiodinium* phylotypes and only C1 had significantly ($p < 0.05$) higher abundance of 18:4 (Fig. 2.5 d). They appeared mostly associated with chloroplast exclusive lipids (MGDG, DGDG). In contrast, the docosahexaenoic acid (DHA) (Fig. 2.5 c) was significantly more concentrated, representing 17% of total lipid fatty acids in phylotypes A1 and C1. However, this percentage was significantly less representative in phylotype B1, in which DHA stood for only 6% of total lipid fatty acids (Fig. 2.5 d). Differently from the other mentioned omega -3, the only membrane lipid polar head highly associated with DHA (n-3) was the betaine lipid 1,2-diacylglyceryl-3-(O-carboxyhydroxymethylcholine) (DGCC). DHA was also abundant as free fatty acids, but only appeared with statistically significant concentrations in all phylotypes when associated with phosphatidylcholine (PC) and cholesterol esters (CE) as it has been previously highlighted on heatmap.

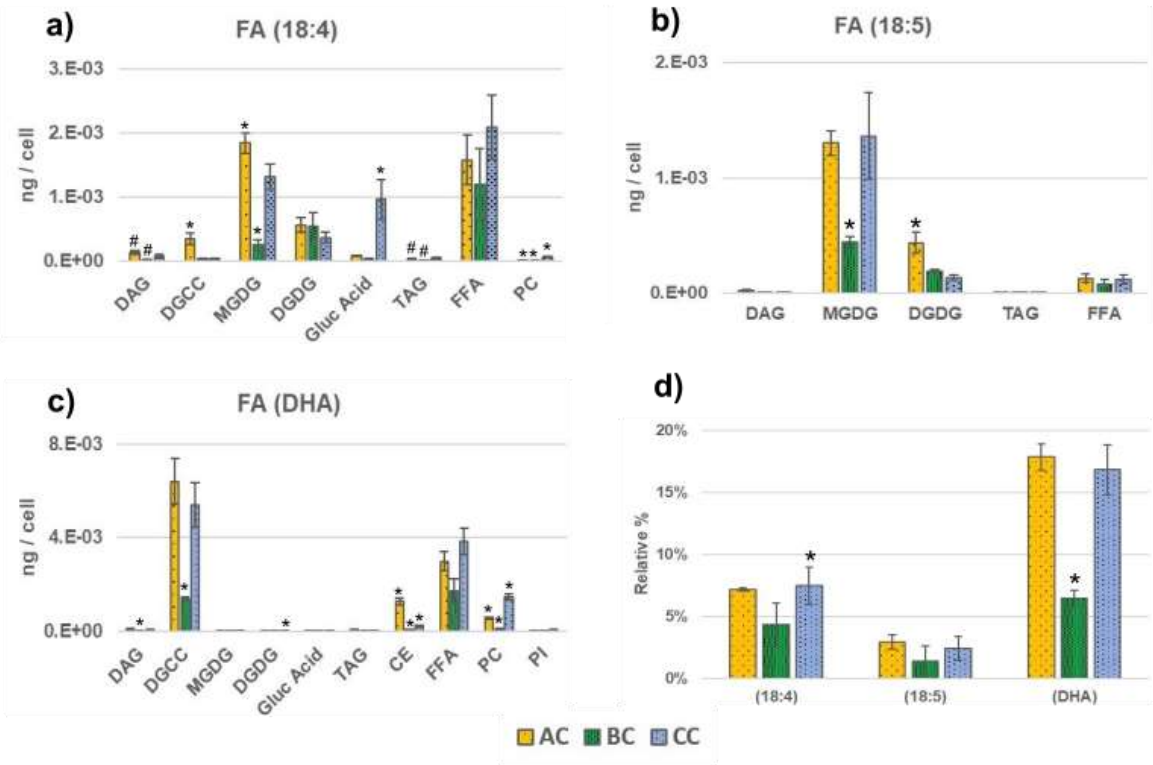

Figure 2.5: Distribution of main omega 3 (n-3) fatty acids found in *Symbiodinium* phylotypes A1, B1 and C1 on their respective lipid polar heads. a) octadecatetraenoic acid (18:4) distribution; b) octadecapentaenoic acid (18:5) distribution; c) docosahexaenoic acid distribution; d) relative percentage of main omega 3 (n-3) fatty acids to total lipids in the 3 studied phylotypes. Means showing * are significantly different ($p < 0.05$) from other mean values inside their respective lipid groups. Means showing # are exclusively significantly different from each other (same $p < 0.05$). Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

4. DISCUSSION

Many studies have investigated lipids and pigments in *Symbiodinium* and other marine flagellate microalgae using the combination of liquid chromatography and mass spectrometry techniques (Leblond *et al*., 2000, 2006, 2015; Gray *et al.*, 2009; Awai *et al*., 2012; Armada *et al*., 2013; Dahmen *et al*., 2013; Anesi & Guella, 2015; Anesi *et al.*, 2016). However, none has ever reported quali- and quantitative global lipidome and pigment characterization made with single analytical runs. Our phenotype data combined the concentration of fatty acids, popularly used in lipid studies (Mansour *et al.*, 1999) with robust polar and neutral lipid heads and pigment characterization, including compounds known to represent specific cell compartments (i.e., glycolipids and plastoquinone – chloroplast). Thus, our results might be useful to understand microalgae lipid metabolic pathways and might serve as guidelines for future investigation of lipids in *Symbiodinium* and other species.

Although concentrations of total lipids and total pigments were not significantly different among *Symbiodinium* phylotypes, both membrane and storage lipids occurred in lower concentrations ($p < 0.05$) in B1. B1 lower membrane concentration was determined by phosphatidylcholine (PC) and 1, 2 -Diacylglycery l - 3 - (O - carboxyhydroxymethylcholine) - DGCC mainly associated with omega 3 (n-3) fatty acids (especially docosahexaenoic acid - DHA). They were also two of the most abundant classes of membrane lipids together with monogalactosyldiacylglycerol (MGDG), which did not vary among phylotypes. Therefore, total concentration of n-3 fatty acids determined more than half of the difference between *Symbiodinium* phylotypes. Other less abundant membrane lipid compounds also accounted for the remaining differences. A1 and C1 phylotypes were less different regarding omega 3 concentrations, but sphingolipids and diacylglyceroltrimethylhomoserine (DGTS) were highly upregulated only in A1.

Photosynthetic eukaryote chloroplast membranes are mainly composed of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). Block *et al.* (1983) demonstrated that together these three glycolipids stand for more than 90 per cent of total

glycolipids, being responsible for the support of photosynthetic machinery (Jones, 2007). Both MGDG and DGDG are neutral lipids with sugar heads usually presenting high PUFA content (Mongrad *et al*., 1998; Boudiere *et al*., 2012, 2014). Uncommon C18 derived PUFAs are often reported with these glycolipid heads in dinoflagellates, defining their unique character not found in any other phytoplankton group (Leblond *et al*., 2000; Guschina & Harwood, 2009). In our *Symbiodinium* spp. samples, both octadecatetraenoic (18:4) and octadecapentaenoic (18:5) acids were very abundant. Also, DGDG was almost the only fatty acid present with similar amounts among phylotypes. DGDG is a bi-layer forming lipid (Shimojima *et al*., 2009) and its double sugar head might help on the stabilization of the membrane, once its PUFAs assign very low viscosity. However, MGDG is a cylindrical forming lipid which supports xanthophyll cycles of violaxanthin and diadinoxanthin (Goss *et al*., 2005; Yamamoto, 2006) that are very important in the chloroplast antenna permitting protein complexes in the thylakoid membrane (Nisar *et al*., 2015). SQDG and PG are both anionic lipids supposed to add negative charges favoring curvatures in the chloroplast membranes (Sato *et al*., 2000, 2004; Bastien *et al*., 2016). Similarly to that found for pigments, SQDG and PG concentrations were not significantly different among phylotypes. Thus, our study defined that membrane lipids and pigments were very conservative in the chloroplast of all *Symbiodinium* phylotypes, suggesting that the bulk of their photosynthetic structures might have been conserved during evolution to keep lipid homeostasis functional enabling optimal bioenergetic rates.

While *Symbiodinium* spp. photosynthetic structures were not much different in terms of lipids, phylotype A1 has a significantly higher concentration, specially all described molecules of sphingolipids and diacylglyceroltrimethylhomoserine (DGTS). Sphingolipids are important components of the plasmatic membrane and they might serve as cell signaling molecules for vital physiological activities (e.g., control of apoptosis) (Wada & Murata, 2009). On the other hand, DGTS is part of the aminolipids group and its metabolism and physiological role in the cell are poorly described. DGTS was also reported in other microalgae groups, lower plants, fungi and bacteria (Vogel & Eichenberger, 1992; Kato *et al*., 1996; Kunzler & Eichenberger, 1997; Schubotz *et al*., 2009; Evans *et al*., 2017), in which it could work as a precursor of another aminolipid 1,2-

diacylglyceryl-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)-fl-alanine) – DGTA (Vogel & Eichenberger, 1992), that was, however, not present in our samples.

Furthermore, differences between *Symbiodinium* phylotypes were mainly determined by higher n-3 fatty acid concentrations in supposedly non-thylakoid membrane lipids in phylotypes A1 and C1. Part of it was in phosphatidylcholine (PC), which is another bi-layer forming lipid dominant in all membranes other than chloroplasts, but not characteristic of any specific cell compartment (Shimojima *et al.*, 2009). Quantitatively more representative, 1, 2 -Diacylglycery l - 3 - (O - carboxyhydroxymethylcholine) – DGCC was another aminolipid to which most of the docosahexaenoic acid (DHA) was connected. Khozin-Goldberg & Cohen (2006) suggested that, under phosphate starvation, PC polar heads can be replaced by DGCC since they are both zwitterionic lipids, i.e., double charged. The same behavior was found in environmental studies, especially in the oligotrophic ocean gyres, where phytoplankton communities were mostly composed of marine flagellate microalgae with lipid phenotypes similar to *Symbiodinium* spp. (Van Mooy *et al*., 2009, 2010; Becker *et al*., 2018). Thus, PC and DGCC might stablish the same role inside cell membranes and their relative proportion could be defined by either nutrient conditions and/or, most likely, be evolutionary determined considering that organisms inhabit oligotrophic ocean regions (i.e., gyres and coral reefs areas).

The most intriguing aspect of DGCC and PC is their connection with omega 3 fatty acids, which are suggested to compose energy transferring membranes boosting electron transport and, consequently, enabling more energy output (Valentine, 2004, 2009). Plastoquinone, which is responsible for electron transport exclusively in chloroplast membranes (Lambreva *et al*., 2014), was only upregulated in phylotypes A1 and C1. This raises the possibility it might have at least partially both PC and DGCC omega 3 compounds present in the chloroplast membranes. Moreover, DGCC concentrations were higher than MGDG, which was supposed to be the most abundant lipid not only among glycolipids, but in some cases, among all subclasses of microalgae complete lipidomes. Armada *et al.* (2013) showed that it was true for *Diacronema vlkianum*, whereas *Pseudochrysis paradoxa* followed the same pattern noticed in *Symbiodinium*

spp. Thus, it is possible that this could be potentially true for other phylotypes of related microalgae. The universal biochemical principle of risk-benefit proposed by Valentine (2004, 2009) in which higher number of double bounds decrease membrane viscosity making it more susceptible to reactive species attack (i.e., most reactive oxygen species – ROS). In order to protect membrane structure, organisms must have concomitantly evolved effective protection strategies. All cell pigments are in the chloroplast; carotenoids, for instance, together with plastoquinone can act as efficient antioxidants protecting thylakoid membranes (Krinsky *et al*., 1989; Ramel *et al*., 2012; Havaux *et al*., 2013; Nisar *et al*., 2015) allowing the presence of many double bounds in the membrane.

The theory proposed above definitely needs more information regarding *Symbiodinium* spp. and other microalgae aminolipid metabolism. Eichenberger & Gribbi (1997) suggested that aminolipids, including DGTS and other relatives not found in the present study, are likely participating in the metabolism of glycolipids, since intermediate polar heads for acyl fatty acid chains ultimately connect to glycolipids heads. However, this theory is not confirmed so far and the fatty acid profiles of amino- and glycolipids classes from our samples were quite distinct and do not indicate this. If both metabolisms are correlated, many enzymes and protein structures might be needed in order to change elongation and saturation of fatty acid chains. Thus, we strongly highlight the necessity of aminolipid metabolism studies to better comprehend distinct physiological boundaries among *Symbiodinium* phylotypes when they are both free-living or associated with coral hosts.

Last, we have shown that our phenotypical lipid data support the investigation of lipid metabolism in cell physiology and can also be applied in *Symbiodinium* spp. and other phytoplankton ecological studies. Distinct basal lipidome profiles presented here might be explained by evolutionary different ecological temperature niches that they have been occupying until nowadays. Thus, they might indicate that *Symbiodinium* phylotypes are likely to respond differently to environmental variations in abiotic factors (i.e., temperature, osmolarity of culture media, carbon chemistry). Putting into perspective the current scenario of global warming, we decided to investigate in chapter 2 how their profiles would be altered after a heat shock event and how lipid data would connect or not to

Symbiodinium phylotypes thermal tolerance. Investigation will also complement discussion and speculations already proposed above.

Supporting Information

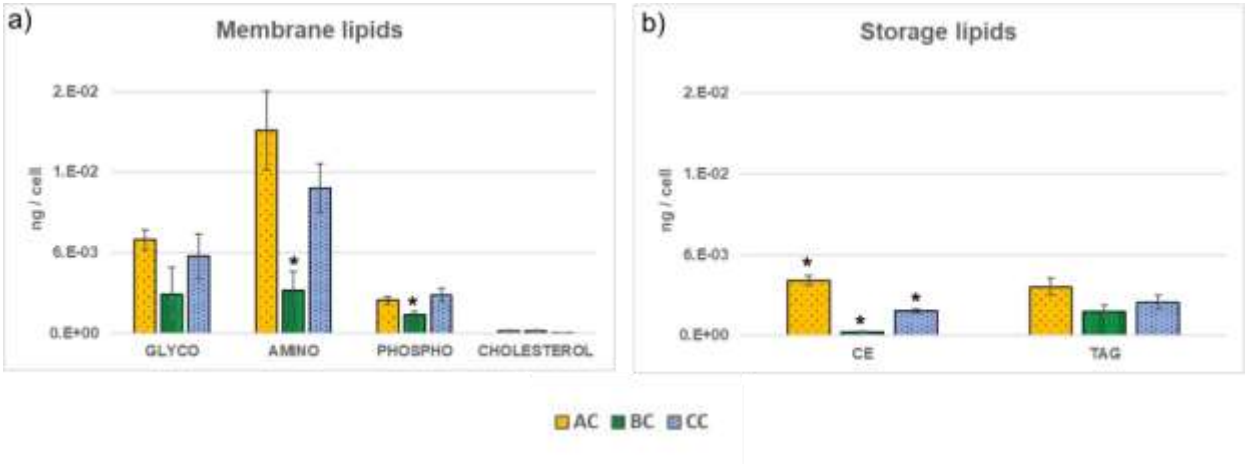

Figure S2.1: Membrane and storage lipid subclasses in *Symbiodinium* phylotypes A1, B1 and C1. a) main membrane lipids subclasses. Sphingolipids are not represented here because their concentrations were lower than 10^{-4}; b) storage lipid subclasses. Means showing an * are significantly different ($p < 0.05$) from other mean values inside their respective lipid subclasses. Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

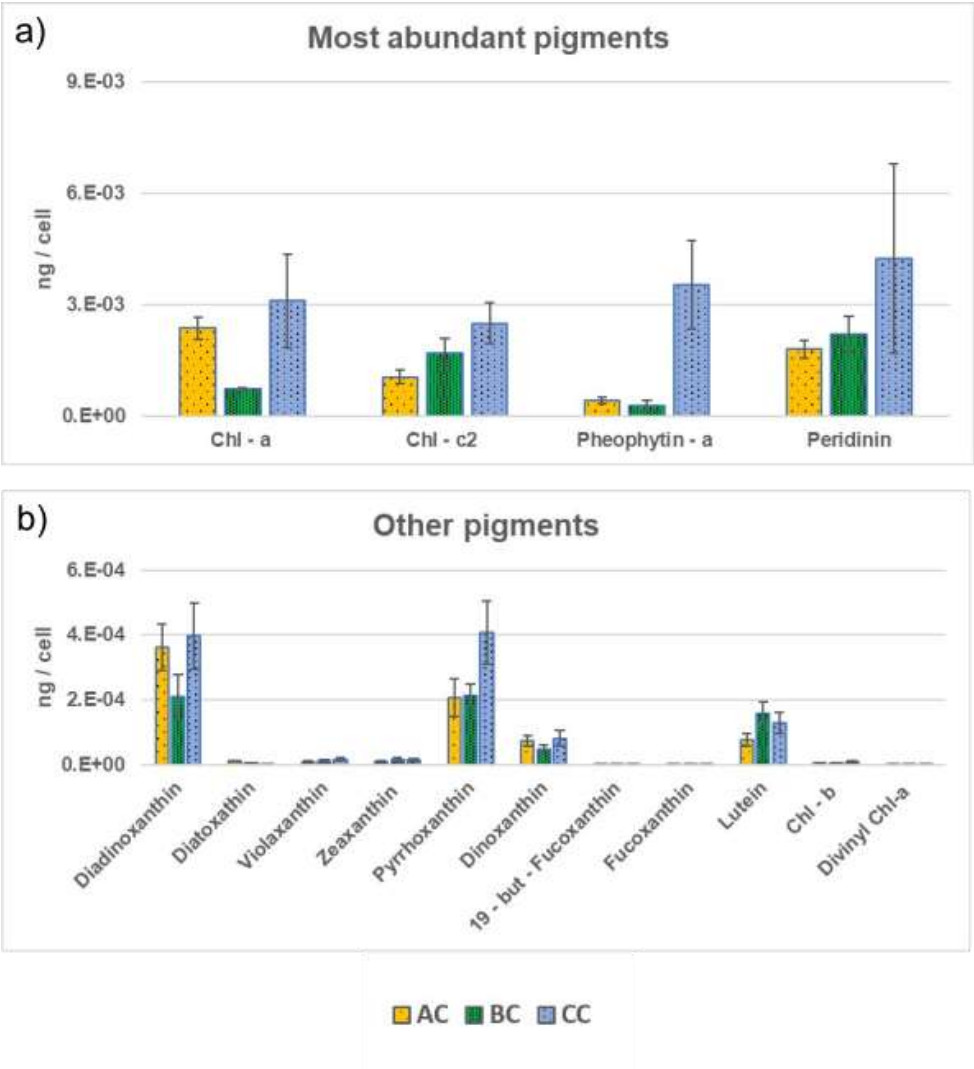

Figure S2.2: Pigments in *Symbiodinium* phylotypes A1, B1 and C1. a) most abundant pigments; b) minor representative pigments. Means were not significantly different. Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

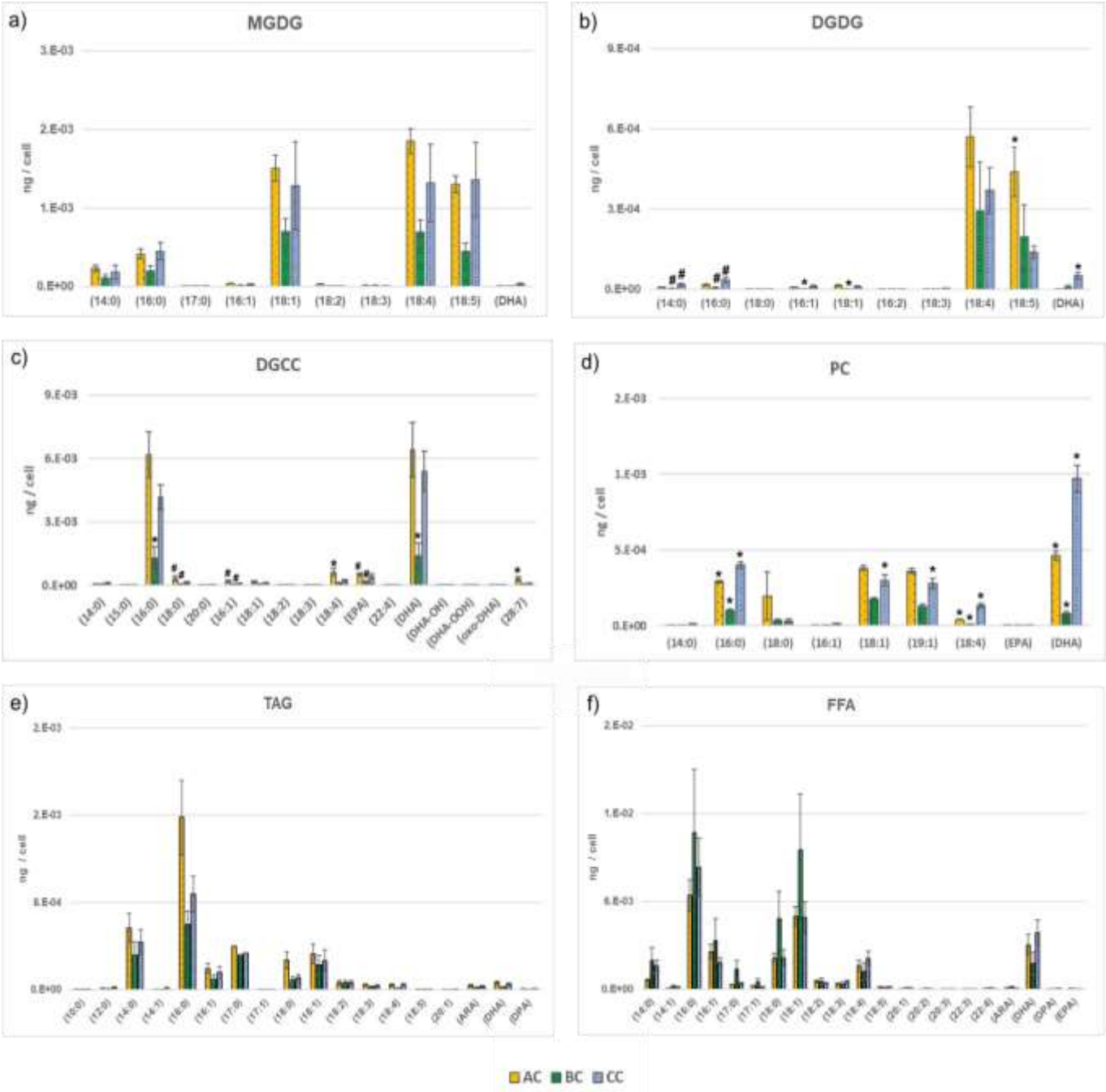

Figure S2.3: Most abundant lipid subclasses in *Symbiodinium* phylotypes A1, B1 and C1. a) monogalactosyldiacylglycerol (MGDG); b) digalactosyldiacylglycerol (DGDG); c)1,2-diacylglyceryl-3-(O-carboxyhydroxymethylcholine) – DGCC; concentrations of oxo-DHA were significantly lower in phylotype C1, plus concentrations of DHA-OH were significantly higher in B1; d) phosphatidylcholine (PC); e) triacylglycerol (TAG); f) free fatty acids (FFA). Means showing an * are significantly different ($p < 0.05$) from other mean values inside their respective lipid subclasses. Means showing # are exclusively significantly different from each other (same $p < 0.05$). Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

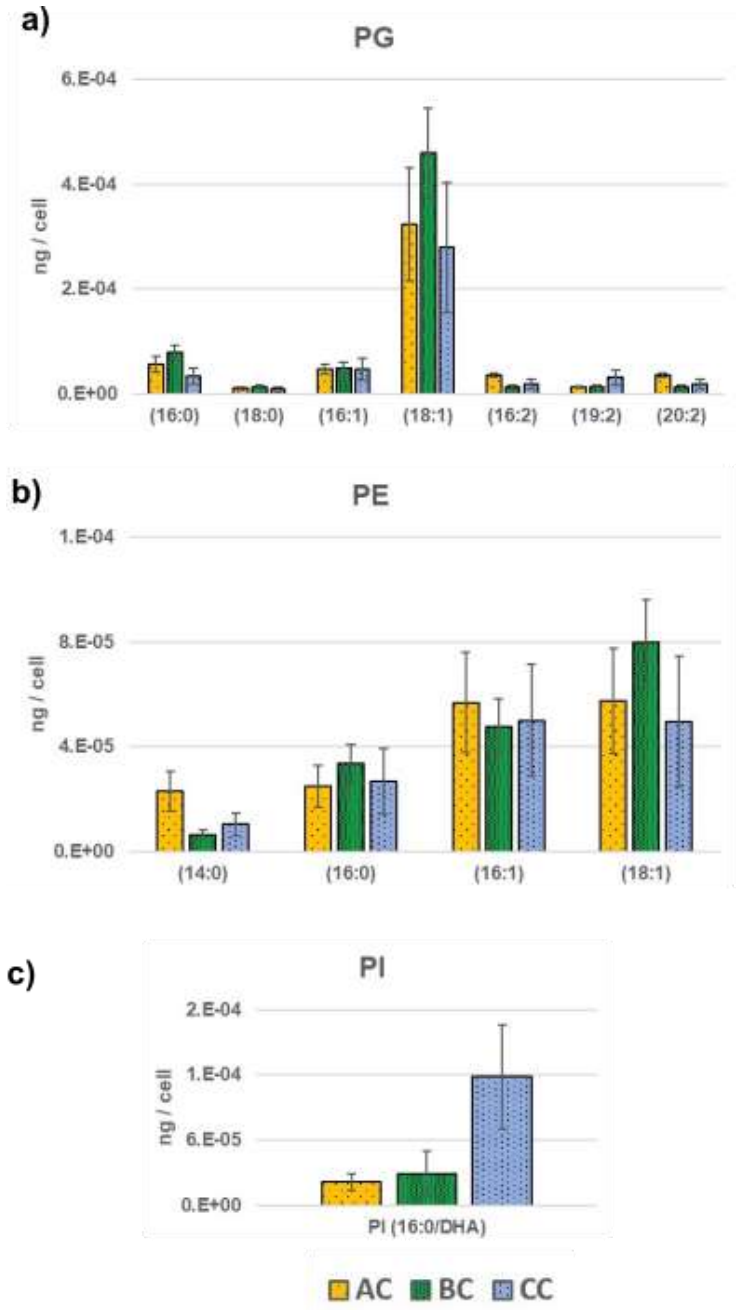

Figure S2.4: Minor abundant phospholipids in *Symbiodinium* phylotypes A1, B1 and C1. a) phosphatidylglycerol (PG); b) phosphatidyletanolamina; c) phosphatidylinositol. Means were not significantly different. Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

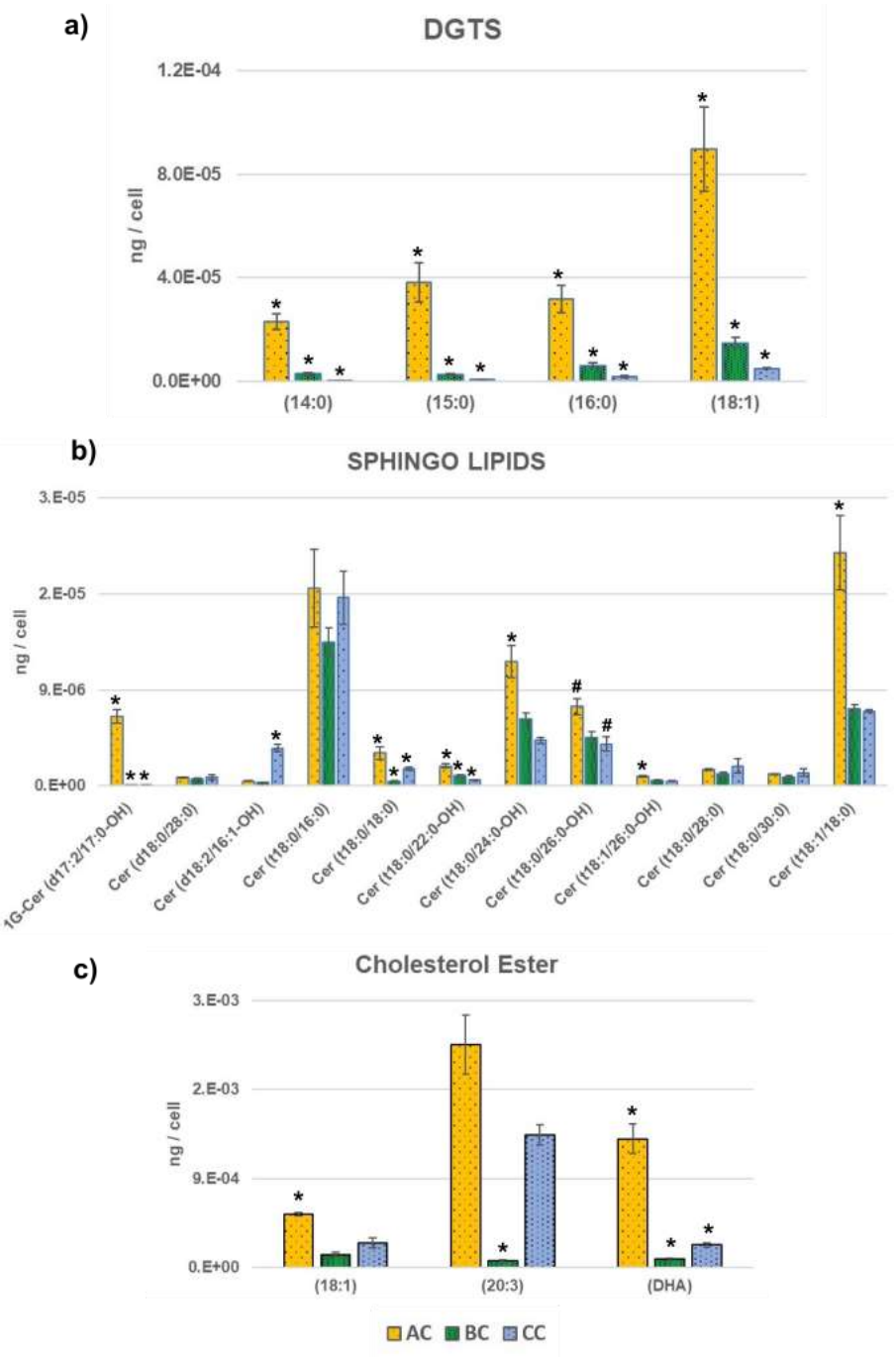

Figure S2.5: Minor compounds significantly different in *Symbiodinium* phylotypes A1, B1 and C1. a) diacylglyceroltrimethylhomoserine (DGTS); b) Sphingolipids; c) Cholesterol ester (CE). Means showing an * are significantly different ($p < 0.05$) from other mean values inside their respective lipid subclasses. Means showing # are exclusively significantly different from each other (same $p < 0.05$). Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

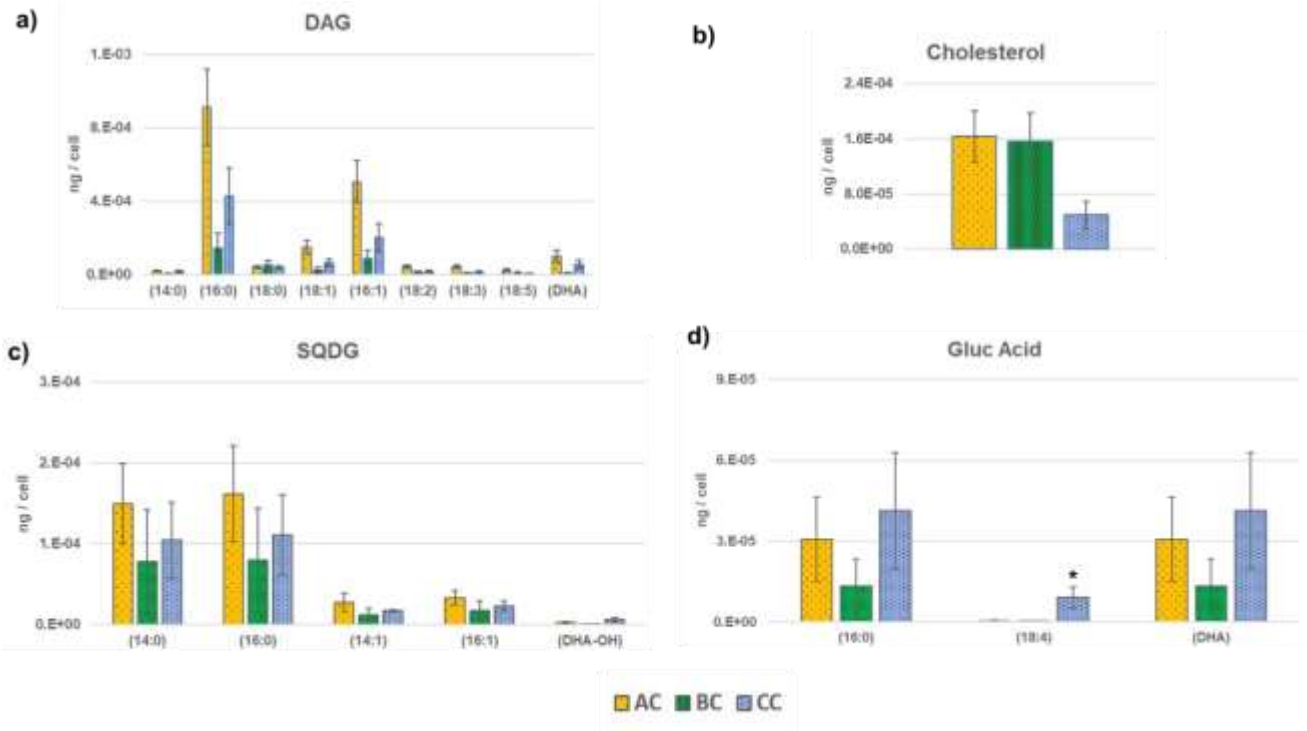

Figure S2.6: Other minor compounds not significantly different in *Symbiodinium* phylotypes A1, B1 and C1. a) diacylglycerol (DAG); b) Cholesterol; c) sulfoquinovosyldiacylglycerol (SQDG); d) Glucoronic acid (Gluc Acid). Vertical lines represent the standard error. AC, BC and CC stand for control samples of A1, B1 and C1, respectively.

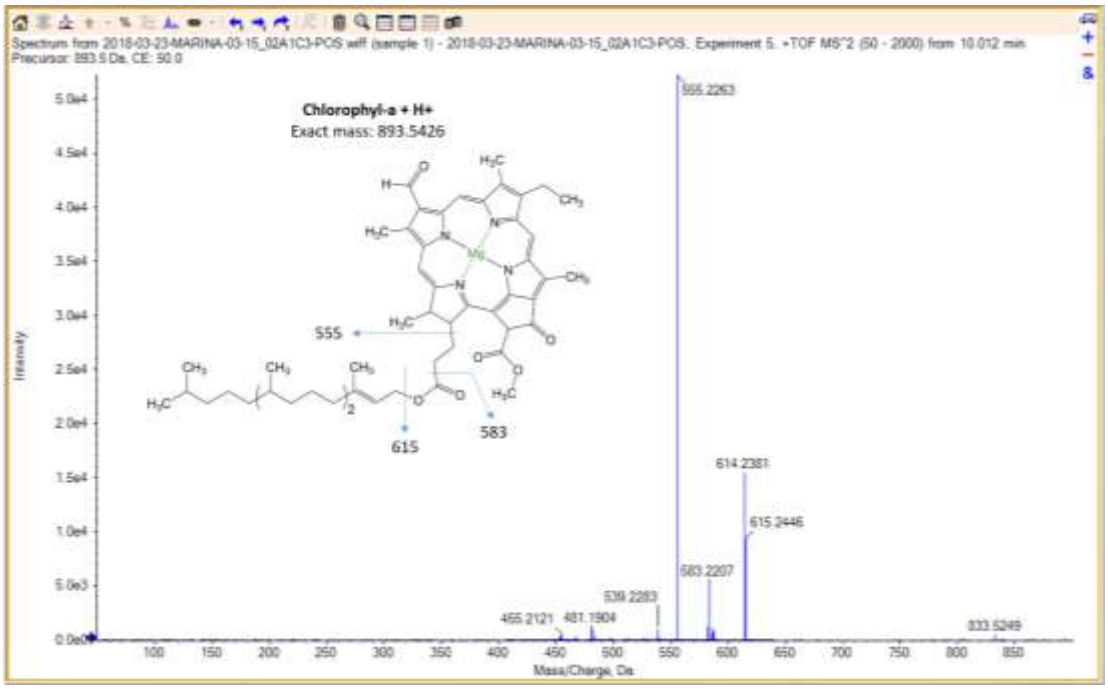

Figure S2.7: Chlorophyll-*a* molecule fragments break spectrum. Molecule draw and exact mass calculations were performed with ChemDraw.

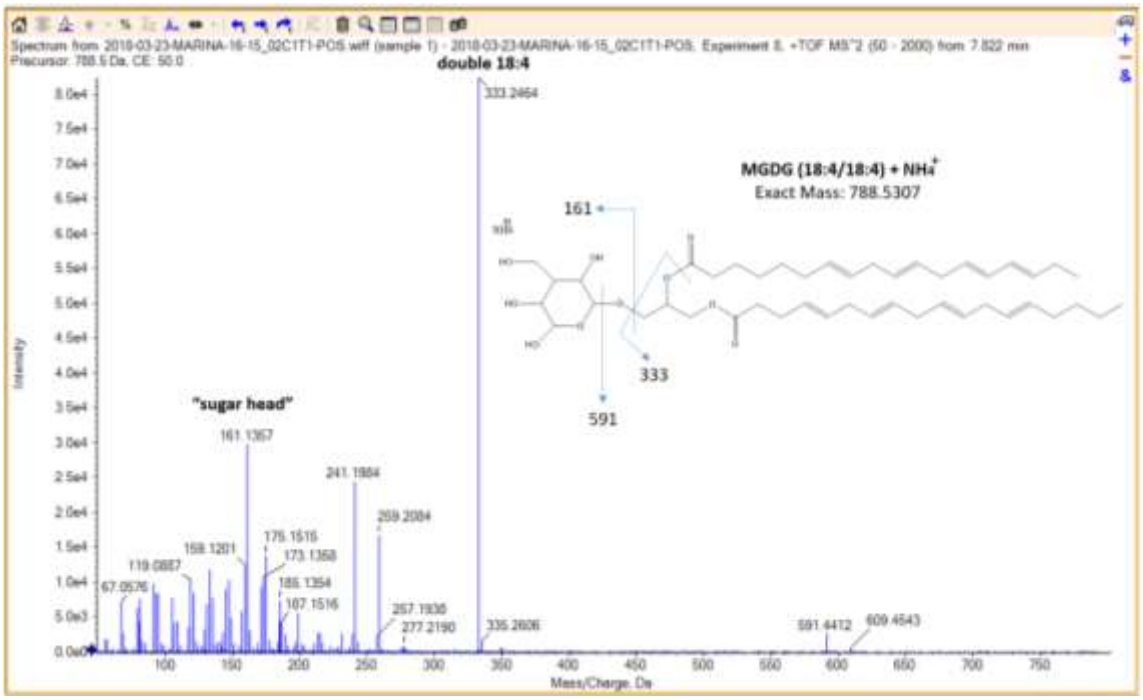

Figure S2.8: Monogalactosyldiacylglycerol molecule ionized with ammonium fragments break spectrum. High peak of 333 indicates double 18:4 fatty acids and 161 represents sugar head. Molecule draw and exact mass calculations were performed with ChemDraw.

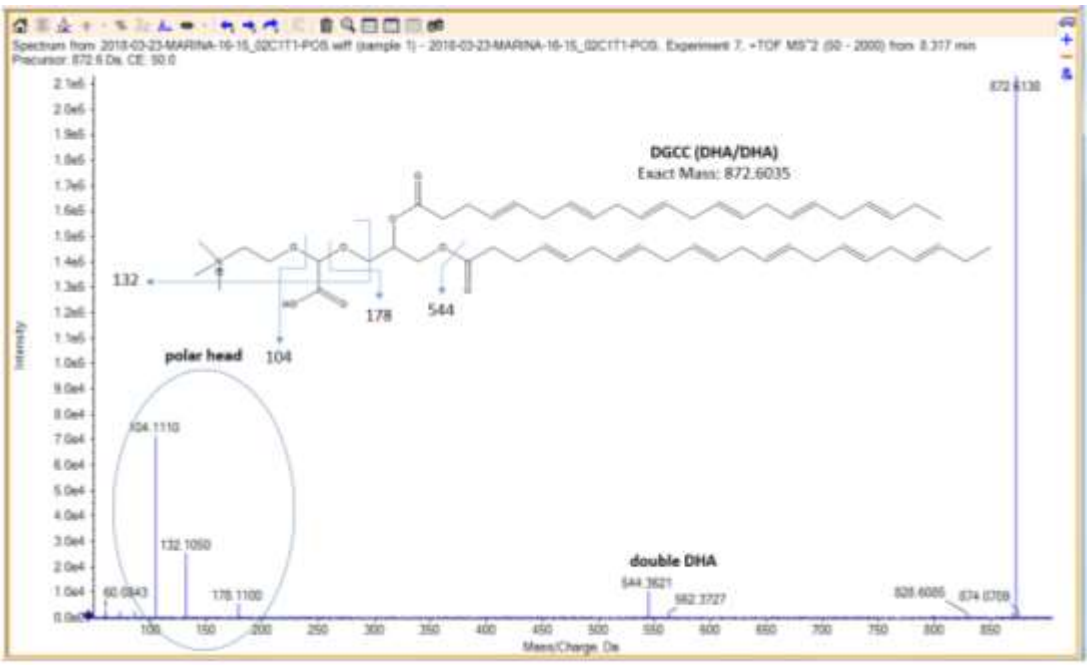

Figure S2.9: 1,2-diacylglyceryl-3-(O-carboxyhydroxymethylcholine) molecule fragments break spectrum. 562 represents loss of DHA fatty acids and 544 represents loss of DHA plus water. Molecule draw and exact mass calculations were performed with ChemDraw.

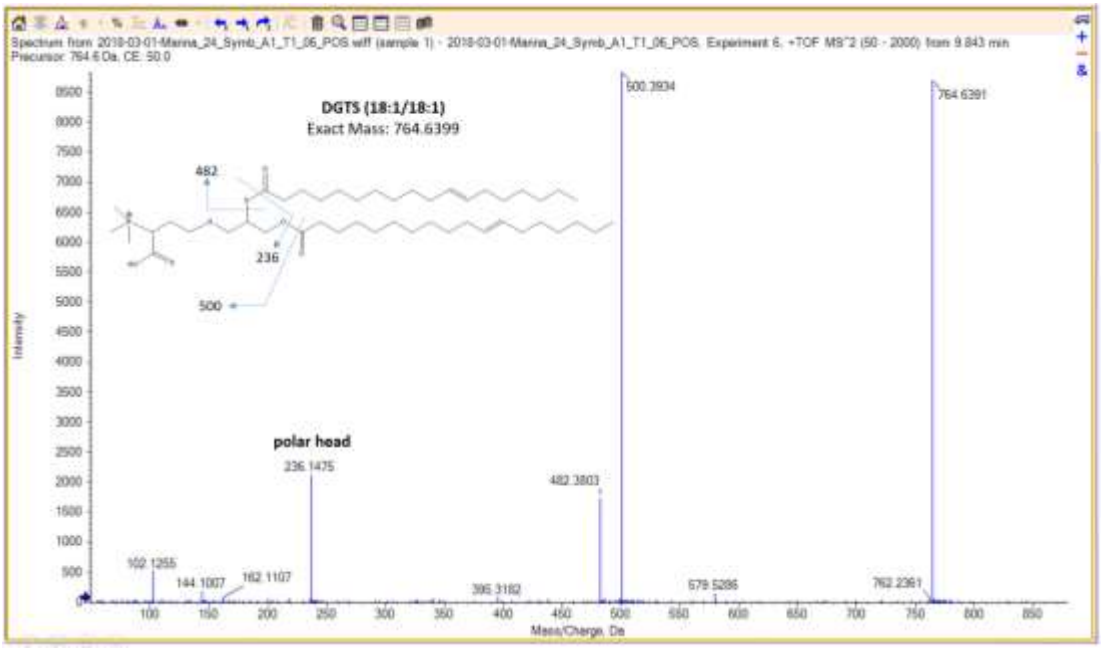

Figure S2.10: Diacylglyceroltrimethylhomoserine molecule fragments break spectrum. 236 represents polar head and 500 represents loss of 18:1 fatty acids. Molecule draw and exact mass calculations were performed with ChemDraw.

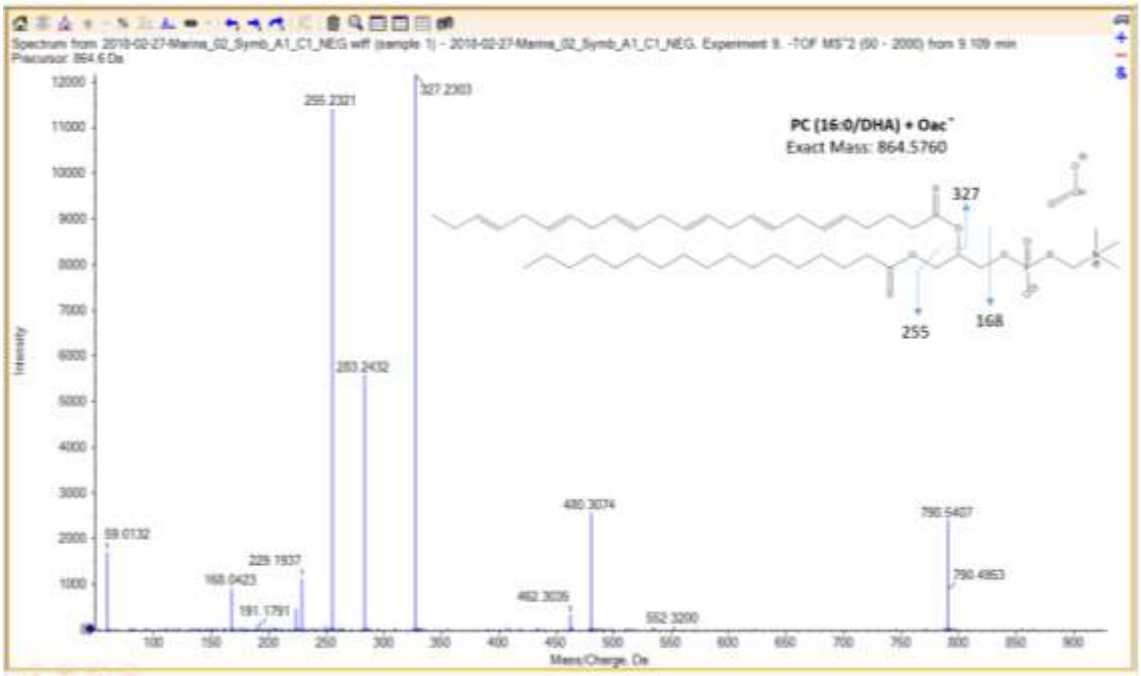

Figure S2.11: Phosphatidylcholine molecule ionized with acetate fragments break spectrum. 168 represents its polar head and each indicated arrow on figure indicate fatty acid losses. Molecule draw and exact mass calculations were performed with ChemDraw.

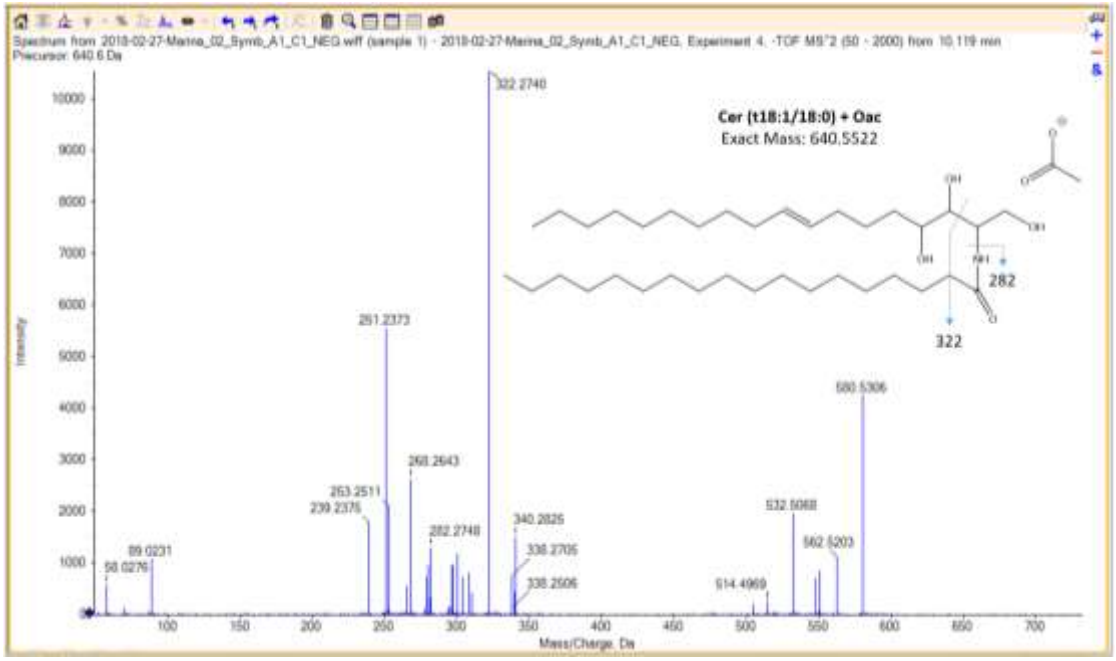

Figure S2.11: Phytoceramide molecule fragments ionized with acetate break spectrum. Molecule draw and exact mass calculations were performed with ChemDraw.

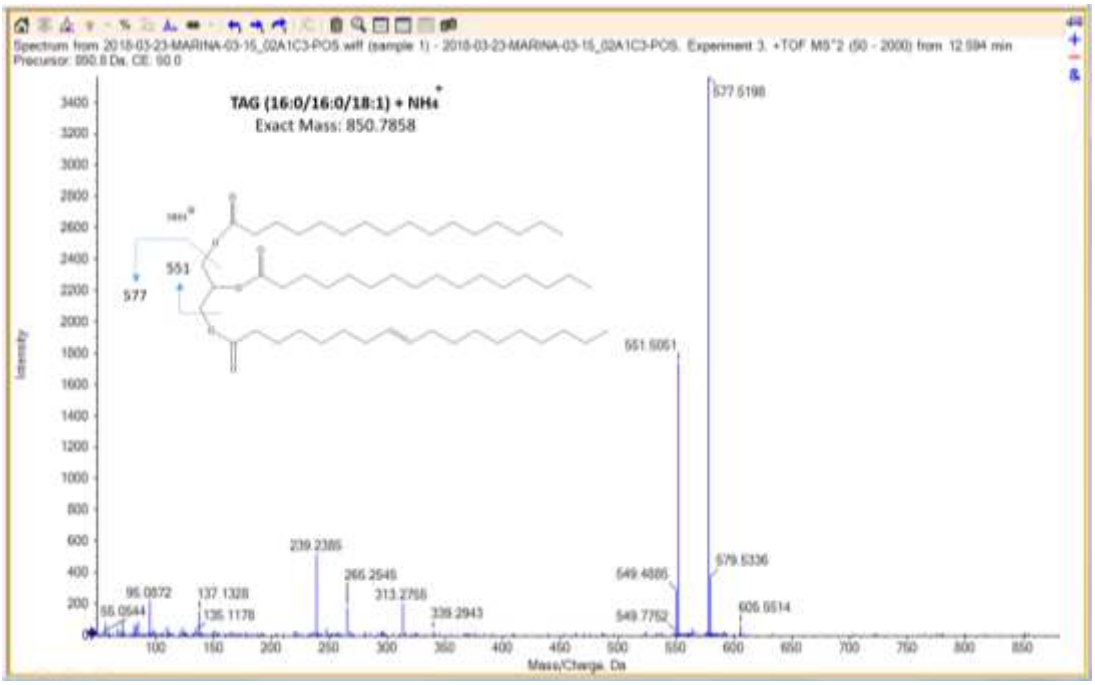

Figure S2.12: Triacylglycerol molecule ionized with ammonium fragments break spectrum. Fatty acids were determined based on exact mass loss. Higher peak of 577 indicates double loss of 16:0 and 551 peak indicates loss of 18:1. Molecule draw and exact mass calculations were performed with ChemDraw.

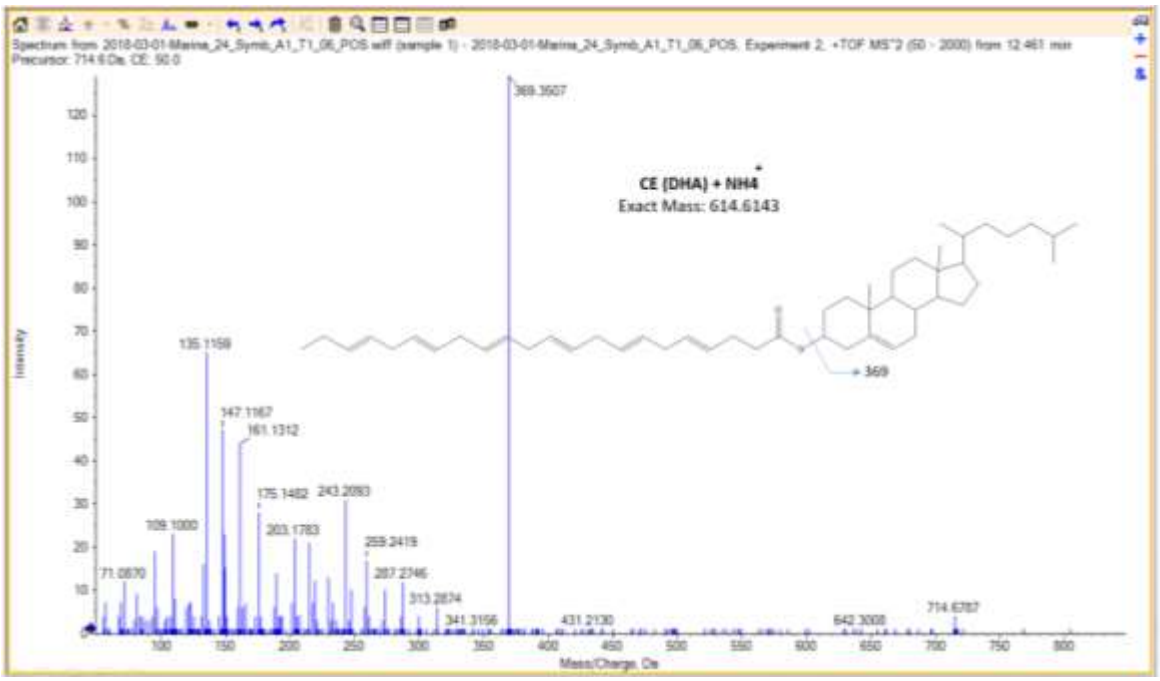

Figure S2.13: Cholesterol ester molecule ionized with ammonium fragments break spectrum. Molecule draw and exact mass calculations were performed with ChemDraw.

Table S2.1: Internal standards used for quantification of sphingolipids, phospholipids, storage lipids and plastoquinone described in the material and methods section.

Internal Standard	Work concentration (µg / mL)	Quantified lipid classes	Source
Ceramide (d18:1/17:0)	10	Cer, aminolipids	Avanti Polar Lipids
PC (17:0/17:0)	10	PC, PI, FFA	Avanti Polar Lipids
PE (17:0/17:0)	10	PE	Avanti Polar Lipids
PG (17:0/17:0)	10	PG	Avanti Polar Lipids
Lyso PC (17:0)	10	lyso PC, pigments	Avanti Polar Lipids
TAG (17:0/17:0/17:0)	10	TAG, DAG, glycolipids	Avanti Polar Lipids
CE (10:0)	10	CE	Avanti Polar Lipids
PA (17:0/17:0)	10	N/A	Avanti Polar Lipids
SM (d18:1/17:0)	10	N/A	Avanti Polar Lipids
Lyso PE (17:1)	10	N/A	Avanti Polar Lipids
CL (14:0/14:0/14:0/14:0)	10	N/A	Avanti Polar Lipids
PC (14:0/14:0)	10	N/A	Avanti Polar Lipids
PE (14:0/14:0)	10	N/A	Avanti Polar Lipids
TAG (14:0/14:0/14:0)	10	N/A	Avanti Polar Lipids

Table S2.2: Additional standards used for quantification of glycolipids, aminolipids and pigments described in the material and methods section.

Standard	Lipid class	Dilution curve range (µg / mL)	Internal std used for quantification	Correction index (α)	Source
Glyco lipids					
MGDG (14:0/16:0)	MGDG	2.5 - 0.078	TAG (17:0/17:0/17:0)	0.5633	Marum - Bremen
DGDG (16:0/18:0)	DGDG	2.5 - 0.079	TAG (17:0/17:0/17:0)	0.7282	Marum - Bremen
SQDG (17:1/17:2)	SQDG	2.5 - 0.078	TAG (17:0/17:0/17:0)	0.8922	Marum - Bremen
Amino lipids					
DGTS (16:0/16:0)	DGTS	2.5 - 0.078	Cer (d18:1/17:0)	1.4484	Marum - Bremen
DGTS (16:0/16:0)	DGCC	2.5 - 0.079	Cer (d18:1/17:0)	1.4484	Marum - Bremen
Pigments					
Chlorophyll-a	Chlorophyll-a	3.0 - 0.093	Lyso PC (17:0)	0.691	DHI Lab Products
Chlorophyll-b	Chlorophyll-b	3.0 - 0.093	Lyso PC (17:0)	0.2725	DHI Lab Products
Chlorophyll-c2	Chlorophyll-c2	3.0 - 0.093	Lyso PC (17:0)	0.1572	DHI Lab Products
Divinyl chlorophyl a	Divinyl chlorophyl a	3.0 - 0.093	Lyso PC (17:0)	0.691	DHI Lab Products
Pheophytin - a	Pheophytin - a	3.0 - 0.093	Lyso PC (17:0)	0.691	DHI Lab Products
Peridinin	Peridinin	3.0 - 0.093	Lyso PC (17:0)	0.6518	DHI Lab Products
Lutein	Lutein	3.0 - 0.093	Lyso PC (17:0)	0.1652	DHI Lab Products
Diatoxanthin	Diatoxanthin	3.0 - 0.093	Lyso PC (17:0)	1.14	DHI Lab Products
Diadinoxanthin	Diadinoxanthin	3.0 - 0.093	Lyso PC (17:0)	0.6518	DHI Lab Products
Dinoxanthin	Violaxanthin	3.0 - 0.093	Lyso PC (17:0)	0.6518	DHI Lab Products
Pyrroxanthin	Violaxanthin	3.0 - 0.093	Lyso PC (17:0)	0.6518	DHI Lab Products
Violaxanthin	Violaxanthin	3.0 - 0.093	Lyso PC (17:0)	0.6518	DHI Lab Products
Zeaxanthin	Zeaxanthin	3.0 - 0.093	Lyso PC (17:0)	0.1652	DHI Lab Products
Fucoxanthin	Fucoxanthin	3.0 - 0.093	Lyso PC (17:0)	1.6438	DHI Lab Products
19-but-fucoxanthin	19-but-fucoxanthin	3.0 - 0.093	Lyso PC (17:0)	0.9074	DHI Lab Products

Table S2.3: All identified lipid compounds including their exact mass and retention time in both positive and negative modes. File is in excel spreadsheet available in Research Gate profile website.

CHAPTER 3

Concentration of omega 3 fatty acids in membrane lipids determine *Symbiodinium* phylotypes (A1, B1 & C1) fate after temperature stress

1. INTRODUCTION

Symbiodinium sp., also known as zooxanthellae, are photosynthetic dinoflagellates found as yellow-brownish vegetative cysts in a mutualistic endosymbiosis with many marine invertebrates and protists, including, flatworms, molluscs, sponges, ciliates and foraminiferans and most importantly, corals (Pawlowski *et al*., 2001; Stat *et al*., 2006). When associated with scleractinian corals, their photosynthetic products enable coral growth, reproduction and maintenance (Muscatine & Porter, 1977), underpinning reef productivity and skeleton calcification. Their symbiotic association supports the most diverse marine habitats with paramount importance for mankind, including fisheries, tourism, aquarium trades and drug development (Moberg & Folke, 1999; Salvat, 1992; Alves de Lima *et al.*, 2010; Olivotto & Simões, 2011).

The rise in abiotic stressors over the past decades resulted in extensive areas of coral reef degradation worldwide (Graham *et al.* 2008; Wilkinson, 2008). In this context, elevated seawater temperatures are known for being the main cause of coral bleaching events (Hoegh-Guldberg & Smith, 1989; Gates *et al.*, 1992; Hoegh-Guldberg & Bruno 2010). They are defined by mass expulsion of the zooxanthellae from coral host and/or the loss of photosynthetic pigments within individual zooxanthellae, potentially leading to coral death (Glynn 1991; Lesser, 1996, 1997 and 2019), once they evolved extremely dependable on the metabolites transferred by their endosymbionts (Muscatine & Porter, 1977).

A recognized explanation for the phenomenon of bleaching was first proposed in Lesser (1997) and coined by Downs *et al*. (2002) as “the oxidative stress theory of coral bleaching”, in which thermal stress and light uncouples photosynthesis by enabling the scape of high energy electrons in the thylakoid membranes of the endosymbiotic

dinoflagellate genus *Symbiodinium* leading to the production of reactive oxygen species (ROS) (Lesser 1996, 1997, 2019; Downs *et al.*, 2002; Smith *et al.*, 2005; Ragni *et al.*, 2010; Roberty *et al.*, 2015; Goyen, 2017). In molecular terms, production of ROS triggers the oxidation of biomolecules such as pigments, proteins and lipids, which are key components of both symbiont and host cells. Lipids are particularly important because they are major constituents of host tissue (Anthony *et al*., 2002; Grottoli *et al*., 2004). When in the membrane, lipid oxidation initiates a positive feedback cascade of ROS production that may kill the intracellular symbionts and damage the host cells (Tchernov *et al*., 2004; reviewed again in Lesser, 2011). In the past years significant body of data supported Lesser's theory suggesting strong links between thermal stress leading to ROS production and the lipid ecology of coral-dinoflagellate symbiosis (Lesser, 1997; Brown, 1997; Downs *et al.*, 2002; Weiss, 2008).

Lipids are pivotal for coral reef symbiosis and essential components of both coral and *Symbiodinium* spp. membranes (Matthews *et al.*, 2018). Strong evidence suggests that *Symbiodinium* spp. can change the lipid composition and architecture of cell and organelle membranes in order to keep the homeoviscous stability and adapt to environmental alterations (Hazel *et al*., 1995; D'amico *et al*., 2006). In addition, *in vitro* and *in vivo* experiments have shown that a higher concentration of saturated fatty acids (SFA) relative to polyunsaturated fatty acids (PUFA) enhanced the stability of thylakoid membranes of *Symbiodinium* in response to thermal stress (Tchernov *et al*. 2004; Bachok *et al*. 2006; Tolosa *et al*. 2011). The rationale is that thylakoid membranes are enriched in PUFAs, which are highly susceptible to oxidative damage by ROS (Wada, 1994; Lesser, 2006; Catalá, 2009). By lowering the unsaturation levels of its lipids, *Symbiodinium* may protect the photosynthetic membranes against ROS and thus acquire thermal tolerance. Indeed, phylogenetic analyses of *Symbiodinium* phylotypes isolates have shown that thermal tolerance is not associated with a single, monophyletic phylotype, but rather with the level of saturation of their membrane lipids (Tchernov *et al.,* 2004).

In addition, Widomska *et al*. (2019) showed that pigments, i.e. carotenoids, present a dual function of helping in the stabilization of photosystems in the thylakoid membranes and

as anti-oxidants in the presence of ROS. He showed that singlet oxygen, one of the highest energy ROS, exclusively generated by light can only be quenched by carotenoids. On the hand, other ROS, i.e. superoxide and hydrogen peroxide, can be either quenched by carotenoids or enzymes (superoxide dismutase – SOD and peroxidase – APX, respectively) (Krinsky *et al*., 1989; Havaux *et al.,* 2013; Krueger *et al*., 2014). Specific carotenoids from the xanthophyll cycles can switch between epoxy and de-epoxy molecules adapting to either chloroplast redox state and distinct light conditions (Jahns *et al*., 2012; Havaux *et al*., 2013), so their relative concentrations might be used as an indicator of oxidation.

Past studies have suggested alterations in chloroplast membranes promoted by heat stress based on fatty acids analysis of either *Symbiodinium* spp. only or coral-associated *Symbiodinium* (Zhulkova & Titlyanov, 2003; Tchernov *et al*., 2004; Diàz-Almeyda *et al*., 2011, 2017; Kneeland *et al*., 2013). However, this technique generates only total lipid profiles and does not indicate where inside the cells fatty acids came from. Thus, suggested alterations in membrane lipids with thermal stress presented in these studies were just speculative and could not mechanistically explain anything about how fatty acid synthesis or exchanges between cell compartments after thermal stress occurs. Aiming to fill this gap we chose to investigate the molecular mechanisms of coral bleaching by monitoring global lipids and pigments of three *Symbiodinium* phylotypes (A1, B1 and C1) that are mostly associated with scleractinian coral hosts (LaJeunesse, 2005; Berkelmans & van Oppen, 2006; Pochon & Gates, 2010).

Modern lipid characterization, also known as lipidomics, using mass spectrometer-based analytical techniques presented in this study enabled, for the first time, qualitative and quantitative lipid and pigments monitoring of *Symbiodinium* phylotypes isolates before and after a heat shock. Characterized molecules include specific markers of chloroplast-derived lipids such as glycolipids and plastoquinones, and putative ROS subproducts such as peroxidized fatty acids. Then, it is shown whether lipid composition of *Symbiodinium* sp. thylakoid membranes is in fact a good determinant of phylotypes (A1-C1) fates after heat stress. Additionally, we revealed limitations on using lipid profiles as proxies of thermal stress, considering that the membrane oxidative stability and energy

output are also dependent on enzymes and antioxidant components not measured in our study.

Moreover, our data show that pigments can be used as good proxies of oxidation and indication of photosystems viability. Our findings also suggested that *Symbiodinium* phylotypes followed different physiological strategies to resist high temperature obeying a risk-benefit principle. This principle states that the higher the number of double bounds in fatty acids the higher is the amount of electron and energy boosters, which in turn makes thylakoid membranes more susceptible to ROS attack. Therefore, phylotype cells must supply the energic demands and protect themselves against oxidative stress at the same time. Understanding such biochemical and universal principle may help comprehending other temperature-related ecological transformations in the marine environment (i.e., changes in primary production rates, variations in the carbon cycle) and to further knowledge on the role of oxidative stress in coral bleaching and phylotype-related host response.

2. MATERIALS AND METHODS

2.1. Experiment design

Symbiodinium sp. cultures of phylotypes A1-C1 were brought from University at Buffalo (NY-USA) and kept inside BMAK microalgae facility in the Oceanographic Institute of Universidade de São Paulo (IO-USP). They were grown in triplicates with autoclaved natural sea water in f/2 nutrient conditions (Guillard & Ryther, 1962). Room temperature was kept at 22 ºC; light conditions of 80 µmol photons m^{-2} s^{-1} into a 12L:12D cycle. Growth rates were monitored overtime and heat shock experiments started when cultures were in the exponential phase (ca. 10 days of cultivation). *Symbiodinium* spp. were kept at 34 ºC for a period of 4 hours to simulate a heat shock event. Immediately after that, the first sampling took place utilizing sterile pipets. Since cultures had different cell densities, volumes were calculated estimating filtration equivalent to a number greater than 1 million cells per filter. Volumes were then filtered in sterile pre-combusted GF/F filters (5 minutes under 300˚C) for lipid and pigment extraction and analysis. After 24h and 240h (10 days) cultures were sampled again following the same protocol. Sampling time aimed to describe cells lipidome and pigments alterations in both short- and long-term response to temperature stress.

2.2. Population growth rates

Cell density were determined with a Neubauer counting chamber under light microscope. Cultures growth rates (***µ***) were calculated with the equation below:

$$\mu = \frac{(\ln c_1 - \ln c_0)}{t_1 - t_0}$$

where c_0 and c_1 denote the number of cells at the start (t_0) and end (t_1) of the incubation period (expressed in days).

Cell densities triplicates data from each strain were plot overtime to calculate growth curves and their respective growth rates (***μ***) values. Comparing the obtained ***μ*** values for control and stressed cultures we also estimated the percentages of loss in growth caused by heat shock.

2. 3. Standards, lipid analysis and data processing

Detailed in Chapter 2.

2.4. Statistical analysis

All lipid statistical analyses were performed with Metaboanalyst (website: www.metaboanalyst.ca). Data were log transformed and the statistical significance ($p < 0.05$) evaluated by t-test analysis comparing temperature stressed cells (T) x control cells (C) in each sampling time. Differences were evaluated through Volcano-plot adjusted for false discovery rate (FDR) also considering p values below 0.05 for significance.

For comparison of lipids and pigments profiles of control samples (AC, BC and CC), data was also log transformed, and the statistical significance ($p < 0.05$) was evaluated by one-way ANOVA followed by Tukey's post-hoc test.

3. RESULTS

3.1. Heat shock experiment revealed two resistant *Symbiodinium* spp. and drastic changes in lipids and pigments profiles

The first physiological proxy used to understand how *Symbiodinim* phylotypes react to heat shock was growth rate changes after stress. Control bottles from phylotypes A1 and B1 presented the same μ value of 0.32, whereas for C1 was a little higher 0.34 for the same control conditions (Fig. 3.1). However, after a heat shock, B1 (BT) was not resistant and its biomass was heavily reduced. As a result, B1 phylotype growth rates could no longer be calculated owing to the very low final cell density (Fig. 3.1 c). Phylotypes A1 and C1 (AT and CT) also presented considerable growth rate reduction of 12% and 22%, respectively (Fig. 3.1 a, c). However, both phylotypes survived and were still growing after the heat shock event.

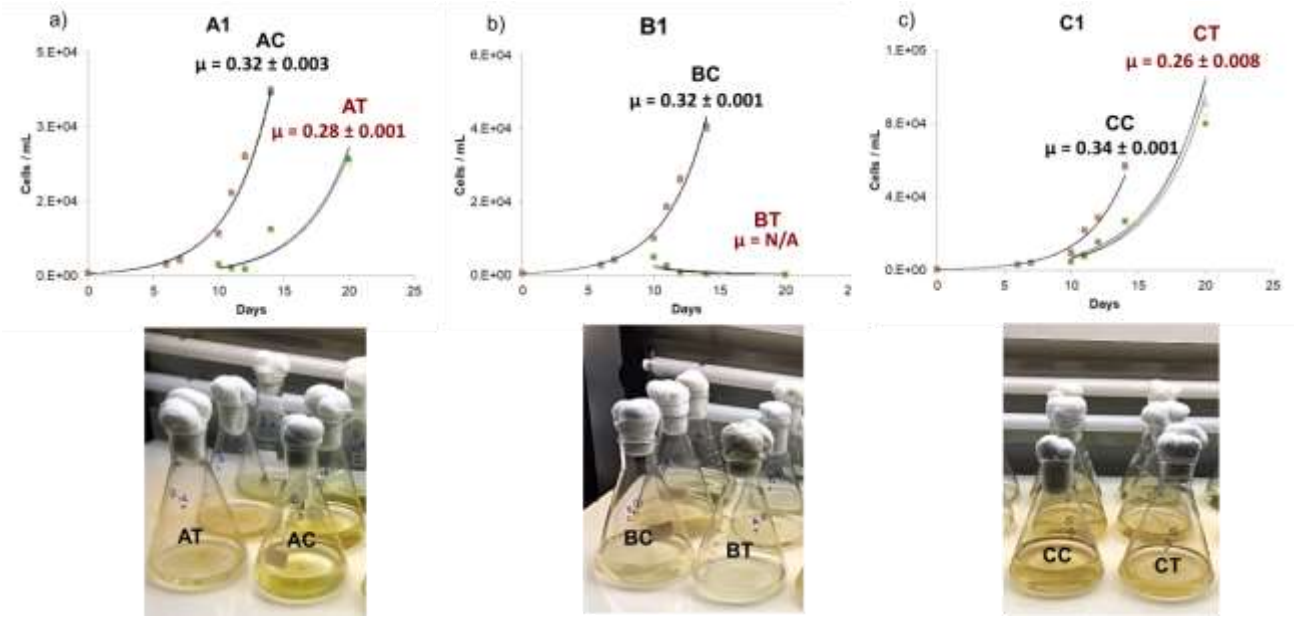

Figure 3.1: Growth rate curves of control (AC, BC, CC) and temperature stressed *Symbiodinium* phylotypes cultures (AT, BT, CT) with its respective *μ* values and standard deviation. Regression curves were obtained with n=3 (triplicates). a) Growth rate curves of phylotype A1; b) Growth rate curves of phylotype B1; c) Growth rate curves of phylotype C1. Images below graphs show final culture appearance. Note that lighter coloration of cultures denotes lower cell densities.

3.2. Changes in lipid composition after heat shock

Changes in the growth rate values strongly reflected lipid profile modifications in all *Symbiodinium* spp. after the heat shock event (Fig. 3.2). Although phylotype B1 was not heat shock resistant, and its culture (BT) had high reduction of biomass, lipid analysis was still doable, but it was not possible to separate dead cells from living cells and analysis consisted on total available biomass. All lipid alterations were evaluated with Volcano-Plot statistics considering a fold change (FC) of 1.5 and p values below 0.05 adjusted for false discovery rate (FDR). Lipid compounds that had its concentration changed at least 1.5 times higher or lower than the control and obeyed p values and FDR determined in the test were considered statistically significant changed.

The first reduction of growth rate noticed in B1 was right after heat shock (4h) and it resulted in the downregulation of 29 lipid compounds (Fig. 3.2 a) composed of the main

membrane lipids groups – amino, glyco and phospholipids that were highly associated with n-3 fatty acids (Fig. 3.2 b; Table S3.1). B1 was the phylotype with the lowest number of altered lipids at the first sampling time, however, its lipidome changed drastically after 10 days when 91 compounds were significantly different. All identified sphingolipids were upregulated and hidroxy-phytoceramides had fold changes six times higher than the control. Besides, all membrane and storage lipids highly associated with SFAs and monounsaturated fatty acids (MUFA) were upregulated. Glycolipids (exclusively from chloroplast membranes) were an exception and their absence is highlighted with an asterisk in figure 3.2 b. Therefore, all remaining cells of phylotype B1 presented intense lipid remodeling in the end of the experiment. A high abundance of PUFA and specially n-3 fatty acids were replaced by MUFA and SFA, in which palmitic (16:0), palmitoleic (16:1), stearic (18:0) and oleic (18:1) acids were outstanding (Table S3.1).

On the other hand, phylotypes A1 and C1 had fast and more intense remodeling of their lipid profiles comparing to phylotype B1 after heat shock. They presented 56 and 79 altered compounds, respectively (Fig. 3.2), and main alterations were also regarding downregulation of membrane lipids associated with n-3 FAs (Table S3.1). After 24 hours, phylotype A1 had 35 modified species, upregulating the less abundant phospholipids phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) associated with either SFAs or MUFAs (Table S3.1). Phylotype C1 fast recovered its lipidome showing no significant changes compared to its initial control profile. The main distinctions regarding phylotypes A1 and C1 lipidomes were noticed in the long term 10 days after the heat shock event. While phylotype A1 had 22 altered lipid species, phylotype C1 had nearly 70% of all its monitored lipids (alteration of 166 molecules over 241 monitored) significantly changed including compounds from all lipid classes (Fig. 3.2 b). Phylotype C1 was the phylotype with the highest lipid remodeling in the long term and, therefore, with the highest phenotypic plasticity.

Drastic changes of common lipid compounds were also noticed in all studied *Symbiodinium*. Diacylglyceroltrimethylhomoserine (DGTS), triacylglycerol (TAG) and sphingo lipids were both upregulated in either 4 or 24 hours after heat shock. DGTS and

TAG species were nearly exclusively associated with palmitic (16:0) and other SFAs (Table S3.1).

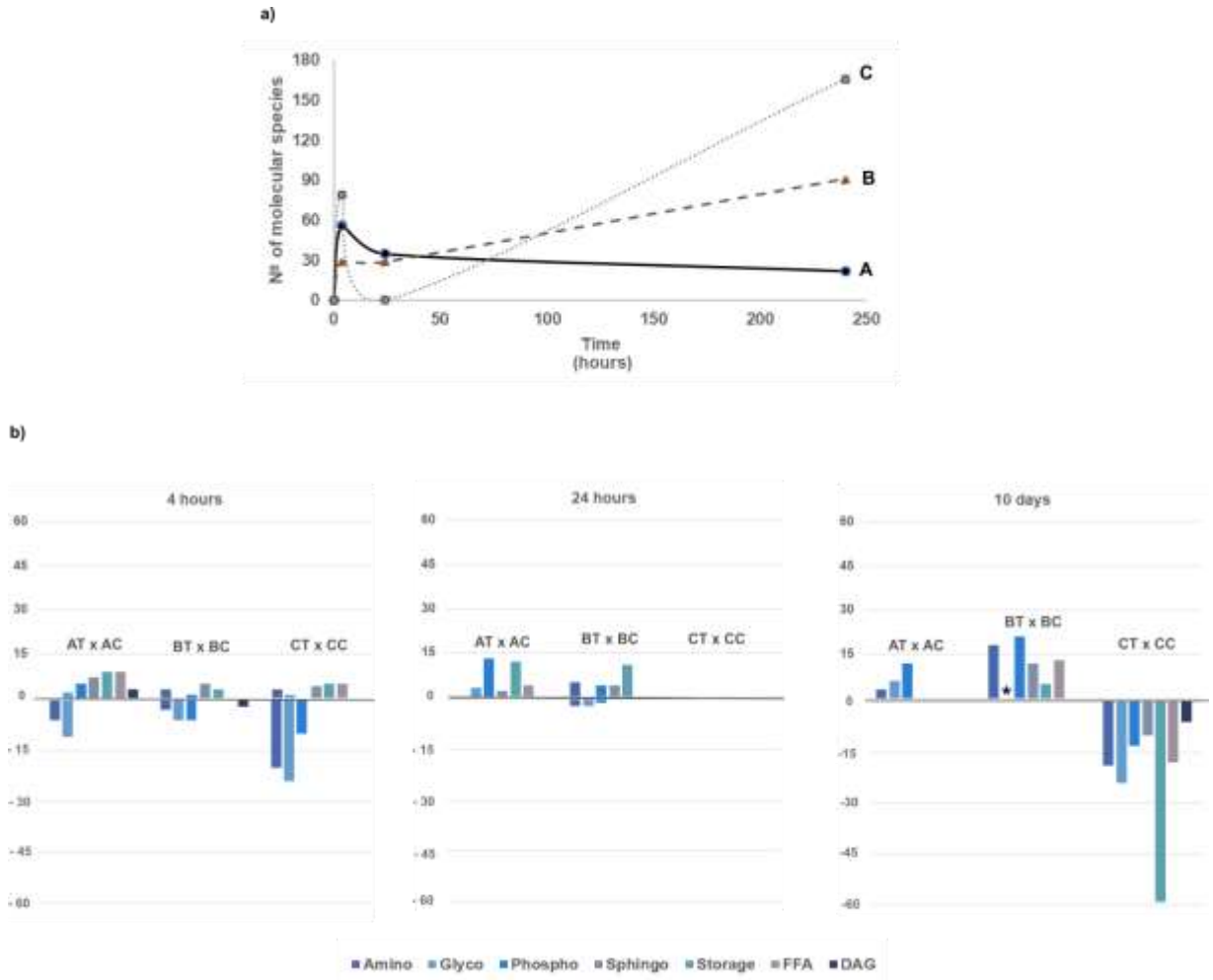

Figure 3.2: Lipid remodeling data indicating distinct phylotype response time that are correspondent to growth rate curves behavior. a) number of significant modified lipid species overtime after heat shock event comparing samples from stressed cultures against samples from control (C x T). X axis presents time in hours after heat shock event. Data points were based on the cultures sampling made 4 h, 24 h (1 day) and 240 hours (10 days) after stress; b) same significant modified compounds sorted into the main lipid groups and the same sampling times. Bars above zero show species that were "upregulated" compared to control; whereas bars below zero show lipids that were "downregulated" compared to control. The * represents no upregulation of glycolipids in phylotype B1 after 10 days. Differences were evaluated through Volcano-Plot analysis (FC = 1.5 and $p < 0.05$; FDR adjusted). Detailed data and molecular lipid compounds are presented on Table S3.1.

3.3. Polyunsaturated fatty acids in membrane lipids were pivotal for *Symbiodinium* spp. long term survival

PUFAs were mostly made of n-3 fatty acids and its fast downregulation after heat shock was independent of *Symbiodinium* phylotype. However, what varied was their time of response on stablishing again, or not, their same control values necessary for survival. Figure 3.3 shows that all phylotypes decreased their total n-3 concentration after stress, but only phylotypes A1 and C1 fast recovered and kept concentrations similar to the control throughout the experiment. On the other hand, phylotype B1 highly reduced its n-3 concentration and did not resisted in the long term, though it had a slower response in the short term.

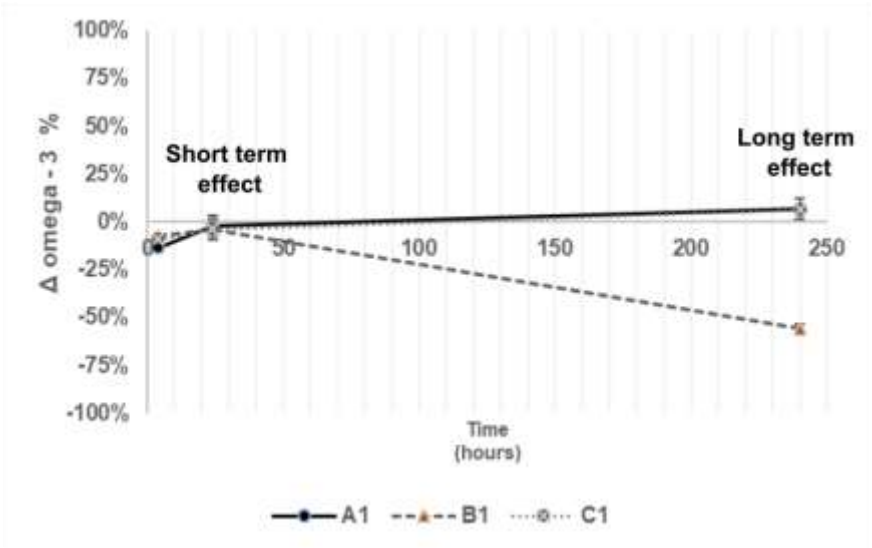

Figure 3.3: Total variation of omega 3 polyunsaturated fatty acids over time. Relative percentage of all omega 3 (n-3) fatty acids (DHA, EPA, DPA, 18:3, 18:4 & 18:5) was considered for each *Symbiodinium* phylotype considering 4- and 24-hours variations as short term and 10 days as long-term effect. Variations were calculated based on values from stressed minus control lipid data in each sampling time. Bars show standard error values obtained from triplicates.

The most abundant membrane lipids: glycolipids (specially monogalactosyldiacylglycerol – MGDG), 1,2-Diacylglyceryl-3 - (O-carboxyhydroxymethylcholine) (DGCC), and phosphatidylcholine (PC) had more than half of their side chains made of PUFA. There was a correlation between plastoquinone, responsible exclusively for the electron transport inside chloroplast membrane and PUFA from the most abundant membrane lipids in all *Symbiodinium* samples (Fig. 3.4). As expected, the highest R^2 value was noticed for glycolipids, highly indicating PUFA presence in chloroplast membrane lipids. In addition, a surprising true positive correlation was noticed for DGCC ($R^2 = 0.58$ and $p < 0,05$), which location inside the cell is not known, but our data suggests that it could also potentially be present in the chloroplast. The main distinctions were regarding specific PUFA species connected to distinct polar heads. Glycolipids had mostly octadecatetraenoic (18:4) and octadecapentaenoic (18:5) acids, whereas DGCC had docosahexaenoic acid (DHA). Therefore, we highlight the indication of omega 3 fatty acids high concentration and diversity in chloroplast membranes. The lowest R^2 was found for PC and high p value (7.E-02) indicates a non-significant positive correlation. Therefore, phosphatidylcholine with PUFA side chains are probably not a major membrane component of chloroplasts.

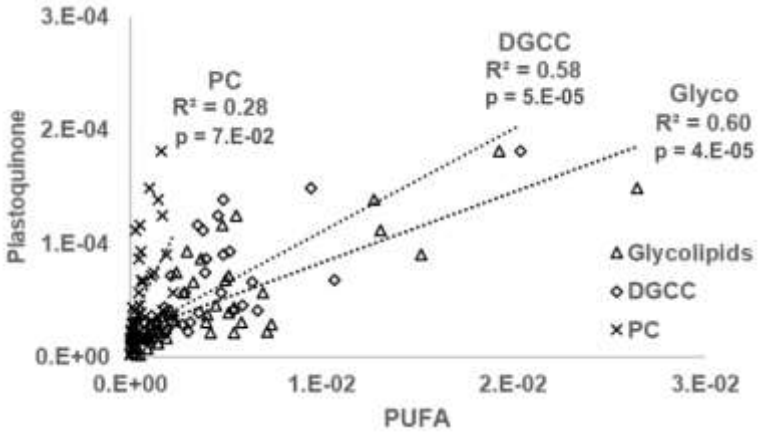

Figure 3.4: Correlation of polyunsaturated fatty acids (PUFA) in the most abundant membrane lipids and plastoquinone. Respective R^2 and p values are represented together in each graph. Glycolipids were calculated considering monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and glucoronic acid (GlucA). 1,2-Diacylglyceryl-3-(O-carboxyhydroxymethylcholine) was the only aminolipid associated with PUFAs, as well as phosphatidylcholine (PC) for phospholipids. All lipid data is represented in ng per cell.

3.4. Changes in pigments and oxylipins as evidences of oxidative stress caused by heat shock

Although 16 distinct pigments were monitored during the experiment, we chose to present only data from the ones that changed significantly after the heat shock. Chlorophyll-a and pheophytin-a are both located in the reaction center of PSII and, therefore, necessary for the establishment of photosynthesis. Chlorophyll-a can be converted into pheophytin-a and we used their relative abundance as a proxy of health chloroplast status (Hendry *et al*., 1987). There was a tendency of ratio downregulation for all phylotypes when comparing stressed cultures (T) against control (C) (Fig. 3.5), but it was only statistically significant for B1 in the last sampling time - 10 days (Fig. 3.5 c) and Table S3.1).

In addition, the ratio chlorophyll-a/ carotenoids was used as another health proxy because other groups of thermotolerant microalgae have presented higher carotenoid concentration when facing high temperature stress (Havaux & Tardy, 1996; Shi & Chen, 2001). Phylotypes A1 and C1 had statistically significant faster cell response considering carotenoid increase (Fig. 3.5 a), but in phylotype B1 the ratio was only significantly

downregulated after 10 days (Fig. 3.5 c). In this case, however, downregulation was due to low chlorophyll concentrations instead of carotenoid increase.

Other carotenoids located in the chloroplast antenna also varied considerably. Violaxanthin is important for the “violaxanthin cycle”, in which it is converted into zeaxanthin. We also noticed diadinoxanthin, which is present in the “diadinoxanthin cycle” and converted into diatoxanthin under higher light conditions. Both cycles consist on epoxy molecules turning into de-epoxy molecules via enzymatic pathways in order to protect the photosystem II against oxidative stress (Havaux *et al*., 2013). Higher temperatures are also expected to threat algae physiology via oxidation, so we are likely to see higher concentrations of de-epoxy xanthophylls also as a response to heat shock. This was true for all *Symbiodinium* spp. samples, but only statistically significant for phylotypes B1 and C1 in 4 hours after stress (Fig. 3.5 a) and, especially for phylotype B1 after 10 days (Fig. 3.5 c). Downregulation of all B1 pigment ratios indicated that it was the phylotype most affected by oxidative stress and, possibly, it did not recover its photosystem after heat shock.

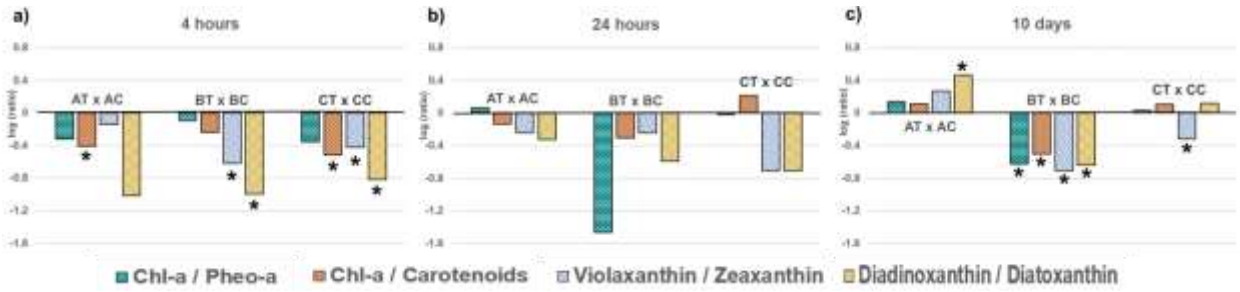

Figure 3.5: Pigments ratios that changed significantly after heat shock. Ratios are represented in logarithmic scale to evidence its up or downregulation comparing temperature stressed cultures (T) and control cultures (C) in three distinct sampling times: a) 4 hours; b) 24 hours; c) 10 days after heat shock event. * represent samples statistically significant altered based on Volcano Plot analysis (FC = 1.5 and $p < 0.05$) and details are on Table S3.1.

To test whether lipids, especially the ones containing polyunsaturated fatty acids (PUFA) were affected by reactive oxygen species (ROS) generated by heat shock, we searched for less abundant polar lipids containing oxidized fatty acids (oxylipins). Several amino, glyco and phospholipids were found esterified to oxylipins (primarily composed of mono-hydroxide and hydro-peroxide fatty acids) derived from the most abundant PUFA, i.e. 18:4, 18:5 and DHA (Fig. 3.6). Given these results, we also searched for free fatty acids (FFA) composed of mono-hydroxide and hydro-peroxide PUFAs and found them abundantly distributed among our samples (Fig. 3.6). Of note, we have determined also that both singlet oxygen and radical derived isomers were present among the oxylipins (data not shown).

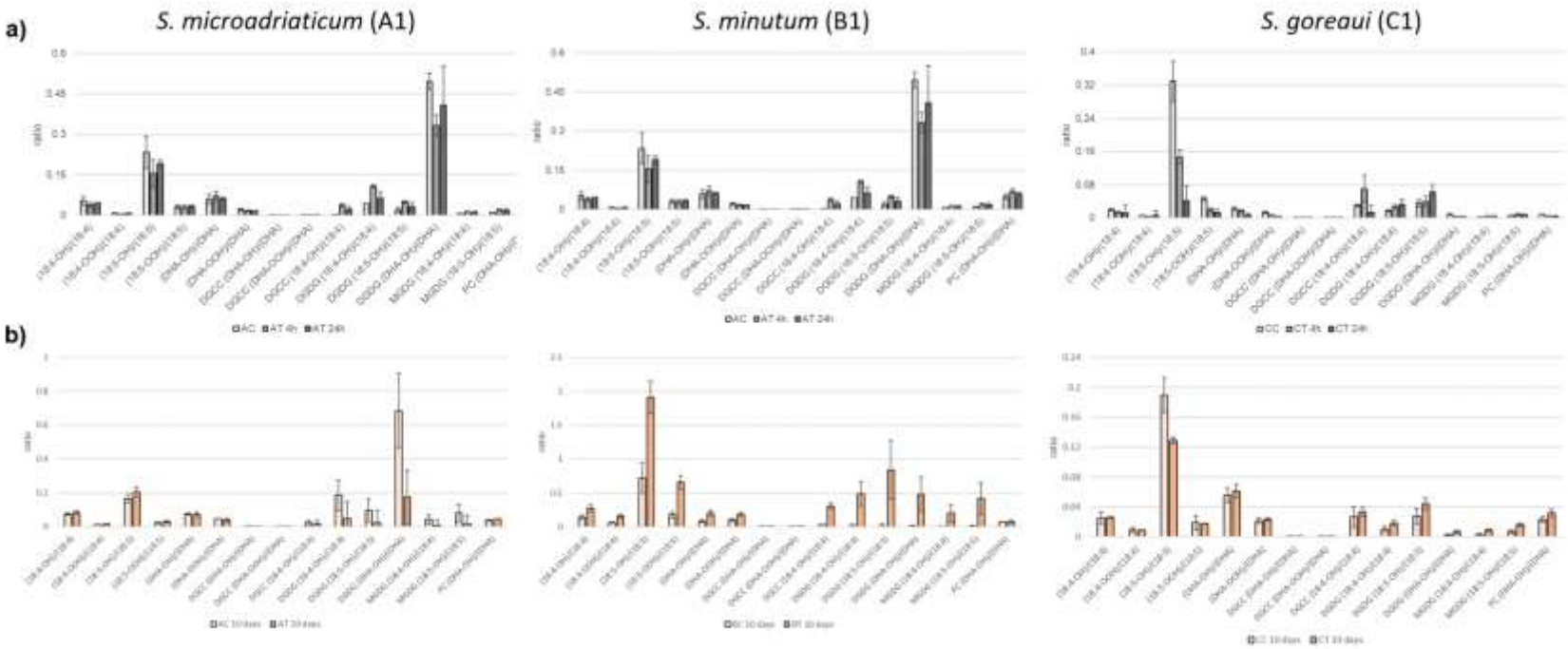

Figure 3.6: Ratios of total concentrations of oxidized fatty acids and their precursors from polar lipids (PLs) and free fatty acids (FFAs). 15 molecules represented by free fatty acids (6) and polar lipids (8) were identified and monitored during our experiment. Ratios considered total concentration of each identified fatty acid in either free or membrane associated forms. a) short term (4h and 24h after heat shock); b) long term (10 days after heat shock). Bars represent average concentrations with respective standard errors. Temperature stressed samples values are represented by (T), while control samples are represented by (C).

It is important to mention that oxidized PUFAs were observed in control samples at considerable levels, accounting for 1 to 50% of precursor molecules (Figure 3.6. a)). However, when exposed to heat shock all *Symbiodinium* phylotypes displayed a trend of increased amounts of oxidized lipids. This trend was more conspicuous for B1 than C1 and A1, in which oxidized lipids were significantly more abundant at 24 hours and 10 days relative to control samples (Fig. 3.6). In many instances, the levels of oxidized fatty acids observed in B1, considering both polar lipids and free fatty acid pools, suppressed the content of their precursor molecule. A trend of modest increase in concentrations of oxidized lipids was observed after heat shock experiments for both A1 and C1 phylotypes. However, in general, the lowest levels of oxidized lipids were found in C1, whereas A1 displayed intermediate levels (better represented in Fig. 3.7).

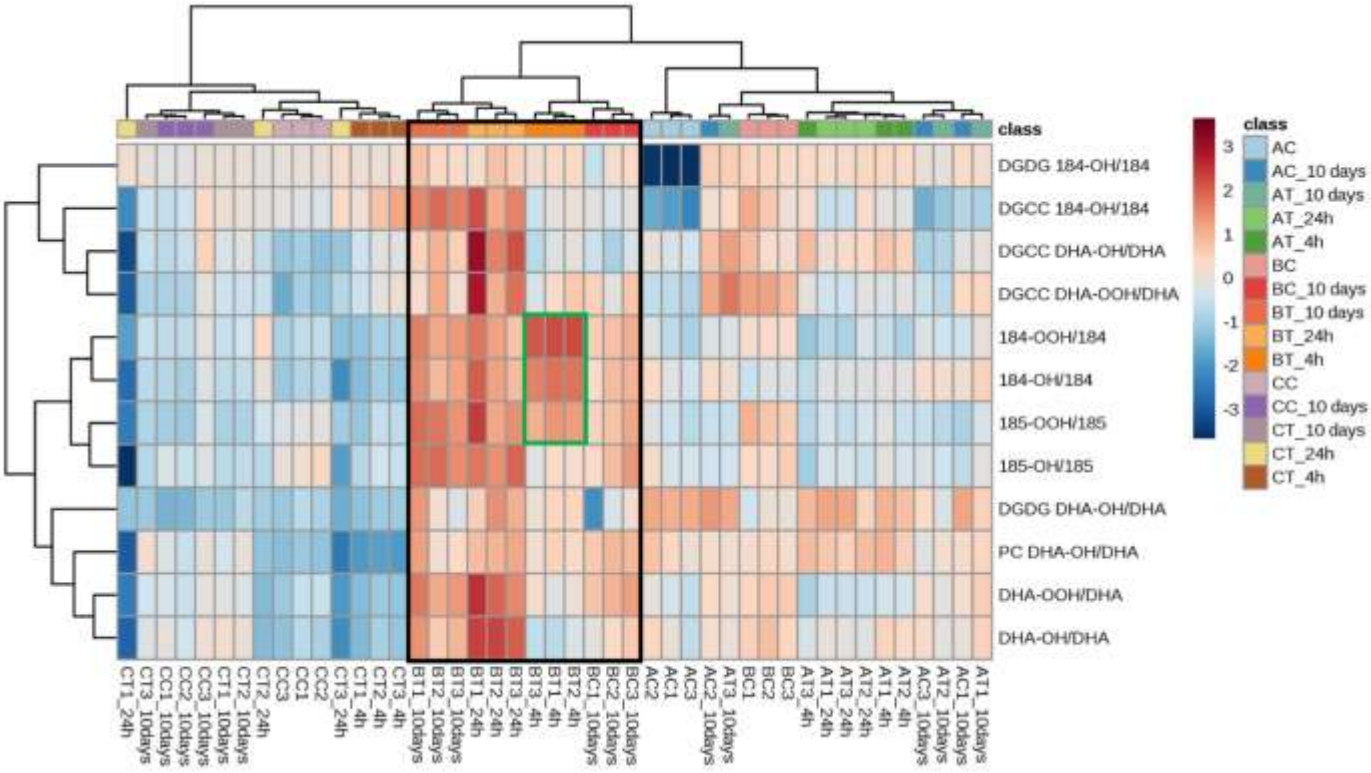

Figure 3.7: Heatmap of oxylipins ratios that changed significantly after heat shock. In total, 12 oxylipins ratios are shown in rows and samples in columns. Statistical significance was evaluated by one-way ANOVA followed by Turkey's post-hoc test ($p < 0.05$) using Metaboanalyst. Distance was measured in Pearson, and Ward´s clustering algorithm. Each colored cell on heatmap corresponds to normalized concentrations. Log transformation was used for data normalization values. Red color indicates upregulation, whereas blue means downregulation of oxylipins ratios. AC, BC and CC samples represent controls. AT, BT and CT represent temperature stressed samples. 4h, 24h and 10 days represent sampling times after heat shock for both stressed (T) and control (C) samples. Middle black rectangle groups phylotype B1 samples after heat shock and green rectangle highlights single higher concentrations of FFA oxylipins.

4. DISCUSSION

4.1. Chloroplast bioenergetics and oxidative stress: a risk – benefit story

Heat shock caused prompt downregulation of omega 3 fatty acids (n-3) in *Symbiodinium* spp*.* accompanied by drastic changes in lipids and pigments. However, modifications were only lethal for survival of phylotype B1, which suffered downregulation of PUFAs mostly represented by n-3 compounds from glyco- and betaine lipids, probably from the chloroplast membrane. Such changes were not reversible in the long term but, instead, n-3s were replaced by higher concentrations of MUFAs and SFAs. B1 had also downregulation of all its pigment ratios and compiled data indicate loss of chloroplast viability. In contrast, heat shock resistant phylotypes A1 and C1 showed little final alterations of glyco- and betaine lipids regarding their relative concentrations of n-3 fatty acids. Therefore, we conclude that chloroplast energy transduction was not compromised by phenotypical lipid changes in these heat shock resistant phylotypes.

Our study was the first one to approach higher temperature effects on *Symbiodinium* spp*.* through global lipids analyses, in a way that it was possible to target which and how differently cell compartments were affected with stress. We showed how chloroplast-derived lipids were differently changed in A1, B1 and C1 phylotypes in both short and long terms confirming our hypothesis of temperature leading to oxidative stress harm on cell bioenergetics. Previous research had already suggested heat leading to oxidative stress as the main cause of *Symbiodinium* spp. physiological alterations potentially leading to bleaching events in coral hosts (Lesser 1996, 1997, 2006, 2011, 2019; Warner *et al*., 1999; Fitt *et al.*, 2001, 2001; Tchernov *et al*., 2004; Yamashiro *et al*., 2005). In our study, we have analytically confirmed that for *Symbiodinium* spp. isolates an increase in oxidized fatty acids (oxylipins) is observed when phylotypes are exposed to heat shock. In addition, variations in pigment ratios also went along with membrane lipids evidences for determining chloroplast viability. Jahns & Holzwarth (2012) suggested that xanthophyll composition is regulated by light intensity and chloroplast redox status in plants. Photosystem II needs to respond rapidly in terms of its xanthophyll composition (epoxy or de-epoxy molecules) in order to not destabilize thylakoid membranes and keep

them functional (Deeming-Adams, 1996; Nisar *et al*., 2015). We noticed fast upregulation of zeaxanthin and diatoxanthin after heat shock in *Symbiodinium* spp. However, in the long term, all pigments ratios were significantly downregulated only in phylotype B1, in which the low concentrations of chlorophyll would probably disrupt the electron transport chain and photosynthesis. Carotenoids can function as antioxidants under oxidative stress conditions and xanthophyll cycles are dependent on enzymatic activity (Nisar *et al*., 2015). Supposedly, singlet oxygen generation could also lead to formation of ß-carotene endoperoxides through a non-enzymatic pathway (Terao *et al*., 2010), but we did not find ß-carotene among the most abundant described pigments, neither its endoperoxides.

Omega 3 concentrations were rapidly downregulated in *Symbiodinium* spp. in the short term (4h), but heat shock resistant phylotypes responded rapidly in 24h showing either few or no alterations. Besides, A1 and C1 almost did not change their relative n-3 concentrations compared to final control values after 10 days. However, the analysis of their full lipidomes suggest that A1 and C1 used distinct survival strategies. A1 rapidly invested on the synthesis of all lipid classes when compounds were downregulated. Similar fast responses were also shown by other authors who reported A1 fast acclimation to higher temperatures and fast production of antioxidant enzymes in the presence of ROS (Dìaz-Almeyda *et al.*, 2017; McGinty *et al*., 2012, respectively). Besides, Mansour *et al.* (2018) reported A1 possesses high phenotypic plasticity to temperature alterations. Consequently, evidence suggests A1 as heat shock resistant responding to impact through a fast lipid synthesis. On the other hand, C1 is reported to present high basal levels of antioxidant enzymes independent of ROS production (McGinty *et al*., 2012) and not to alter its fatty acid profile under high temperatures (Dìaz-Almeyda *et al*., 2011). We also noticed C1 poorly altering its lipid profile in the short term probably because it could count on basal efficient antioxidant machinery to protect itself against ROS attack. In the long term, C1 might have followed a more conservative strategy downregulating all lipid classes together with plastoquinone, but only slightly altering the relative n-3 concentration in its chloroplast membrane lipids. Lower growth rate in C1 compared to A1 after stress also suggests C1 is more conservative, but still capable to survive and keep growing after stress.

Budin *et al*. (2018) showed that bacteria and yeast cultures growth rates were highly dependable on their lipid unsaturation levels. Budin *et al*. (2018) argue that higher concentrations of unsaturated fatty acids decrease membrane viscosity enabling more efficient electron transport chain by diffusion of electrons through the protein complexes transported by quinone-derived molecules. This promotes higher respiration rates in either yeast mitochondria and *E. coli* membranes enhancing growth. Other authors suggested cell survival and growth as a process universally mediated by membrane viscosity and proposed that various organisms might present similar adaptations to maintain cell respiration rates (Sinensky *et al*., 1974; Hazel *et al*., 1995; Valentine, 2009; Kellermann *et al*., 2016; Yoshinaga *et al*., 2016). In our case, *Symbiodinium* sp. is a microalga, so we propose that respiration is sustained by ATP production in its chloroplast membranes and controlled by plastoquinone and viscosity mostly stablished by n-3 concentrations. Thus, phylotypes survival was defined by their capacity of maintaining energy efficient output. Higher temperatures alter thylakoid membrane viscosity and motion enabling scape of high energy electrons generated by water molecules split. High energy electrons easily associate with oxygen creating ROS and other relative compounds (i.e., reactive nitrogen and sulphur species) that can potentially cause lipid peroxidation and protein denaturation (Dietz *et al*., 2016). These events would rapidly impair chloroplast membrane energy production and lead to cell death if not contained by its antioxidant machinery (i.e., enzymes and pigments) (Fitt *et al*., 1999; Leggat *et al*., 2011; Krueger *et al*., 2014, 2015a, b) and/or synthesis *de novo* of important temperature downregulated (oxidized) lipids (Nanjo *et al*., 2010) in order to keep constant the structural viability of cell membranes.

Symbiodinium spp. glycolipids were formed by higher concentrations of unsaturated C18 derivate n-3 fatty acids (18:4 and 18:5) associated mostly with MDGD and DGDG. This peculiar lipid profile is only seen in marine flagellate microalgae (Guschina & Harwood, 2006; Leblond *et al*., 2000, 2010; Boudiere *et al*., 2012, 2014) from diverse genera including few other symbionts (e.g. *Amphidinium klebsii*) and many free-living (e.g., *Pavlova* sp.; *Amphidinium carterae*; *Heterocapsa* sp.; *Peridinium aciculiferum*) organisms (Leblond *et al*., 2000, 2010, 2015; Gray *et al*., 2009; Dahmen *et al*., 2013; Flaim *et al*., 2014; Anesi & Guella, 2015). Therefore, n-3 presence in chloroplast membranes is highly

reported in all organisms. In addition, considering our high correlation results of plastoquinone and PUFA in DGCC, we suggest that high concentrations of DHA (n-3) are also likely taking part of *Symbiodinium* spp. chloroplasts membranes when connected to DGCC betaine polar head. 1, 2 -Diacylglycery l - 3 - (O - carboxyhydroxymethylcholine) is commonly reported only in that same groups of microalgae which commonly inhabits oligotrophic zones where phosphate is very limiting for primary production. Then, usual phosphatidylcholine (PC) tend to be replaced by DGCC that is also a zwitter charged lipid generalist in all cell membranes (Guschina & Harwood, 2009; Van Mooy *et al.*, 2009, 2010; Becker *et al.*, 2018; Shemi *et al*., 2016; Canavate *et al*., 2016). Most mentioned studies were made in ocean gyres where winds and currents generate downwelling making surface waters scarce of nutrients. When considering coral reefs areas, nutrient conditions are quite similar, therefore similar lipid membrane adaptation is plausible, an ecological case of convergent evolution. Not much is known about DGCC role in microalgae lipid metabolism, but we strongly suggest, at least, its partial presence in chloroplast membranes, because it was also one of the most abundant polar lipids, and chloroplast lipid biomass normally stands for about 40 per cent of cell total lipids in microalgae (Shimojima *et al*., 2009). Our *Symbiodinium* spp. samples only respect this universal standard if we consider glycolipids (~ 18% of total lipids, data not shown) and partial betaine DGCC (~ 20%) concentrations together (~ 38%), admitting that they are both most represented by PUFA acyl chains. Thus, *Symbiodinium* spp. chloroplasts membranes are likely to be enhanced in PUFA n-3 containing not only 18:4, 18:5, but also DHA.

Chloroplasts are organelles originated from a cyanobacterial ancestor in the marine environment (Raven & Allen, 2003). Chloroplasts have supposedly evolved to perform at maximum efficiency of energy output, which is leveraged by the presence of highly unsaturated n-3 fatty acids (HUFA) (i.e. 18:4, 18:5, EPA, DHA) (Valentine, 2009). Electrons must go through a long distant transport in the thylakoid membranes and higher n-3 concentrations boost electron transport enabling more energy output. On the other hand, it also makes membranes more susceptible to the attack of free radicals (i.e., ROS) due to the higher unsaturation levels. We identified and monitored several molecular species of oxidized fatty acids (mainly from the omega-3 family), including both polar

lipids and FFA already present in considerable amounts in control samples. When exposed to heat shock, we revealed that phylotype B1 displayed the highest concentration of oxidized lipids relative to phylotypes A1 and C1. These findings support previous studies demonstrating a limit capacity of B1 to cope with oxidative stress (McGinty *et al*., 2012; Goyen *et al*., 2017; Lesser *et al*., 2019). The lowest concentrations of oxidized lipids in C1 even after heat stress is also supported by its higher concentrations of antioxidant enzymes reported by McGinty *et al* (2012) and Krueger *et al* (2014). Finally, intermediate levels of oxidized from A1 phylotype after the heat shock support its fast response in upregulate antioxidant enzymes under high concentrations of ROS evidenced by McGinty *et al* (2012).

In summary, we conclude that a risk-benefit principle has been honed by evolution, in which chloroplast membranes evolved efficient protection mechanisms against free ROS (i.e., antioxidant enzymes, pigments – mostly carotenoids) to guarantee viable membranes for high-energy production (Valentine, 2009). In our study, we propose that phylotype B1 did not survive the heat shock because it probably has a poor efficient antioxidant machinery (also suggested by McGinty *et al.*, 2012). McGinty *et al.* (2012) demonstrated the poor capacity of B1 to upregulate antioxidant enzymes in response to ROS. Although B1 downregulated n-3 concentrations, which made it less susceptible to ROS attack, it did not fulfill the requirements for cells bioenergetic demands in the long term.

As a limitation of our study we highlight that B1 low final biomass and cell densities were possibly underestimated due to cell counting techniques. Its lipid data was then, possibly overestimated, but did not compromise our conclusions. In order to obtain a more precise quantitative data from phylotype B1, the experiment must be performed using more sensitive cell counting techniques (i.e., cytometer instead of counting chambers). In the cytometer, each analysis is performed with 200 microliters per count, whereas in the Neubauer chamber it is only 1 microliter per count.

Finally, we noticed minor alterations of less abundant lipid compounds that have been previously reported in stress signaling pathways of other eukaryotic organisms including marine algae. Heat shock and other abiotic stressors can induce higher production of

ceramides that in some cases tend to cause cell apoptosis (Vigh *et al*., 2005, 2007). Diverse ceramides were upregulated in *Symbiodinium* spp. after stress and the highest changes were found in phylotype B1, which was also the only one which did not survive. Triggered cascade reactions caused by symbiont and host communication that might lead to coral bleaching are one of the least understood mechanisms in symbiosis physiology which definitely requires further investigation.

4.2. Ecological implications

4.2.1. Lipid ecology of zooxanthellae in coral reefs symbiosis and global warming perspectives

Reef building scleractinian corals hold high levels of fat in their tissues, which is dominantly provided by zooxanthellae (Harland *et al*., 1991, 1993; Grottoli *et al*., 2004; Imbs *et al*., 2007, 2012) representing their major sources of energetic metabolites (Grottoli *et al*., 2004; Teece *et al*., 2011). Lipid analysis has been considered an important tool to answer questions with distinct physiological and ecological purposes, such as defining coral host trophic relationships and diagnosing *Symbiodinium* phylotypes and host susceptibility to thermal stress (Treigner *et al*., 2008; Seeman *et al*., 2013; Tchernov *et al*., 2004; Dìaz-Almeyda *et al*., 2011, 2017; Kneeland *et al*., 2013; Towle *et al*., 2017). However, their symbiotic fat metabolism and lipid exchanging mechanisms are still poorly understood mainly because there are missing data defining which specific cell compartments are involved in the exchange and storage processes. Information on how they contribute when interacting with each other are also needed.

Although our study was limited to the lipidome of three *Symbiodinium* phylotypes, it represents a first step to better understand their lipid metabolism and survival strategies facing thermal stress when free-living and, potentially, when associated with the coral host. C1 was reported as the most common phylotype in scleractinian corals with worldwide distribution (Van Oppen *et al*., 2001; Goulet *et al*., 2008; Yang *et al*., 2012). Its lipid data indicate a very conservative strategy: growth rate is reduced together with its

total lipid content while essential fatty acids are maintained in the membrane at similar relative concentrations, guaranteeing the bioenergetic demands of cells. C1 drastically changed its lipid profile downregulating nearly 200 compounds including plastoquinone, though relative concentrations of omega 3 fatty acids poorly changed. Lipid and growth rate data indicate that C1 was harmed by heat shock and its photosystem efficiency was downregulated. Lesser (2019) had similar founds in his study and he suggested C1 as an intermediate temperature tolerant phylotype. C1 strategy to survive may explain its large distribution spectrum. Besides, it also means that, when in endosymbiosis, coral host might need to associate at the same time with other phylotypes to supply its energetic necessities (Cooper *et al*., 2011), since the coral metabolism is highly dependable on lipid availability. Host flexible association with different phylotypes has been proposed to attend distinct energetic requirements (Rowan *et al*.,1997) and also during temperature stress events (Silverstein *et al*., 2017; Tong *et al*., 2017; Matthews *et al*., 2018).

Considering phylotype A1, its fast lipid synthesis response may be a mechanism of fast acclimation to temperature variations when living associated with the host (Dìaz-Almeyda *et al*., 2017; Mansour *et al*., 2018). Caribbean corals are known to typically associate with both phylotypes A1 and C1 (and sometimes B1), as well as the Brazilian endemic brain coral *Mussismilia* spp. (Manning & Gates, 2008; Parkinson *et al*., 2015; Picciani *et al.*, 2016). This combined association might guarantee intense lipid translocation from phylotype A1 to host assuming that lipid exchange obeys similar rates of lipid synthesis in *Symbiodinium* sp. At the same time, concomitant association with phylotype C1 could be explained by its elevated production of antioxidants enzymes (i.e., SOD, APX, CAT) (McGinty *et al*., 2012; Krueger *et al*., 2014). These may also be transferred to coral host and guarantee a stable symbiotic association even under higher temperatures.

Nevertheless, our data from phylotype B1 show its low tolerance to higher temperatures and corroborates ecological evidence of restricted distribution in coral hosts, mostly in Caribbean specific regions (Andras *et al*., 2011; Parkinson *et al*., 2015). McGinty *et al.* (2012) showed that B1 was uncapable of upregulating antioxidant enzymes under higher concentrations of ROS, which is likely to happen in a heat shock event. Recent evidences again showed B1 as a thermal sensitive phylotype also due to the same limitation of

upregulating its antioxidant enzymes (Goyen *et al*., 2017; Lesser, 2019). B1 *de novo* synthesis pathway enzymes of essential lipids (i.e., omega 3 compounds), highly targeted by ROS, may also have been desaturated in the long term and explains why it did not survive. We suppose that B1 low phenotypic plasticity in the free-living mode reflects its lower temperature limit, which might even suggest that it realized narrower niche regarding temperature compared to strains A1 and C1.

At last, we showed for the first time where *Symbiodinium* spp. essential fatty acids provided to the coral host are located and relatively distributed inside cell compartments. Many studies suggested their translocation via lipid droplets (Gordon *et al*., 2018; Matthews *et al*., 2018), which are highly made of storage lipids - triacylglycerol (TAG) and cholesterol esters (CE). However, in Chapter 1 we showed *Symbiodinium* phylotypes basal profiles and they were not abundant in storage lipids compared to other lipid classes. Storage lipids neither presented high concentrations of essential fatty acids). Thus, when important omega 3, mainly DHA, is translocated to the coral host, which metabolic pathway is used? Could it happen via free fatty acids (FFA) flux rather than TAG? In our samples, DHA was nearly exclusively associated with DGCC betaine lipid polar head and very few is known about its importance in the symbiont, let alone in the coral host. We believe that answering these questions and further investigating both symbiont and coral host lipid metabolism combining also “other omics” techniques (i.e., genomics, transcriptomics and proteomics) are crucial to understand the symbiosis association and how it might be affected by global warming. Additionally, it could mechanistically explain differences in the thermal resilience of distinct symbiont phylotypes and their hosts.

Supporting information

Table S2.1: Description of lipids and pigments compounds either up or downregulated after heat shock for *Symbiodinium* phylotypes A1, B1 and C1 after 4h, 24h and 10 days. File is in excel spreadsheet available in Research Gate profile website

FINAL REMARKS

In Chapter 2 we verified the use of lipidomics as a precise tool for description, quantification and monitoring of total lipids and pigments of *Symbiodinium* phylotypes A1, B1 and C1 growing under optimum conditions of temperature, light and nutrients. We also showed that statistically significant differences among phylotypes were mainly determined by their membrane lipids. Although A1 and B1 were more similar based in the heatmap analysis, A1 was more alike to C1 considering their higher concentrations of omega-3 polyunsaturated fatty acids and plastoquinone.

In Chapter 3 we analyzed variations in *Symbiodinium* growth rates and both short and long terms lipidomes after a heat shock event summarized in Figure F1. Phylotype B1 was not heat shock resistant and its high decrease in biomass and cell death occurred after a downregulation of essential membrane omega-3 fatty acids and all identified pigments from chloroplasts. Thus, lipidome and pigments changes of B1 could not guarantee its cell's energetic requirements. However, phylotypes A1 and C1 both resisted mainly because they were capable of maintaining higher concentrations of essential omega-3 fatty acids in the thylakoid membranes and supply cells energetic requirements in the long term.

Combined information from both chapters demonstrated lipidomics as a functional tool to comprehend cell physiological alterations caused by thermal stress by a unified concept. We noticed that functional thylakoid membrane lipid structure cannot vary much in order to feasibly supply the cell bioenergetic demands. Therefore, the fate of organisms is likely to be determined by the cell antioxidant machinery, which were not analyzed in the present study, but that might protect membranes against ROS and keep efficient energy output even under stress conditions.

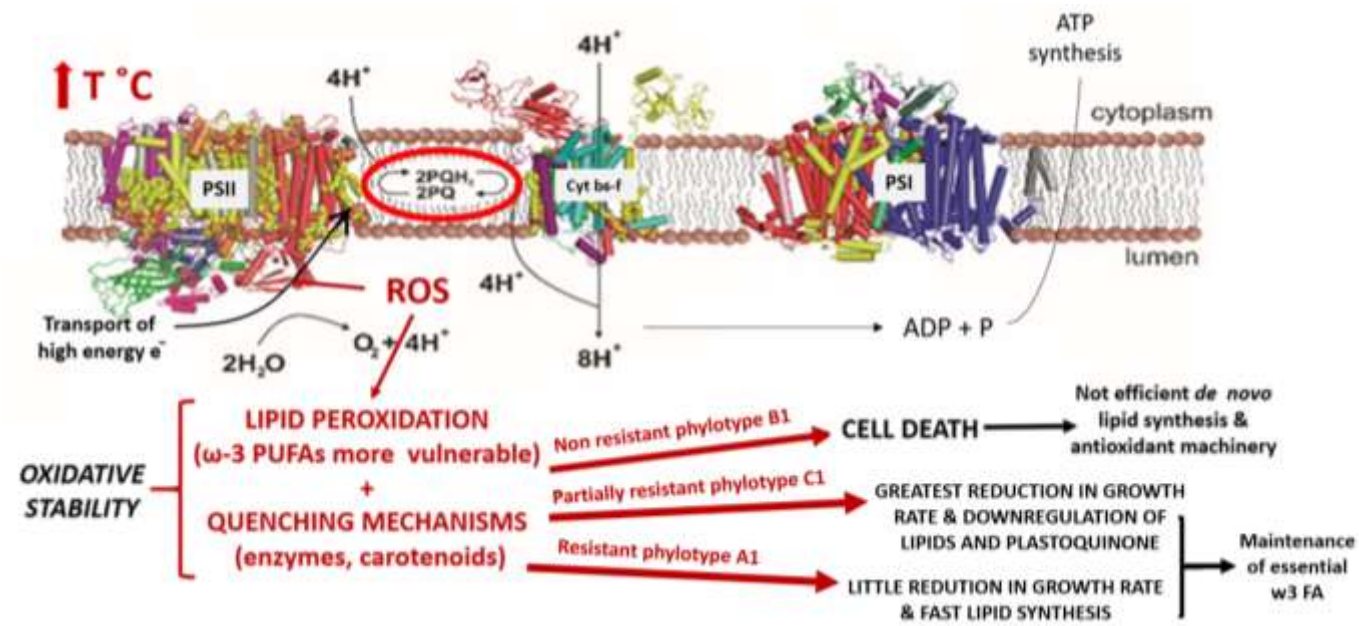

Figure F1: Summary of *Symbiodinium* phylotypes cell's proposed mechanisms caused by upregulation of ROS. Temperature alters membrane viscosity and enable scape of high energy electrons from electron transport chain. This might be responsible for upregulation of ROS that will concomitantly lead to the peroxidation of most vulnerable omega-3 polyunsaturated fatty acids and activate quenching mechanisms. We propose that phylotype B1 did not survive because it could not avoid lipid peroxidation of essential ω-3 in the membranes, neither synthesize *de novo* epoxidized lipids. On the other hand, it was not true for phylotypes A1 and C1. Both kept efficient energy output, although we suggest that they followed distinct strategies already discussed in chapter 3. Protein complexes photosystem II (PSII), cytochrome b_6-f (cyt b_6-f) and photosystem I (PSI) were highlighted. Plastoquinone pool responsible for electron transport from PSII to cyt b_6-f was also highlighted in red. Scape of high energy electron leading to ROS was highlighted in dark red. Source: Adapted from Wada & Murata, 2009.

Finally, we attempted to elucidate specific hypothesis delineated in this dissertation/thesis:

1) "*Symbiodinium spp. growth rates are negatively affected by heat shock"*

Growth rates were all reduced after heat shock. Besides, response was different between phylotypes. *S. minutum* (B1) did not survive stress, whereas *S. microadriaticum* (A1) and *S. goreau* (C1) were resistant to heat shock but decreased their growth rates.

2) "*Changes in lipids and pigments are different between Symbiodinium phylotypes after heat shock"*

Symbiodinium spp. lipidomes and pigments profiles were differently altered by heat shock. However, downregulation of n-3 fatty acids 4 hours after stress was common in all phylotypes. After 24 hours and in the long term considered as 10 days, each phylotype had a specific survival strategy and fate summarized in figure 4. This demonstrates the essential role of omega-3 fatty acids for cellular energy as suggested by Valentine and Valentine (2004).

3) "Lipidome and pigments profiles of Symbiodinium spp. after stress are good indicators of oxidative stress caused by heat shock experiment"

We considered variations in the pigments ratios as indicators of chloroplast "health status" and chlorophyll decrease strongly indicated damage in the chloroplasts caused by heat shock. This data together with variations in epoxy/de-epoxy xanthophylls suggest that damage was likely caused by oxidative stress but could not prove it. However, oxidized fatty acids in the free form and connected with polar lipids analytically proved damage provoked by oxidative stress.

4) "*Lipid peroxidation preferably occurs in polyunsaturated fatty acids (PUFAs) from chloroplast membranes"*

Lipid peroxidation was evidenced by the presence of oxidized fatty acids mostly derived from omega 3 polyunsaturated fatty acids in free (FFA) and membrane associated (PL) forms of MGDG, DGDG, DGCC and PC. Glycolipids (MGDG and DGDG) are well known structural thylakoid membrane lipids, plus, evidences discussed in chapter 3 strongly indicated DGCC presence also in the chloroplast. Therefore, chloroplast membranes were main targets of lipid peroxidation. Plus, significant changes in DGCC and DGDG compounds suggest that they might be specifically located closer to chloroplast ROS

formation sources when compared to MGDG because they did not change significantly after heat shock.

Importance of phytoplankton lipids in the global carbon budget and bottom-up effects in the marine food chains

Symbiodinium sp. takes part of the Dinophyceae class and many lipid compounds characterized and monitored in our work have been previously reported in the phenotypes of relatives from the same class and other microalgal classes that inhabit oligotrophic ocean gyres (e.g., Crytophyceae, Haptophyceae) (Van Mooy *et al*., 2009, 2010; Shemi *et al*., 2016). We suggest our temperature-related alterations in the lipidome of *Symbiodinium* spp. are similar to those that may occur in phytoplankton communities considering a universal biochemical principle of lipid composition in bioactive membranes.

Becker *et al.* (2018) showed that in the North Pacific subtropical gyre (NPSG) nearly half of the relative abundance of organisms was composed of Dinophyceae and Haptophyceae (ca. 25.3% each). This region comprises 40% of Earth's total surface area, representing the world's largest biome (Emmerson *et al*., 1997; Sarmiento *et al*., 2004). Local and global alterations in phytoplankton communities were noticed with elevated temperatures and other climate change stressors in the past years leading to alterations in global primary production and carbon sinking (Sarmiento *et al*., 2004; Behrenfeld *et al*., 2006; Schmittner *et al*., 2008; Nagelkerken & Connell, 2010), but the physiological mechanisms responsible for such alterations were poorly discussed. We suggest that changes are likely to happen because of phytoplankton photosynthetic structure vulnerability to oxidative stress considering that they are the major DHA producers in the biosphere (Valentine, 2009). High concentrations of DHA were reported by Becker *et al*. (2018) in the NPSG community associated with DGCC betaine polar head. Their most abundant DGCC had C38:6 – 800.6035 m/z - acyl chains of palmitic acid (16:0) and docosahexaenoic acid (DHA), which was also the most abundant feature in our samples. Photosynthesis and phytoplankton growth in phenotypes with high DHA concentration can be impaired through mechanisms already discussed in our work.

Furthermore, the high abundance of DHA in *Symbiodinium* phylotypes and in other flagellate microalgae (Leblond *et al.,* 2000, 2006, 2015; Gray *et al*., 2009; Awai *et al*., 2012; Armada *et al*., 2013; Dahmen *et al*., 2013; Anesi & Guella, 2015; Anesi *et al.,* 2016) sustains the idea proposed by Valentine (2009): phytoplankton represents the global stock of DHA production in the marine ecosystem. Life cycle of zooplankton species depend on the nutritional quality of phytoplankton, which is defined by lipid content (Søreide *et al*., 2010). Ingestion of DHA and other omega 3 enables development of their sensorial mechanisms essential for survival, growth and reproduction (Müller-Navarra *et al*., 2000; Falkowski & Oliver, 2007). Higher in the food chains, other organisms also evolved with the same dependence in n-3 and their populations may either decrease or present individuals with neurological and sensorial deficiencies if n-3 consumption is low (Davis *et al.,* 1992; Budge *et al.,* 2001; Jonasdottir *et al.,* 2002). Consequently, we highlight the importance of DHA ingestion in the marine food chains and how they are also likely to be impacted by global warming. Behrenfeld *et al.* (2006) showed that from 1999 to 2004 the average global primary productivity dropped by about 200 tons a year. Local changes had a decrease as high as 50% (see also Bopp *et al.*, 2013). If ocean temperatures keep increasing progressively as predicted by climate models (Hansen *et al*., 2010; Rogelj *et al*., 2012; Cabré *et al*., 2015), omega 3 producing phytoplankton might follow an opposite way and promote a bottom-up effect in all marine food chains that could potentially lead to ecological collapse of the whole ecosystem

Omega-3 presence in microalgae membranes mediate cell death cascades with temperature changes in the environment (Valentine, 2009). It was highly evidenced in our experiments with *Symbiodinium* spp. and generated valuable data for coral reef symbiosis and bleaching research. Besides, the concomitant omega-3 presence in phytoplankton populations with large distribution patterns enhances our insights of large detrimental effects in marine food chains following ocean warming. Therefore, we highlight the importance of the present study in mechanistically explaining a universal biochemical principle for all living creatures, from algae cells to more complex organisms. A principle useful to understanding cell physiology and also how slight modifications can impact the whole environment.

Additional considerations

The study of the interface between future projected models of physical-chemical alterations in the marine environment and its effects in the physiology of organisms is still challenging because stressors can act synergistically, antagonistically and additively at the same time (reviewed in Gunderson *et al*., 2016). Sea surface temperature is expected to change together with carbonate chemistry, oxygen content, nutrients and light, thus future scenarios are projected considering multiple stressors (Wohlers *at al.*, 2008; Bopp *et al*., 2013; Gunderson *et al*., 2016). Biological responses are non-linear variables and it is still hard to precisely stablish future numbers since physiological boundaries and mechanisms are not completely understood. However, when we propose a universal biochemical principle of cell membrane bioenergetics determining life's fate, we can also speculate about possible future alterations regarding other climate change stressors, assuming that they will somehow all lead to oxidative stress and, again, survival will be dependable on adapting meanwhile guaranteeing energetic requirements.

Higher temperatures are expected combined with higher luminosity and low nutrient availability in the ocean gyres. Plus, in some coral reef areas nearby very populated shorelines eutrophication might also happen. Higher light intensity can potentially damage the microalgae photosynthetic system due to the generation of singlet oxygen which would also initiate a cascade oxidation of biomolecules and potentially lead to death. Carotenoids are reported as the only components capable of quenching such high energy ROS (Widomska *et al*., 2019). Therefore, phytoplankton species with higher carotenoid concentrations might cope better and resist, while others may not. In chapter one we showed that total pigment content was not significantly different among *Symbiodinium* phylotypes, however, this situation changed after heat stress (presented in chapter 2). Thus, beyond basal carotenoid levels, phytoplankton distinct ability to trigger higher carotenoid production might be an additional mechanism of dealing with light stress and it should be an issue of further investigation.

Alterations in the nutrient availability might also determine which lipid polar head type will be present connected with fatty acid acyl chains in membranes. Oligotrophic environments tend to be more depleted in phosphorus, so that their derived lipids are

replaced by betaine lipids. If ocean gyres become even more oligotrophic, they might be scarce not only in phosphorus, but also in nitrogen. Therefore, in this case they might need to reduce cell volume and growth in order to maintain a suitable membrane energetic structure. In contrast, coastal coral reef areas where nutrient concentration may increase due to higher run-offs and sewage discharges. If nutrient concentrations increase, symbionts may be upregulated in phospholipids and either do not present or downregulate betaine lipids. In this case, it is harder to speculate about how *Symbiodinium* spp. and phytoplankton in general would respond. More studies are needed in terms of systems biology to better understand metabolic pathways and make more precise future predictions of organism physiology and community structures.

FUTURE PERSPECTIVES

More investigation is needed to prove our theory regarding DGCC lipids associated with omega-3 fatty acids presence in the thylakoid membranes of chloroplast. We aim to hopefully soon isolate chloroplasts of *Symbiodinium* phylotypes and analyze their lipidomes. Working with *Symbiodinium* mutant phylotypes in which DGCC synthesis gene is either knocked out or silenced would also be helpful for proving its importance for cells bioenergetics. Unfortunately, complete genomes of *Symbiodinium* phylotypes still scarce and production of mutants has never been reported so far, but it is still a goal in our future studies.

In addition, we are working on oxylipidomics analyses in order to determine their formation due to singlet oxygen and/or radical derived oxidation and we aim to reveal these data soon. Formation of n-3 fatty acids oxylipins from DGCC molecules due to singlet oxygen oxidation will be another strong evidence that this lipid class is located in the chloroplast because singlet oxygen formation is dependent on triplet chlorophyll formation, which happens exclusively in the chloroplast photosystems.

At last, we aim to perform lipidomics studies in the same *Symbiodinium* phylotypes when in symbiosis with coral hosts. We strongly believe that our technique is helpful for better understanding metabolic exchanges in the symbiotic association and possibly determining endosymbiont and coral host fates in the current scenario of climate change.

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