Effect of temperature-induced stress on dimethylsulphide (DMS) and dimethylsulphoniopropionate (DMSP) in members of the Symbiodiniaceae family and the anemone *Exaiptasia pallida*

Michela Lever

A thesis submitted for the degree of PhD in Marine Biology

School of Life Sciences

University of Essex

July 2022

Impact of COVID-19

The Covid-19 outbreak denied having access to the lab from March to July 2020, when I was ready to start a key experiment after substantial preparation and growing sufficient anemones for several months. During the lockdown, all *Exaiptasia pallida* specimens died and I had to source (Dr. Nick Aldred) and regrow a minimum stock. After this, it has taken several months to grow sufficient numbers of anemones to proceed with experimentation.

Taken together, this has resulted in a reassessment of initial plans (abandoned measurements of reactive oxygen species), a decrease in parameters quantified during experimentation (e.g. chlorophyll concentration in anemones), and fewer replicates in most analyses than initially planned.

“JUST KEEP SWIMMING.”

Dory, Finding Nemo

Disney - Pixar 2003

Summary

Coral reefs are among the most biodiverse ecosystems on Earth and they are showing a global decline due to different causes, including temperature increase caused by climate change. Corals survive thanks to the obligate symbiotic relationship between the animal host and their algal endosymbionts from the Symbiodiniaceae family. These autotrophic dinoflagellates are strong producers of dimethylsulphoniopropionate (DMSP) and dimethylsulphide (DMS) which are important in global climate, because they assist with the formation of cloud condensation nuclei and temperature homeostasis. Chapter Two focuses on the DMSP concentration in the coral *Porites astreoides* and macroalgae that grow in its proximity, giving an insight of DMSP-production potential along future tropical coasts where corals might be replaced by macroalgae. The following chapters use two clonal isolates of the model anemone *Exaiptasia pallida* (strains CC7 and H2) and their respective Symbiodiniaceae species (*Symbiodinium linuchae* and *Breviolum minutum*) in a laboratory setting. Chapter Three reports a short-term experiment on increased temperature and showed that anemones bleach at 34°C, but unlike previous research DMSP/DMS did not increase in stress conditions, it decreased instead. Chapter Four describes the development of bio-imaging techniques to quantify the actual Symbiodiniaceae loss within the anemones over the same temperature profile as in Chapter Three. It was confirmed that both anemone species suffer significant algal symbiont loss. Experimentation described in the final data chapter subjected the organisms to a more realistic temperature increase. Anemones did not withstand the increased temperature as well their algal symbionts and started to die off after the temperature reached 32°C. The concentration of DMSP and DMS in both experiments was closely related to the numbers of Symbiodiniaceae present in the anemones, and also in this occasion it did not increase with stress. This thesis highlights the decrease of DMSP/DMS concentration in both algae and anemones at high temperature stress and advances our understanding of these organisms' behaviour in a warming planet.

Declarations

I declare that this thesis is the result of my own work and was written entirely by myself. Any specific chapter declarations, where any specific work was undertaken by others, is as follows:

- Chapter Two: Cody Panton, from the Department of the Environment – Government of the Cayman Islands, has assisted with sample collection and preservation. Dr Croy McCoy has assisted with the diving and provided additional financial support to the project.
- Chapters Three and Five: Dr Brisneve Edullantes developed microscopy methodologies as part of his PhD project. He instructed me on the use of a Leica research microscope and shared his methodology on the semi-automated counting of algal cells (R/FIJI script included).
- Chapter Four: Dr Benoît Chatin advised on the methodology with preparing the imagery for quantitative analysis.

Chapter 1 Introduction

1.1 The importance of dimethylsulphide (DMS) in climate change

Marine trace gases are important for the atmosphere but also vital for the marine environment. Dimethylsulphide (DMS) is a biogenic sulphonium compound that is the most abundant volatile sulphur compound produced in the sea (Charlson et al., 1987; Steinke et al., 2006). Volatile DMS is also the main source of organosulfur in the atmosphere with an annual sea-to-air flux of approximately 30 teragrams of sulphur (Lana et al., 2011). It is a product of dimethylsulphoniopropionate (DMSP), and it is generated by planktonic algae and bacteria in the ocean (Curson et al., 2017). Once in the atmosphere, it can be oxidised to form sulphate aerosols and enhance cloud formation (Charlson et al., 1987; Lovelock et al., 1972). DMS has received much interest in the past 35 years, mostly because of its importance to the CLAW hypothesis. In a seminal paper in 1987, Charlson, Lovelock, Andreae and Warren formulated the CLAW hypothesis that postulates that the climate would self-regulate changes in temperature and light via DMS production and its subsequent flux to the atmosphere (Charlson et al., 1987). They further suggested that the most important climatic role of DMS is to increase cloud albedo over the tropical oceans where the largest flux of DMS occurs. They concluded that because when DMS is oxidized to sulphate aerosols, it can form cloud condensation nuclei (CCN) that increase the albedo (reflectance) of tropospheric clouds. Hence, lowering solar radiation and sea surface temperature at a local scale (Figure 1-1). Once the oceans were to cool, the flux of DMS would decrease and this would constitute a negative feedback (Charlson et al., 1987).

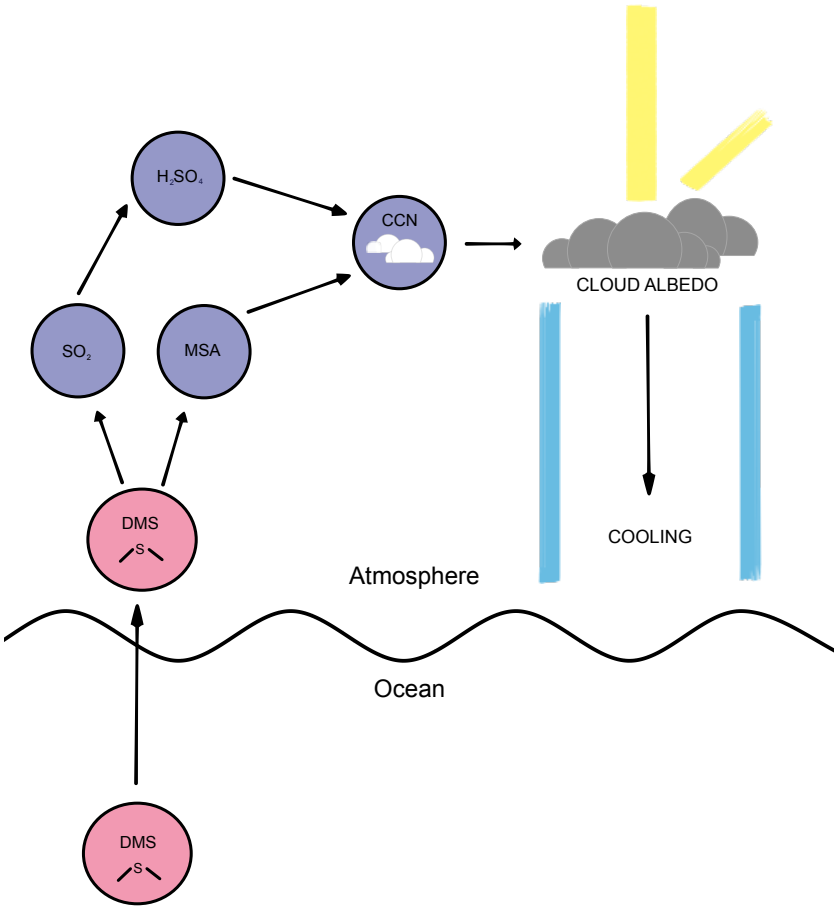


Figure 1-1. The CLAW hypothesis including the key processes linking DMS to CCN and cloud albedo. DMS is produced by biological activity in the ocean and it reaches the atmosphere where it is oxidised to products of low volatility including methanesulphonic acid (MSA) and sulphuric acid. These compounds can stimulate the production of CCN that can increase the reflectance of clouds, reverting more sunlight back to space (Cox, 1997).

In order to counteract global warming due to the increasing amount of greenhouse gases including CO_2 in the atmosphere, it is estimated that the number of CCN would have to double. When considering possible climate change feedback mechanisms, it is important to also take into account that the highest rate of DMS emission to the atmosphere is in areas of the ocean that are characterised by high solar radiation, high salinity waters and high sea-surface temperature (but also in the Arctic (Levasseur, 2013), while the rate of primary

production on its own does not seem to be directly linked to overall DMS (Galí et al., 2011; Toole et al., 2006). Lovelock et al. (1972) collected water samples from the Atlantic Ocean and measured an average DMS concentration of 1.2 x 10^{-11} g mL^{-1}. Over 20 years later, Quinn and Bates (2011) stated that the flux of DMS produced in a hypothetical global warming scenario with a 50% increase of CO_2, would not make a significant change in CCN (only ~0.1% increase at a global scale). After conducting various laboratory and field experiments, they concluded that the CLAW hypothesis is too simplistic, instead, the processes involved in the cycle are complex and still not fully understood (Quinn & Bates, 2011).

1.2 Roles of sulfonium compounds in the ecology and physiology of marine organisms

1.2.1 Dimethylsulphide (DMS) and dimethylsulphoniopropionate (DMSP)

DMS is a compound that fulfils multiple roles in ecosystem processes, not solely related to the atmosphere and climate (DeBose et al., 2008; Steinke et al., 2006). Steinke et al. (2006) demonstrate an aquatic infochemical role, whereby copepods are capable to detect and react to the presence of DMS, proposing that this gas is able to impact both the function and structure of marine food webs. Similarly, DeBose et al. (2008) suggest that planktivorous reef fishes use DMSP as a cue for foraging, by picking up the residual chemical signatures left by their plankton prey. Furthermore, DMSP is also linked to coral disease since (Garren et al., 2014) demonstrate that the coral pathogen *Vibrio coralliiyticus* uses chemotaxis (specific and directed movement in response to a chemical stimulus) and chemokinesis (random and undirected motile response to chemicals) to find

the mucus of the coral. Normally, a healthy coral is covered in mucus and it is continuously produced for cleansing, feeding and defence. DMSP reaches concentrations of 11.9 to 62.2 μM in the mucus of healthy coral colonies, significantly higher than in the surrounding waters (6 to 11 nM) (Broadbent & Jones, 2004). Furthermore, the sulphur compound is not catabolised by the bacteria, indicating that its main role is to act as an infochemical rather than to satisfy metabolic demands in the bacteria. Concentrations of DMS and DMSP were also measured in mucus ropes (patches of mucus that concentrate as a surface film on the sea surface at low tides) and coral mucus from corals in the Great Barrier Reef, Australia. Broadbent and Jones (2004) recorded the highest concentration of DMSP in the marine environment, one order of magnitude greater than the previous record established in the highly productive seawater in Antarctica. Also the DMS concentration in the mucus ropes was a record high (Broadbent & Jones, 2004). These high concentrations of both compounds suggest that coral reefs may play an important role in the global sulphur cycle than has been suggested, given their modest global distribution (Raina et al., 2010). Many functions covered by DMSP have been discovered, but the specific physiological role in corals is still unclear (Gardner et al., 2016).

1.2.2 Dimethylsulfoxonium propionate (DMSOP)

Recently, Thume et al. (2018) discovered a new metabolite in the organosulfur cycle: dimethylsulfoxonium propionate (DMSOP) and demonstrate that this is synthesised by numerous DMSP-producing microalgae and marine bacteria. DMSOP is produced from DMSP and metabolized to DMSO (Figure 1-2). This is a key addition in the marine sulphur cycle (Thume et al., 2018). In surface waters, large quantities of dissolved DMSP and DMS can be detected, but often the concentration of dissolved DMSO exceeds the concentration of each of these two species. DMSO is mainly produced from abiotic (photochemical) and bacterial DMS oxidation, but algal sources of DMSO may also be important (Simó et al., 1998). Similar to DMSP, the oxidized sulphoxonium zwitterion has both a eukaryotic and bacterial origin and this indicates its likely universal distribution in oceanic surface waters. DMSOP was in fact detected at several coastal sites (northwest Pacific Ocean, northwest Atlantic Ocean, Arctic Ocean and Mediterranean Sea) with an average concentration of 0.14 ± 0.18 nM (Thume et al., 2018).

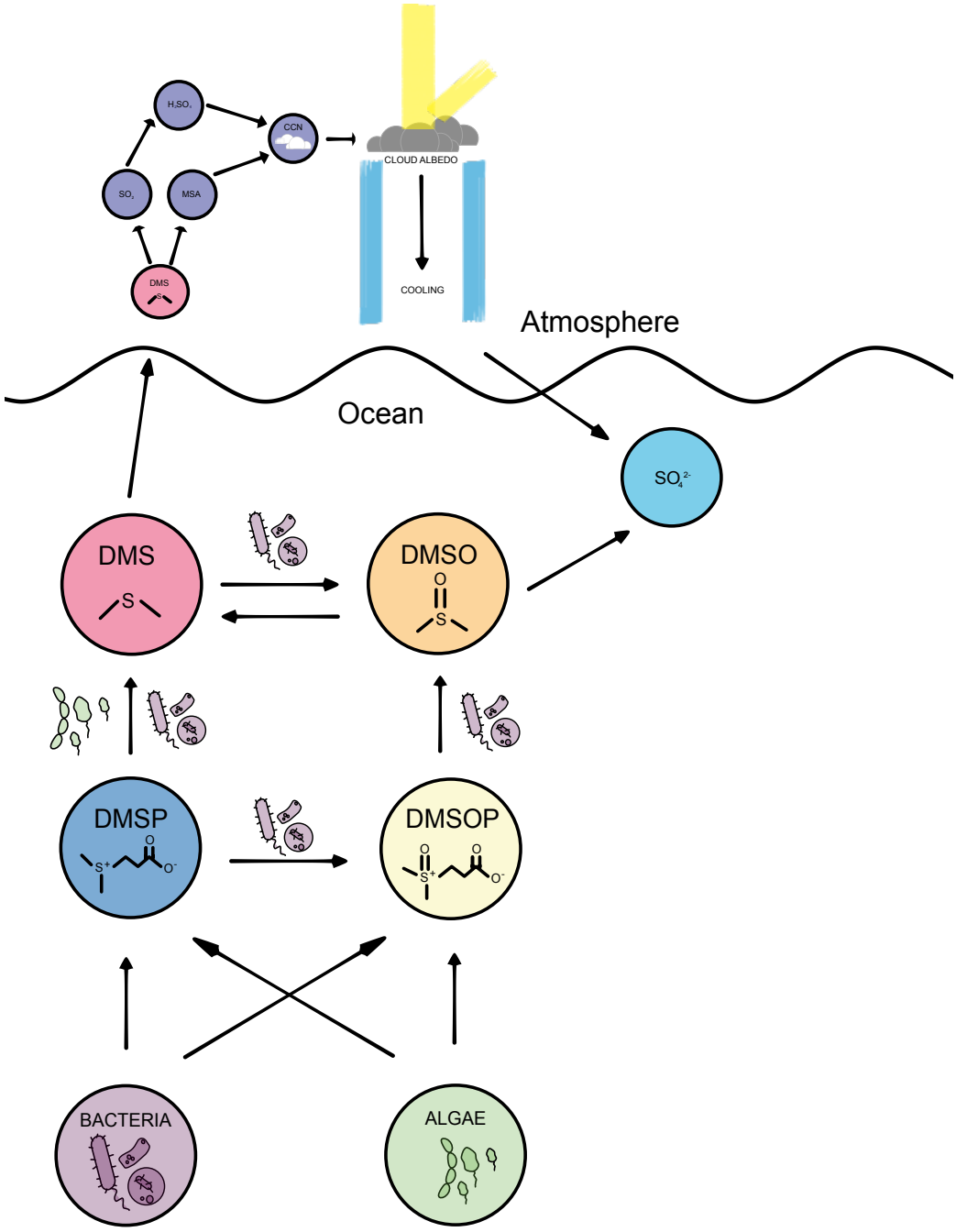


Figure 1-2. Revised and simplified marine sulphur cycle. DMSOP (yellow) is produced in eukaryotic microalgae (green) but also in bacteria (purple). Bacteria metabolize DMSOP and thus it contributes to the marine DMSO pool. Black arrows represent the already established pathways. DMSP is formed by marine algae and bacteria and subsequently cleaved by algal and bacterial DMSP lyases to DMS and acrylate (not shown). Then, biological and photochemical oxidation of DMS to DMSO, sulphate and other products can occur within bacteria, algae, in seawater and the atmosphere (Thume et al., 2018).

1.3 The role of microorganisms in the methyl-sulphur cycle

1.3.1 Bacterial DMSP catabolism

DMSP can be catabolised by bacteria in different ways: the 'demethylation pathway' in which a methyl group is removed, producing methylmercaptopropionate (3-MMPA) and subsequently to methanethiol (MeSH) or mercaptopropionate (MPA); and 'lysis' in which DMS is produced. Some marine bacteria are highly versatile in their metabolism of DMSP, having DMSP lyase, DMSP demethylase and 3-methiolpropionate demethiolase activities (Yoch, 2002). The first step of the demethylation pathway is crucial to the marine sulphur cycle, since it removes a methyl group from DMSP and eliminates DMS as a possible degradation product (Howard et al., 2006). To date, only one prokaryotic gene (*dmdA*) that encodes DMSP demethylase has been identified, while DMS-production pathways have a minimum of four different *ddd* gene types (Johnston et al., 2008; Todd et al., 2007).

1.3.1.1 DMSP demethylase pathway

The DMSP demethylase, *dmdA* is found mainly in two clades of marine Alphaproteobacteria (Roseobacters and *Pelagibacter ubique* (SAR11)), both are very numerous in the ocean and account for the abundant and widespread presence of *dmdA* genes in marine metagenomes (Rusch et al., 2007). *DmdA* enzymes transfer a methyl group to the acceptor molecule (usually tetrahydrofolate - THF) to yield 5,10-methylene-THF, in addition to their deamination activity. Unusual for a member of this family, the methylated product of *dmdA* -mediated demethylation of DMSP is 5-methyl-THF (Johnston

et al., 2016) (Figure 1-3). In the coral-dinoflagellate symbiosis, 70 % of the total amount of DMSP generated seems to undertake the bacterial demethylation pathway, and 30% is cleaved to DMS by bacterial pathways (Raina et al., 2010).

1.3.1.2 DMSP lysis pathway(s)

The lysis pathway can be mediated by at least three classes of '*Ddd*' (DMSP-dependent DMS) bacterial enzymes (Figure 1-3).

- *DddD* Acetyl CoA Transferase: *dddD* is (so far) unique in that its C3 initial product is not acrylate, but 3-hydroxy-propionate (3HP), or a CoA- linked version thereof
- The 'metallo-peptidase' *dddP*: the *dddP* DMSP lyase cleaves DMSP into DMS plus acrylate, and is widespread in bacteria of the Roseobacter and SAR116 clades
- Cupins: The cupin motif comprises a metal-binding beta-barrel and occurs in a great number of polypeptides, with many functions. No less than three different cupin-type DMSP lyases, *dddL*, *dddQ* and *dddW* have been identified to date — again, mostly in the marine Roseobacter (Johnston et al., 2016).

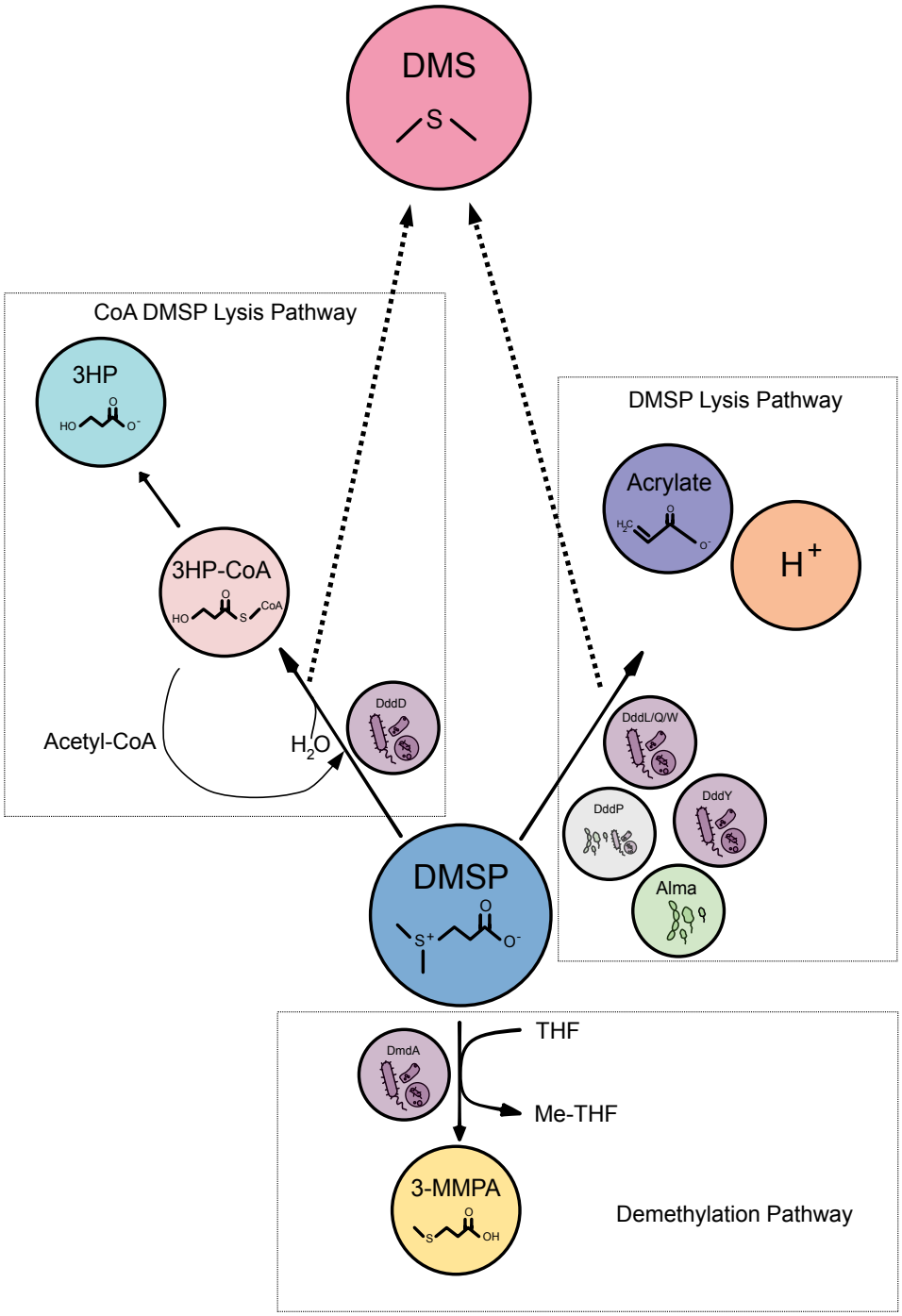


Figure 1-3. Products formed through the action of different enzymes that act on DMSP. DMSP is converted to acrylate, DMS and a proton via the *dddL*, *Q*, *W*, *Y*, *P* and *Alma-1* enzymes of the DMSP lyase pathway. The *dddD* enzyme of the coenzyme A (CoA) DMSP cleavage pathway yields 3-hydroxypropionate (3HP)-CoA and DMS from DMSP, using acetyl-CoA as a CoA donor. The demethylation of DMSP by *dmdA* produces methylmercaptopropionate (3-MMPA) and methyl-tetrahydrofolate (THF). Bacterial enzymes are on pink background, eukaryotic on green, and *dddP*, found in both domains on yellow (Johnston et al., 2016).

Yost and Mitchelmore (2009) tested for the potential DMSP lyase potential activity (DLA) in five cultured strains of *Symbiodinium microadriaticum*, in order to understand if DLA differed among strains. They discovered that *S. microadriaticum* has DLA, but its level varies significantly (under identical conditions) and that one of the strains did not demonstrate this ability. This suggests that DMSP lyase is not a universal enzyme in this species or that they were not able to detect it. In addition, DLA was investigated in non-axenic cultures and it was observed that DLA was primarily associated with the algae and not the bacteria (Yost & Mitchelmore, 2009).

1.3.1.3 DMSP production in bacteria

Until a few years ago, it was believed that only eukaryotes produce significant amounts of DMSP, but it has been demonstrated that many marine heterotrophic bacteria are also able to do so, probably using the same methionine transamination as demonstrated in phytoplankton and seaweeds (Curson et al., 2017). Curson et al. (2017) identified the first DMSP synthesis gene in any organism, *dsyB*, that encodes the key methyltransferase enzyme of this pathway that is also a dependable reporter for bacterial DMSP synthesis in marine Alphaproteobacteria.

They have further investigated the potential functions of DMSP and/or its lyase to DMS and acrylate in *Labrenzia aggregata* LZB033 by monitoring DMSP production and the transcription of the *dsyB* and *dddL* genes in response to diverse conditions. They also concluded that DMSP production can increase due to oxidative stress, but its degradation increases too, possibly by spontaneous reactions of DMSP with radicals. Further investigations conclude that, in contrast

to what has been thought previously, the *de novo* DMSP synthesis does not seem to have a major role in the functions attributed to DMSP in eukaryotes including roles in osmoprotection, cryoprotection, protection against thermal and/or oxidative stress, or its production under conditions where nitrogen is limiting (Curson et al., 2017).

1.3.1.4 Bacteria involved in the degradation of DMS

It was originally believed that the major removal of DMS from the ocean was its flux to the atmosphere, but later studies demonstrate that between 50 and 80% of the DMS produced is directly consumed directly by bacteria (Bates et al., 1994). Principally, DMS is degraded through two different pathways:

- Via an NADH-dependant monooxygenase to form methanethiol and formaldehyde when oxygen is present
- Via a methyltransferase that does not require oxygen as a substrate, thus allowing growth on DMS with nitrate or nitrite as the electron acceptors (Raina et al., 2010).

Raina et al. (2010) discovered that more than 65% of the bacteria genera associated with DMSP/DMS metabolism have also been found in coral-associated communities. Their study showed that corals retain a large number of bacterial strains that are potentially involved in the metabolism of methylated sulphur compounds, further validating that these compounds play a major role in structuring coral-associated microbial communities.

1.3.2 DMSP production in algae

The first molecular genetic information on Alma-1, a DMSP lyase, became available recently (Alcolombri et al. 2015). The enzyme was discovered in the coccolithophore algae *Emiliania huxleyi,* but sequence-homology research has suggested that Alma1 is part of a gene family that is present in major phytoplankton taxa, comprising Symbiodiniaceae (even if at low-level similarity, ~25% identical to Alma1). All enzymes that belong to Alma1 catalyse the abstraction and/or addition of a proton from a carbon next to the carboxylate group. In fact, Alma1 catalyses proton abstraction at the same position, resulting in the release of DMS and acrylate. The discovery of this algal DMSP lyase is essential to fully comprehend the physiological roles of DMS and its place within the marine sulphur cycle (Alcolombri et al., 2015). Alcolombri et al. (2015) determined that the product of catalysis was not 3-hydroxypropionate but acrylate, as the bacteria DddD product (Figure 1-4).

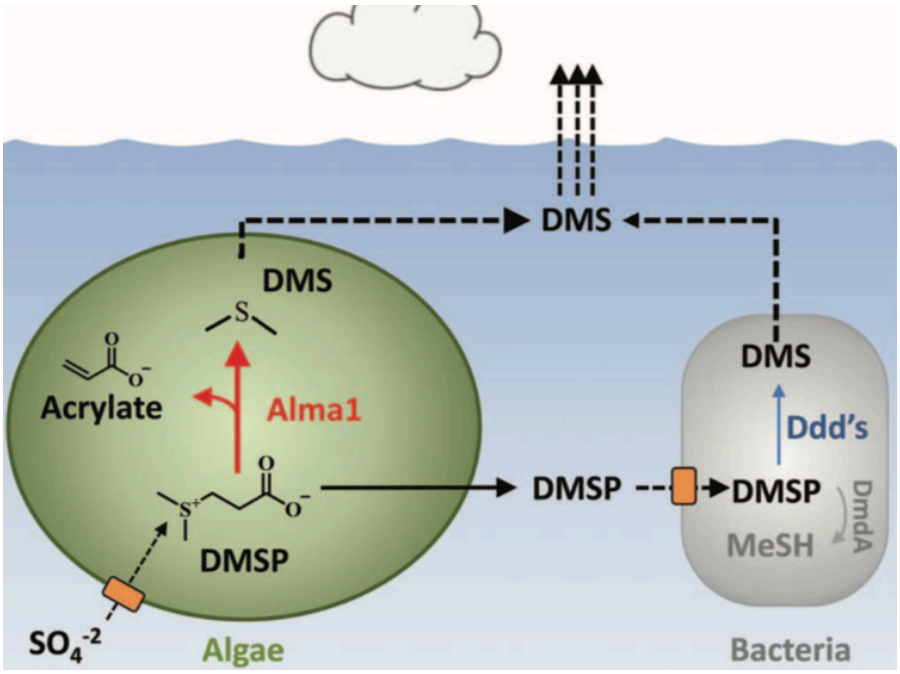


Figure 1-4. DMS catabolism in the ocean is mediated by both algal and bacterial DMSP lyases. DMSP is mainly synthesised by algae. DMSP is released and catabolised by marine bacteria by competing metabolic processes of demethylation (DmdA – no DMS produced) or lyase activity, or additionally algal DMSP can be directly lysed by Alma1 to release acrylate and DMS, which is then emitted to the atmosphere (dashed lines) (Alcolombri et al., 2015).

1.4 DMS vs isoprene

The emission of biogenic volatile organic compounds (BVOC) in tropical marine ecosystems is known to be dominated by DMS while isoprene dominates in terrestrial environments (Steinke et al., 2011). DMS and isoprene can both influence the air quality, cloud dynamics and albedo, therefore making their production and flux to the atmosphere an important area of research (Dani & Loreto, 2017). Corals are known to produce DMS, via their symbiotic dinoflagellates but also from the animal host itself (Franchini & Steinke, 2017; Raina et al., 2013; Van Alstyne et al., 2009); however, corals (Exton et al., 2015) and their symbiotic algae (Exton et al., 2013) are also significant isoprene producers. Exton et al. (2015) showed that similarly to DMS, isoprene production

patterns vary among corals under different stresses. Dawson et al. (2021) have registered an emission rate of 0.97 – 2.24 pmol cm $^{-2}$ h^{-1}. Dani and Loreto (2017) recently showed that marine phytoplankton usually emit either DMS or isoprene and that sea-water surface concentration and emission hotspots of the two compounds have opposite latitudinal gradients. They hypothesised that natural selection favours DMS in cold water and isoprene in warm (tropical) oceans; thus, global warming may expand the geographic range of marine isoprene producers. This phenomenon could have important consequences for future marine-atmosphere interactions (Dani & Loreto, 2017).

1.5 DMS and DMSP production in Symbiodiniaceae and Cnidarians

Symbiodiniaceae are a substantial source of DMSP and DMS in the Great Barrier Reef where the highest concentrations of DMS/P were recorded (Broadbent & Jones, 2004). Both cnidarian hosts and symbionts present a wide range of intracellular DMSP and DMS concentrations (Table 1-1 and Table 1-2).

Table 1-1. Summary of concentrations of DMSP in corals and anemones. Data are from single concentration measurements, concentration ranges or means ± standard deviation using values published from 1994 onwards. NBR = Nelly Bay Reef, KR = Kelso Reef, OTR = One Tree Reef, OI = Orpheus Island, GBR = Great Barrier Reef, # = bleached colonies, Nd = Not Detectable, * = aposymbiotic, ^ = (µmol/g dry weight), ~ = (µmol/g fresh mass), light acclimation level 100 = 100 µmol photons/m^2/s, 400 = 400 µmol photons/m^2/s

Species	Location/ Geographic origin	N. of samples	DMSP (fmol/cell)	DMSP (µmol/cm^3 cell volume)	DMSP (nmol/cm^2)	Reference
Corals						
Acropora formosa	Magnetic Island, GBR		150-270			(Broadbent & Jones, 2004)
Pocillopora damicornis	Magnetic Island, GBR (NBR)	25	99±59	181±114	126±168	(Broadbent et al., 2002)
	Magnetic Island, GBR (KR)	75	179±66	296±110	56±18	
	Magnetic Island, GBR (OTR)	26	89±47	158±83	43±14	
Acropora formosa	Magnetic Island, GBR (NBR)	113	235±113	419±220	330±174	
	Magnetic Island, GBR (KR)	5	641±78	1193±217	533±157	
	Magnetic Island, GBR (NBR) #	5	436	673	572	
	Magnetic Island, GBR (NBR) #	5	171	356	237	
Acropora palifera	Magnetic Island, GBR (NBR)	3	2831±635	5968±984	3842±1237	
	Magnetic Island, GBR (OI)	60	3831±1476	7590±2829	3538±1349	
Lobophytum sp.	Magnetic Island, GBR (OI)	2	43±13	72±22	70±27	
Favites sp.	Magnetic Island, GBR (OTR)	1	21	36		
Acropora pulchra	Magnetic Island, GBR (OTR)	10	40±8	81±28	34±17	
Isopora palifera	Magnetic Island, GBR		2831±635			
Acropora aspera (control)	Heron Island, GBR		43±3			(Deschaseaux et al., 2014)
Acropora aspera (elevated temperature)			1280±354			
Acropora aspera (direct sunlight)			2180±169			
Acropora intermedia	Heron Island, GBR (2001)		152±26			(Swan et al., 2017)
Acropora intermedia	Heron Island, GBR (2002)		141±42			
Acropora intermedia	Heron Island, GBR (2002) #		665±237			
Acropora intermedia	Heron Island, GBR (2003) #		735±200			
Acropora cf. *horrida* ^{100}		15	410±378.5		910.2±628.9	(Hopkins et al., 2016)
Acropora cf. *horrida* ^{400}		9	1054.6±956.9		1618.7±1333.9	
Porites cylindrica ^{400}		11	41.5±45.6		79.7±71.8	
Seriatopora hystrix ^{100}		12	10.1±4		65.4±17	
Seriatopora hystrix ^{400}		6	12.1±2.9		38.1±22.3	

Species	Location/ Geographic origin	N. of samples	DMSP (fmol/cell)	DMSP (µmol/cm^3 cell volume)	DMSP (nmol/cm^2)	Reference
Anemones						
Aiptasia californica		3	934±134	15±1.8 ~		(Van Alstyne et al., 2009)
Aiptasia pallida		2	369±54	3.4±1.2 ~		
*Aiptasia pallida**		3	Nd	Nd		
Aiptasia pulchella		4	483±71	6.3±1.6 ~		
*Aiptasia pulchella**		3	Nd	Nd		
Anthopleura elegantissima		5	311±453	11±2.1 ~		
Anthopleura elegantissima		3	1165±695	0.43±0.11 ~		
Anthopleura elegantissima		3	3375±2157	0.30±0.10 ~		
Aiptasia pulchella		3		54.7±15.2 ^		(Yancey et al., 2010)
Aiptasia cf. *pallida*		6		32.7±6 ^		(Franchini & Steinke, 2017)
Aiptasia cf. *pallida**		6		0.6±0.19^		

Table 1-2. Summary of concentrations of DMSP in Symbiodiniaceae presented in the literature from 2002 to present day (mean±SE, unless otherwise stated).

Symbiodiniaceae	Clade	DMSP	Unit	DMSP	Unit	DMS (mmol/L/CV)	Author
Symbiodinium	A1	98±4.18	(fmol/cell)			0.32±0.112	(Franchini & Steinke, 2017)
Symbiodinium	A2	126.8±8.59				0.06±0.018	
Symbiodinium	A13	85.6±22.03				0.10±0.029	
Symbiodinium	B1	39.3±2.33				0.04±0.025	
Symbiodinium in extract from *Acropora palifera*		3.831	(pmol/cell)	7590	(mM/cell)		(Broadbent et al., 2002)
Symbiodinium in extract from *Acropora palifera*		2.831	(pmol/cell)	5968	(mM/cell)		
Symbiodinium in extract from *Acropora formosa*		0.641	(pmol/cell)	1193	(mM/cell)		
Symbiodinium in extract from *Acropora formosa*		0.436	(pmol/cell)	673	(mM/cell)		
Symbiodinium	D1	50±4.09	(fmol/cell)	140±16.6	(mmol/L/CV)		(Deschaseaux et al., 2014)
Symbiodinium	C1	63±4.67	(fmol/cell)	220±20.1	(mmol/L/CV)		
Symbiodinium – strain 1633	B	68±5.8	(fmol/cell)	450±115	(mmol/L/CV)		(McLenon & DiTullio, 2012)
Unspecified – strain 373				7.6±2.3	(mmol/L/CV)		(Yost & Mitchelmore, 2009) (mean±SD)
Unspecified – strain 374				4.7±2.1	(mmol/L/CV)		
Strain 421	E	201±138.9	(fmol/cell)	353±70	(mmol/L/CV)		
Strain 828	A	126.6±60.7		192±51			
Strain 829	A	81.4±42.9		128±54			
Strain 830	B	33.8		52			
Strain 1633	B	329±193.2		686±59			

1.5.1 The importance of the holobiont in DMS and DMSP production

Previously, it was believed that the source of DMSP in symbiotic animals derives solely from the symbiotic algae, not the animal (Van Alstyne et al., 2009). Van Alstyne et al. (2009) did not find detectable concentrations of DMSP in aposymbiotic anemones, even when grown in both light and dark conditions. DMSP was detected in anemones that were inoculated with Symbiodiniaceae (369±54 to 934±134 fmol/cell) but was not present in those uninoculated, although they were both kept at identical conditions in light. Also, symbiotic anemones that were grown in the dark had less zooxanthellae than animals kept in the light (~100,000 and ~500,000 cells per animal, respectively) and contained respectively lower amounts of DMSP (~ 40 and ~50 fmol/cell respectively). More recently, Raina et al. (2013) demonstrated that coral juveniles produce DMSP even in the absence of algal symbionts (~3 nmol/mm^2 coral surface area). They observed that DMSP concentration increases up to 54% over time in aposymbiotic juvenile corals, and when they subjected the animals to thermal stress DMSP level increase up to 76%. To support their conclusion, previous experiments on Symbiodiniaceae cultures demonstrate that the DMSP concentration per cell volume decreases under thermal stress, indicating that corals have the necessary enzymatic machinery to produce DMSP, without their symbiotic algae (McLenon & DiTullio, 2012). These results suggest that increases in DMSP concentration in thermally stressed corals may not be attributed to the activity of dinoflagellates alone. This also implies that DMSP production is not limited to juvenile life stages, but also takes place at high levels in adult specimens subjected to thermal stress. DMSP is involved in many key

cellular and ecological processes, which might explain why corals produce it directly (Raina et al., 2013).

1.6 Bleaching

Bleaching is the process in which the symbiosis between the animal host and the algae ceases to exist (Weis, 2008). Bleaching is identified as a stress response to environmental disturbance, it is commonly due to several different stressors, such as higher solar radiation, higher temperature, salinity changes and increased nutrient/pollutants or diseases. In order to bleach, an increase of 1-2°C for several weeks or 3-4°C for a few days above the summer sea surface water temperature is sufficient (Brown, 1997; Hoegh-Guldberg, 1999). Bleaching is essentially determined by the measurement of zooxanthellae physiological status and their possible loss from the host organism. The degradation of algal cells and consequent loss is only part of the damage and a destruction of the coral gastrodermis has also been reported in some cases (Brown, 1997). Currently, there are four major mechanisms that have been documented to occur in this process (Figure 1-5) (Bieri et al., 2016). First, algal cells can be degraded *in situ*, this could simply be due to cell death and it has been recorded in corals subjected to natural thermal stress. Second, degraded but also intact algae might be expelled from the host cells by exocytosis or a similar mechanism; this was reported both in the field during natural thermal bleaching and also under laboratory conditions when subjected to higher temperature and/or light (Bieri et al., 2016; Davy et al., 2012). Third, host cells that contain algae can detach themselves from the gastrodermal layer and are then released into the animal's

gut to be later expelled into the environment. This behaviour was observed in mesocosm environments when anemones were exposed to temperature stress or caffeine, but also during a natural thermal bleaching event. Fourth, host cells that contain the algae may die inside the host's symbiosomes, which is the compartment in a host cell that houses the endosymbiont), due to a programmed apoptotic mechanism (programmed cell death) or necrosis (cell injury resulting in premature death); this process was mainly induced at increased temperature. Host-cell death has been attributed to reactive oxygen species (ROS) released by the algae, symbiont/host production of nitric oxide, activation of host innate-immune responses and also the deterioration of the host mitochondria in stress conditions (Bieri et al., 2016; Weis, 2008).

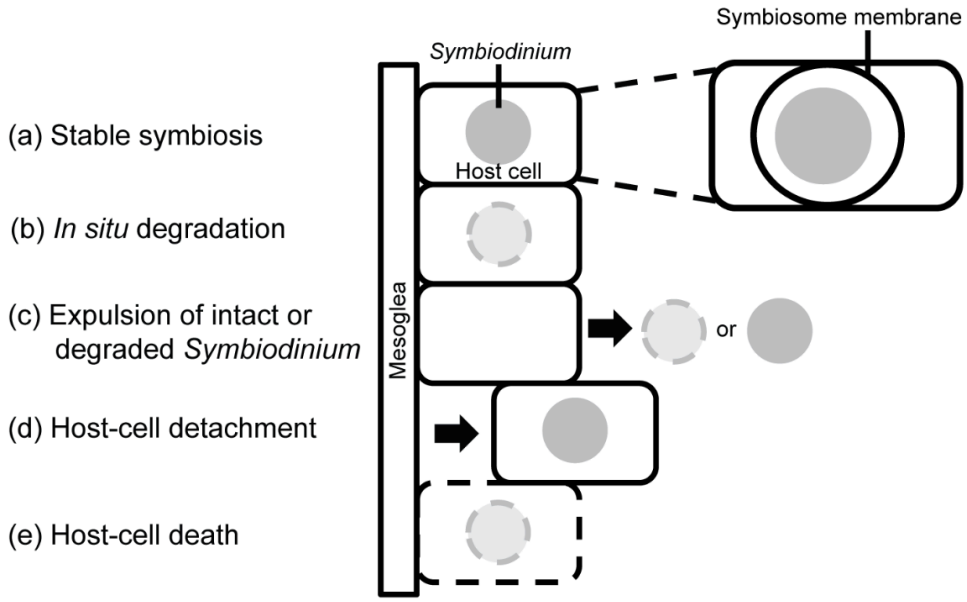


Figure 1-5. Four possible cellular mechanism of cnidarian bleaching under stress. *Symbiodinium* has a stable symbiosis with its host (a), during a stress event the algae can degrade inside the host (b), the algae can survive and be expelled by the animal host (c), both algae and host-cell can detach from the animal (d) and finally the whole host-cell can die (e) (Bieri et al., 2016).

1.6.1 Reactive oxygen species

Photosynthesis, the conversion of light into chemical energy, is a vital process in algae and it releases oxygen as a waste product. Oxygen is normally used in cell respiration, in its elemental form, however it can also become a constant oxidative threat when it is present at cellular level. Increased ROS concentrations can be generated by biotic and abiotic stresses (such as high light or temperature), where the first action takes place in the interior of the cell, within the chloroplast (Wietheger et al., 2018).

Symbiodiniaceae can have high photosynthesis rates in high light environments like those in tropical reefs, hence producing large quantities of dissolved oxygen. High temperature and/or irradiance can cause photoinhibition which damages the chloroplast and the photosynthetic apparatus in at least three related ways that act together and this may eventually start the bleaching mechanism (Figure 1-6). First, the D1 protein, which is part of photosystem II (PSII) within the thylakoid membranes, is easy to destabilise and it is in fact considered the 'Achilles Heel' of the photosynthetic apparatus. However, under normal conditions, there is a system able to repair it and keep the D1 protein functioning. In contrast, the D1 protein may become damaged in stressful conditions and this overburdens the standard repairing mechanisms. Damage to the D1 protein leads to a backlog of excitation energy and photosystem I begins to dysfunction. Second, the damage could affect the ribulose bisphosphate carboxylase oxygenase (Rubisco), which results in reduced consumption of Adenosine triphosphate (ATP) and Nicotinamide adenine dinucleotide phosphate (NADPH) coming from the light reactions, that results in the dysfunction of PSII. Third, high light and heat damage the thylakoid membranes, causing energetic uncoupling of electron transport in

both photosystems. Hence, ATP and NADPH are no longer produced but electrons are still generated. The accumulation of extra electrons by any or a combination of all the above mechanisms within the cell, is believed to result in ROS production in the symbiont. The surplus of electrons reduces O_2, in the Mehler reaction in photosystem I (PSI), to generate a highly reactive ROS, superoxide (O_2^-) (Weis, 2008).

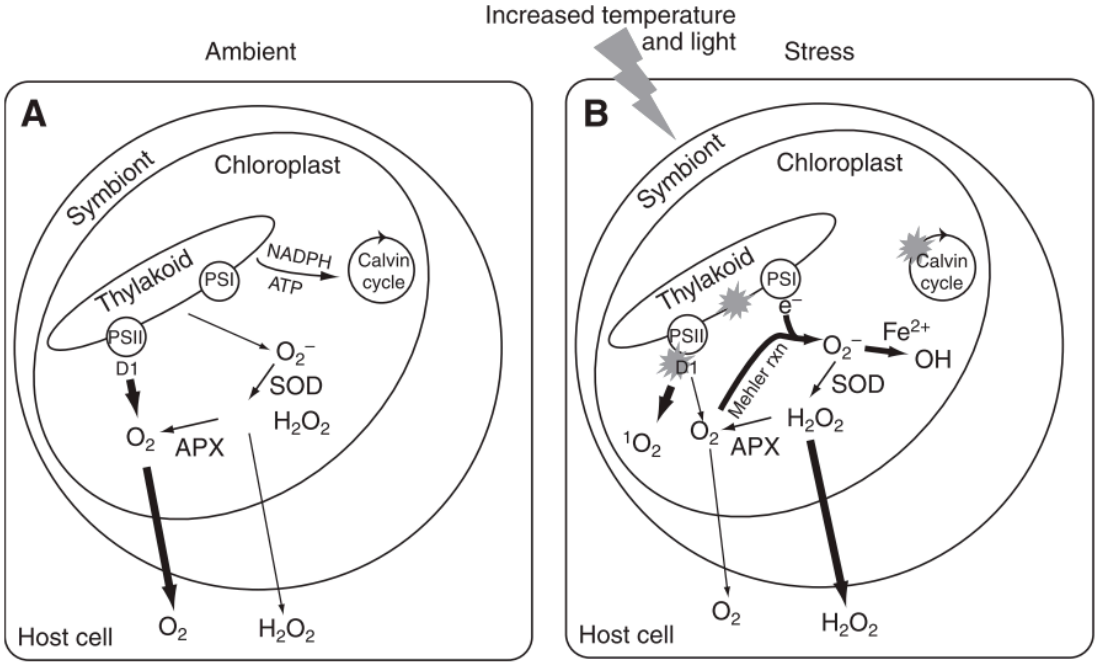


Figure 1-6. Illustration of the oxygen-handling pathways in *Symbiodinium* living in host cells under ambient conditions (A) and during stress events caused by elevated temperature and light. Under ambient conditions, photosystem II (PSII) and photosystem I (PSI) on the thylakoid, functions normally and produces large amounts of oxygen that diffuse into the host. Reactive Oxygen Species (ROS) that are generated, can be enzymatically reconverted to oxygen with superoxide dismutase (SOD) and ascorbate peroxidase (APX). In stress events, the photosynthetic apparatus (PSII and PSI) is damaged in at least three places (indicated by 'flashes' in the figure): D1 protein in PSII; Calvin cycle; and thylakoid membranes. This damage starts to generate large amounts of ROS in the form of singlet oxygen ($^1O_2$) and superoxide (O_2^-) that overwhelm the oxygen-handling pathways. O_2^- gets converted to hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2), that can diffuse into the host tissue (Weis, 2008).

O_2^- can be reduced by superoxide dismutase (SOD) to a less reactive, but nonetheless damaging, hydrogen peroxide (H_2O_2). H_2O_2 can react with ferrous iron (Fe^{2+}) to form a hydroxyl radical (·OH) which is the most reactive ROS. Moreover, extra electrons can react photochemically with O_2 and pigment to also generate singlet state oxygen ($^1O_2$), which worsen the issue by damaging other D1 proteins by reacting with them and bleaching pigments in the photosynthetic system in the thylakoids (Lesser, 2006; Weis, 2008). Once the photosynthetic system is damaged, ROS concentration continues to increase and it can severely damage cells, also oxidizing membranes, denaturing proteins and deteriorate nucleic acids. Both symbionts and host have numerous adaptation strategies to prevent cellular damage from ROS. The symbiotic partners are able to produce high quantities of diverse ROS handling enzymes, like catalase (CAT), ascorbate peroxidase (APX) and various forms of superoxide dismutase (SOD). These enzymes work together to reconvert ROS to oxygen and water. This process is effective if the stress does not exceed the organism's threshold, if the antioxidants defence system becomes overwhelmed, it cannot detoxify the ROS and cells continue to accumulate them. Thus, ROS further damages the photosynthetic membranes in an escalating positive feedback loop. Finally, ROS start to diffuse into the host tissue where further damage incurs and ultimately leads to bleaching (Borell et al., 2016; Gardner et al., 2016; Lesser, 2006; Weis, 2008).

1.6.2 DMS(P)(O) in ROS defence

Studies indicate that corals and Symbiodiniaceae subjected to environmental stresses (high light, increased temperature and hypo salinity) have increased their antioxidant activity, using both enzymatic and non-enzymatic antioxidants in order to protect themselves from the harmful effects of ROS (Gardner et al., 2016; Krueger et al., 2014). DMSP and its breakdown products including DMS, acrylate, dimethylsulphoxide (DMSO) and methane sulphinic acid (MSNA) are effective scavengers of hydroxyl radicals and other ROS and could therefore also be part of the antioxidant system (Sunda et al., 2002). Sunda et al. (2002) demonstrate that under different stresses (solar ultraviolet radiation, CO_2 limitation, Fe limitation, high Cu^{2+} and H_2O_2) the concentrations of DMSP and/or DMS significantly increase in cultured marine algae. The enzymatic cleavage products of DMSP can be even more effective at scavenging ·OH, as are the DMS oxidation products DMSO and MSNA. It has been calculated that, when acting together, these molecules could constitute an antioxidant system even more effective than ascorbate and glutathione (Sunda et al., 2002). An antioxidant function would also explain the observed increase in algal DMSP concentrations at high light as ·OH is produced as a by-product of photosynthesis (Sunda et al., 2002). When DMSP and DMS scavenge ROS, DMSO and DMS_{aq} are produced and a fraction of DMS_{aq} escapes to the sea-surface and reaches the atmosphere (Figure 1-7) (Franchini & Steinke, 2017). Gardner et al. (2016) also show that in stressed corals DMSP and all its breakdown products can scavenge ROS like SOD and glutathione (GS_x). DMS and DMSP react with ROS and oxidize, forming DMSO. DMSP can therefore be a sensitive indicator of stress because if the

DMSP-based antioxidant system works effectively in scavenging ROS, the production of DMSO would increase (Gardner et al., 2016).

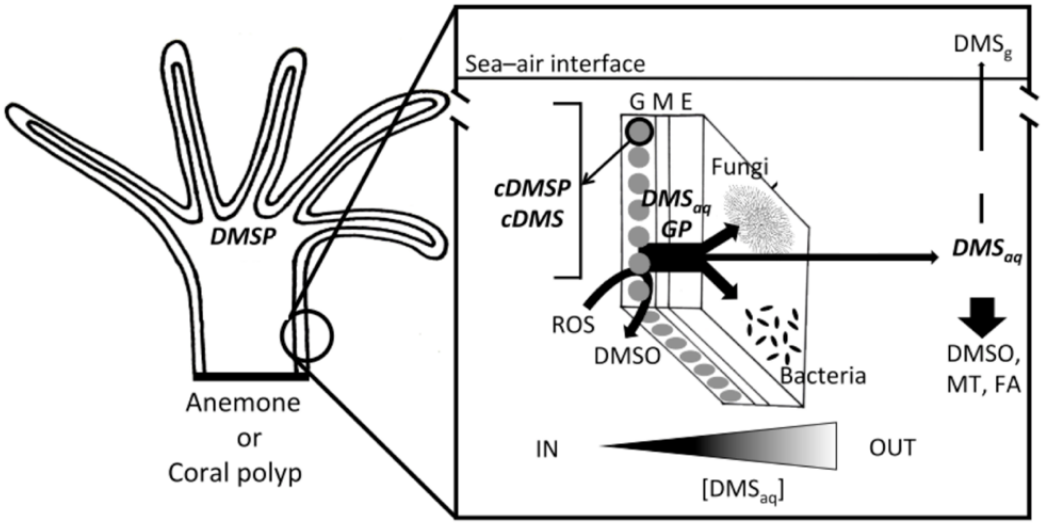


Figure 1-7. Schematic illustration of the production pathways of DMS, from its production by Symbiodinium (grey circles) to its release to the atmosphere. The animal host has a number (N) of Symbiodinium cells that contain DMSP (cDMSP, cellular DMSP) and that produce and release DMS (cDMS, cellular DMS). DMS_{aq} forms and the remaining scavenges ROS and/or is consumed by the bacteria and fungi living on the host surface and once DMS_{aq} dissolves, a portion of it reaches the atmosphere, while the majority is transformed in the water column to DMSO, methanethiol (MT) and formaldehyde (FA). DMS, dimethylsulfide; DMS_g, gaseous DMS; DMS_{aq}, acqueous DMS; DMSO, dimethyl sulfoxide; G, gastrodermis; M, mesoglea; E, epidermis (Adapted - Franchini & Steinke, 2017).

For example, coral *Acropora aspera* was subjected to a range of environmental stressors that can lead to oxidative stress: temperature, light, salinity and air exposure. This experiment allowed to draw a link between the demethylated sulphur compounds (DSC) production and the antioxidant capacity (AOC), showing that under experimental conditions DMS/DMSP/DMSO where produced at higher concentrations, further proving DSC as potential biomarkers of stress levels in coral tissue. The differences in concentrations and partitioning as a consequence of the varying stressors indicate that DSC production and turnover go through different biochemical pathways that depend on the type and severity

of the stress they are under. During osmotic pressure and light depletion, an upregulation of the coral AOC was observed, and it correlated with a significant increase in DMSO:DSC ratio. These results, together with a positive correlation between AOC and DMSO concentrations under osmotic pressure and light depletion, indicate that the DMSP-based antioxidant system is involved in the overall antioxidant regulation of the coral holobiont. An increase of DMS production coupled with a higher DMS:DSC ratio under increased temperature showed that thermal stress trigger DMS formation in coral tissue (Deschaseaux et al., 2014). Gardner et al. (2017) observed similar patterns; the loss of DMSP/(O) could be attributed to an increase in DMSP lyase activity due to heat stress and thus the subsequent oxidation of DMS to DMSO. An alternative, based on Sunda et al. (2002) is that intracellular DMSP could have been used as an antioxidant, with further oxidation to DMSO and MNSA, reducing the available intracellular pools of DMSP and DMSO before any *de novo* synthesis could occur. This could be possible, since DMSP is energetically expensive to produce and if the coral cellular activity is compromised (i.e. lower Fv/Fm, higher ROS and degradation in chlorophyll-a) the production of DMSP by symbiont cells could have been reduced or stopped under acute temperature stress (Gardner et al., 2017b).

Measuring DMSP, coupled with DMS, will give an indication of stress response in organisms and ROS scavenging activities.

1.6.3 Symbiodiniaceae tolerance and 'dark bleaching'

Symbiodiniaceae are known to have various thresholds to thermal and light stress among clades (Rowan, 2004), which can partially explain coral's susceptibility to bleaching (Yost & Mitchelmore, 2009). Yost and Mitchelmore (2009) have hypothesized that if DMSP truly has an antioxidant effect in Symbiodiniaceae, the production of DMSP and the lyase in these dinoflagellates should be correlated with the damage to Photosystem II (PSII) in the algae, since PSII is one of the main mechanisms associated with bleaching (Weis, 2008).

To this date, it is still unclear whether the normally observed correlation of ROS and antioxidants and consequent bleaching is a cause-affect mechanism or if it occurs by chance. Nielsen et al. (2018) suggest that there is not enough evidence to support the former theory and they tried to gather more data. Their results show that coral's dinoflagellates are not critically compromised by heat-induced ROS production and more importantly, when there is no severe photosystem damage, ROS leakage from the dinoflagellates to their host and the following ROS-induced damage does not seem to be the primary trigger for expulsion of the symbionts. Thus, when photosystem damage truly occurs then ROS could really be the trigger for bleaching, but bleaching is likely to occur before the photosystem becomes compromised, possibly due to alterations between host-symbiont nitrogen regulation as a result of stress in the host (Nielsen et al., 2018). Similar conclusions were also drawn by Tolleter et al. (2013) when they discovered that bleaching occurred in the dark, noting a marked increase in singlet oxygen ($^1O_2$) production by photosynthetic electron transport. Their discovery does not contradict the former theory that cnidarian bleaching is primarily triggered during heat stress and the consequent production of ROS, as

a result of photosynthetic activity by light and heat-damaged chloroplasts. They propose that there is an unknown bleaching signal that is not related to photosynthetically produced ROS, since they have observed rapid bleaching in dark conditions (Tolleter et al., 2013). The origin of this signal is still unknown, but the 'dark-bleaching' signal could involve non-photosynthetically produced ROS, nitric oxide production and/or immune reactions to environmental stress. 'Dark-bleaching' at high temperatures could be beneficial to the host, since expelling dinoflagellates at night would lower the amount of these present in the host tissue during the day, when photosynthetically produced ROS ('common bleaching theory') would increase and damage the cells. Thus, this 'system' that triggers bleaching at night could be a different coral adaptation to life with a partner that under stress conditions can become a liability (Tolleter et al., 2013). Similar conclusions were drawn in a similar experiment, where the production of superoxide (O_2^-) in low light and darkness was attributed to the symbiotic dinoflagellates (Zhang et al., 2016).

Recent studies on Symbiodiniaceae showed that unlike originally hypothesized, a phylotype that is thermally sensitive will most likely be sensitive toward oxidative stress, too. However, this was not the case among different phylotypes tested; possibly because each phylotype can invest in a different protective mechanism. The thermally tolerant clade E could have developed mechanisms to have a better tolerance at high temperatures, such as non-photochemical quenching (NPQ - mechanism employed by plants and algae to protect themselves from the adverse effects of high light intensity) and thylakoid membrane stability, thus requiring less oxidative stress defence, like antioxidants. This could mean that thermally tolerant clades could suffer damage more quickly when subjected to

oxidative stress, while the opposite could occur in thermally sensitive clade A1, which could have evolved to deal with thermal stress and subsequent ROS generation by having a more efficient antioxidant system (Wietheger et al., 2018). Wietheger et al (2018) measures the production of ROS (including hydrogen peroxide, superoxide and singlet oxygen) in Symbiodiniaceae under thermal and oxidative stress. Results varied among the examined clades (i.e. A1, B1, E, F1), for example clade E showed no change in any ROS concentration between the control and the high temperature treatment, while in A1 a 10-fold increase was observed. They concluded that both stress responses and the ROS production differed among the examined phylotypes. This difference in quality and quantity of ROS generation suggests that multiple mechanisms are taking place (Wietheger et al., 2018).

1.6.4 Non-enzymatic antioxidants

Unlike enzymatic antioxidants (i.e. SOD, CAT, peroxidase and glutathione peroxidase), nonenzymatic antioxidants have the same function without using enzymatic pathways (Lesser, 2006). Dinoflagellates present in anthozoans mainly contain pigments like peridinin, pyroxanthin, and diadinoxanthin, and in corals zeaxanthin, lutein, and, fucoxanthin are additionally found. The main role of carotenoids in corals is to protect their symbiotic dinoflagellates from irreversible light-induced damage (Galasso et al., 2017). Carotenoids, such as beta-carotene, have additional functions: (i) act as quenchers of singlet molecular oxygen; (ii) convert hydroperoxides into more stable compounds; (iii) can prevent the formation of free radicals, by blocking the free radicals oxidation reactions and by inhibiting the autoxidation chain reaction; and (iv) act as metal chelators,

by converting iron and copper derivates into harmless molecules (Galasso et al., 2017).

In symbiotic anthozoans, up to 30% of the organic carbon produced by photosynthesis can be translocated as lipids from the dinoflagellate to the animal host. Thus, carotenoids could be extruded or leaked from dinoflagellate into intracellular spaces or they may be taken by the host cells. In addition, many symbiotic anthozoan could uptake carotenoids from their diet, by consuming crustaceans and other zooplankton where it is commonly present (Mobley & Gleason, 2003). Mobley and Gleason (2003) investigated the origin of carotenoids in anemone *Exaiptasia pallida* and discovered that it is likely that carotenoids originate from the dinoflagellate and are involved in the photosynthetic system. However, they were no able to understand if carotenoids found in the algae were leaked, sequestered or catabolized in the animal tissue.

The colouration of the majority of Cnidarians is derived from the photosynthetic pigments of their endosymbiotic algae, containing chlorophylls *a* and c_1/c_2 and peridinin or fucoxanthin (or fucoxanthin derivatives); they also contain the photoprotective xanthophylls diadinoxanthin (Dn) and diatoxanthin (Dt). Interconversion of the pigments Dn and Dt is part of a known process of NPQ believed to act as photoprotection and also as prevention of ROS production; β-carotene is also an antioxidant (Venn et al., 2006). In contrast with expectations that these pigments could be beneficial in bleaching events, Venn et al (2006) found no clear correlation between coral bleaching resistance and the abundance of Dn+Dt or β-carotene. They concluded that the impact of bleaching on pigments varies among coral-algal associations (clades A, B and C), and they also

demonstrate that corals could bleach by pigment and by algal density reduction, without showing a significant impact on the chlorophyll-*a* in the remaining dinoflagellates.

Ascorbic acid is used to reduce ROS, it can scavenge H_2O_2 and also O_2^-, ·OH and lipid hyperoxides without enzyme catalysts. Glutathione and tocopherol are also non enzymatic antioxidants (Lesser, 2006).

1.6.5 Osmolytes

Osmoconformers are organisms that use organic osmolytes to keep cellular osmotic pressure equal to that of the external fluid environment they live in. The salts present in ocean (mainly NaCl) have an average concentration of ~1000 milliosmoles/L (1000 mOsm), which is much higher than the ~300 to 400 mOsm created by the solutes in cells (K^+, metabolites, etc.). To avoid osmotic shrinkage, osmoconformers need to obtain the same osmotic pressure as their environment and in order to do so they utilise small solutes to maintain their volume. These solutes are commonly divided in different chemical categories, i.e. small carbohydrates including sugars (e.g. trehalose), polyols (glycerol, inositol, sorbitol, etc.) and derivatives (such as o-methyl-inositol); amino acids (glycine, proline, taurine, etc.) and derivatives (e.g. ectoine); methylamines [such as N-trimethylamine oxide (TMAO) and glycine betaine], and methylsulphonium solutes including dimethylsulphoniopropionate and urea (Yancey, 2005). In algae, the osmolytes more commonly found are sorbitol, glycine, betaine and DMSP. Corals are believed to obtain osmolytes from their endosymbiotic algae, Symbiodiniaceae (Yancey et al., 2010).

Early studies on Anthozoans showed that taurine and glycine are the dominant osmolytes in temperate-zone sea anemones (although non-amino acid were not analysed) and glycine betaine (trimethyl glycine) was found at low levels. In coral *Porites damicornis*, glutamate, serine, alanine and taurine were the main solutes detected in the endosymbiotic dinoflagellate, while glutamate, glycine and taurine were found in the whole coral holobiont. Both temperate and tropical symbiotic anemone, *Anemonia viridis* and *Aiptasia pulchella* respectively, had taurine as their dominant free amino acid (Yancey et al., 2010). DMSP has been indicated as an osmolyte in corals. Yancey et al. (2010) were the first to analyse in detail the osmolytes in adult corals and their respective endosymbionts (clade C) and they presented three major findings. First, methylated solutes - glycine betaine, proline betaine, and DMSP in particular were the major contributors to osmotic pressure, unlike free amino acid and glycerol, that were found at relatively low concentrations in most cases. Second, congeners (i.e. related chemical substances) shared similar osmolyte profiles, possibly meaning that phylogenetic history could be implicated in osmolyte composition. Third, the osmolytes in the symbionts and the host were found at different concentrations, while the methylated amino acids in the dinoflagellates were the same as those present in the host. In 2017, Ochsenkühn et al. (2017) tested whether *Symbiodinium* is capable of synthesising compatible organic osmolytes (COOs), referred to as osmoadaptation, from different strains and clades, both in vitro and in their coral hosts. They identified carbohydrates floridoside, inositol, and mannitol in vitro and in the host; to note that taurine, betaines and DMSP were not measured in this study. These compounds are known to function as both osmolytes and antioxidants, thus are hypothetically capable to convey osmoadaptation to

increased salinities and also to scavenge ROS/free radicals produced under different stress, including heat (Ochsenkühn et al., 2017).

1.7 Symbiodiniaceae

Dinoflagellates in the family Symbiodiniaceae (Figure 1-8) are essential components of coral reef ecosystems in their role as photosynthetic endosymbionts in a large variety of marine organisms, belonging to at least five distinct phyla: Foraminifera, Porifera, Cnidaria, Mollusca, and Platyhelminthes (Pochon et al., 2014). They are commonly found in tropical and subtropical shallow water and inside Cnidaria species, many from the class Anthozoa (e.g. anemones, Scleractinia corals, zoanthids, sea fans etc.), several from the classes Hydrozoa (such as fire corals) and Scyphozoa (jellyfishes). However, only a few groups (Scleractinia corals, foraminifera, gorgonians, and tridacnid clams) have been surveyed using molecular techniques to a degree that can be considered at least marginally representative (Baker, 2003). Symbiodiniaceae can also live freely in the water or in the proximity of sediments where potential hosts are present. Nonetheless, there seem to be no studies that have specifically focused on the diversity of the free-living Symbiodiniaceae in the environment (Baker, 2003; Muller-Parker & Davy, 2001). The proposed new genera in the family Symbiodiniaceae (Figure 1-8) divides the algae in clades (from A to H). The term ‘Symbiodinium’ is no longer used as a generic name to refer to all the species present in this family, *Symbiodinium* is now the genus of clade A only.

Symbiotic algae are widely distributed, and some are found in diverse hosts and appear to be ‘generalist’, instead other seem to be endemic to a specific location and could be restricted to a particular host taxon ‘specialist’ (LaJeunesse, 2002).

There seems to be both host specificity (specificity of host for a specific range of symbionts) and symbiont specificity (specificity of symbionts for a particular range of hosts). Generally, host specificity tends to prevail: the mean number of combinations in which a host is found is normally lower than the mean number of combinations in which a given symbiont is found (Baker, 2003).

Molecular methods show different clades that range from being highly specific to highly flexible. It has been proposed that even if there is a large variety among different species, the one that is more tolerant to thermal stress will eventually take over the rest of the population (Baker, 2003; LaJeunesse, 2002).

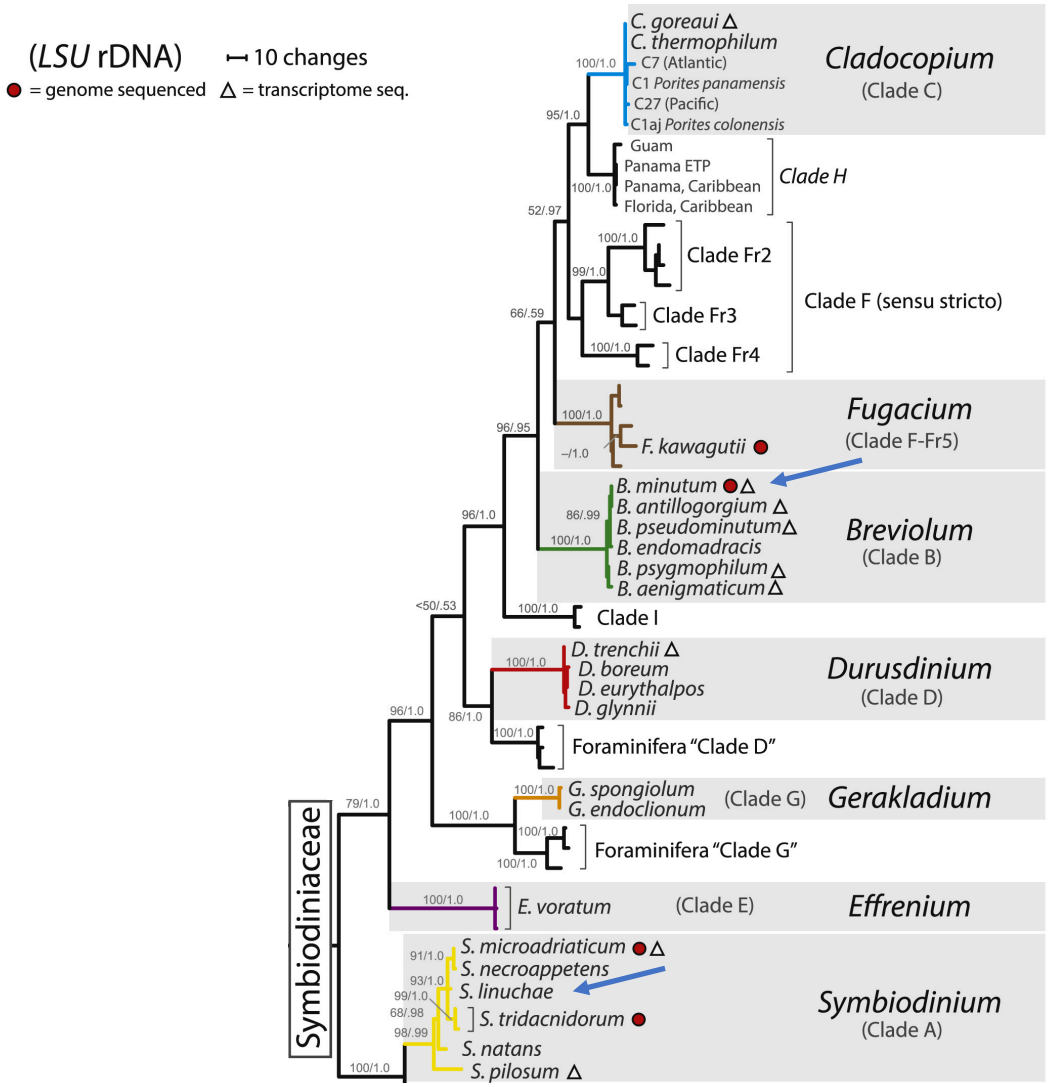


Figure 1-8. Proposed new genera in the family Symbiodiniaceae and large subunit ribosomal ribonucleic acid (LSU rDNA) phylogeny portraying their evolutionary relationships, including remaining lineages that require generic names. Symbols next to terminal branches indicate species whose genomes or transcriptomes are sequenced (red circle) (Adapted - LaJeunesse et al., 2018). Blue arrows indicate the species used in the research.

1. 8 *Exaiptasia*

1.8.1 *Exaiptasia ecology*

Anemone *Exaiptasia* (commonly still known as *Aiptasia*) is a well-known genus among aquarists because of their ability to proliferate and are considered a nuisance species. They are usually accidentally introduced in tropical coral aquaria on rocks, where they attach themselves to (Anderson, 2000). They are able to move around by using a crawling movement, which is unusual compared to other anemones that normally completely detach their foot and swim (Friese, 1972). Grajales and Rodrigues (2014) created a new genus name *Exaiptasia* gen. nov.. The formerly named *Aiptasia pallida* encompasses a single species (now *Exaiptasia pallida*) with organisms from the tropics and subtropics. For this species, they have also erected a new genus (i.e. *Exaiptasia*) based primarily on cnidae (i.e. large secretory organelle used to capture prey), mode of asexual reproduction and symbionts.

Exaiptasia spp. are commonly seen as a pest because they are very difficult to eradicate. In the wild they have a few natural predators. In the Indo-Pacific, raccoon butterflyfish (*Chaetodon lunula*) is known to predate in *Exaiptasia pulchella* while in Hawaii the aeolid nudibranch (*Berghia major*) feeds on *Exaiptasia* spp. (Bertsch & Johnson, 1981). In the Caribbean region, *Exaiptasia tagetes* is commonly found whereas in the Atlantic and Mediterranean *Exaiptasia mutabilis* is prevalent (Anderson, 2000).

In my research, I will use *Exaiptasia* from two different host clonal lines: CC7 and H2 (Figure 1-9). CC7 *Exaiptasia* can form a stable association with a diversity of Symbiodiniaceae, on the contrary H2 *Exaiptasia* show high fidelity to their native

Symbiodiniaceae community (clade B) suggesting a higher selectivity and/or specificity with their symbionts (Grawunder et al., 2015).

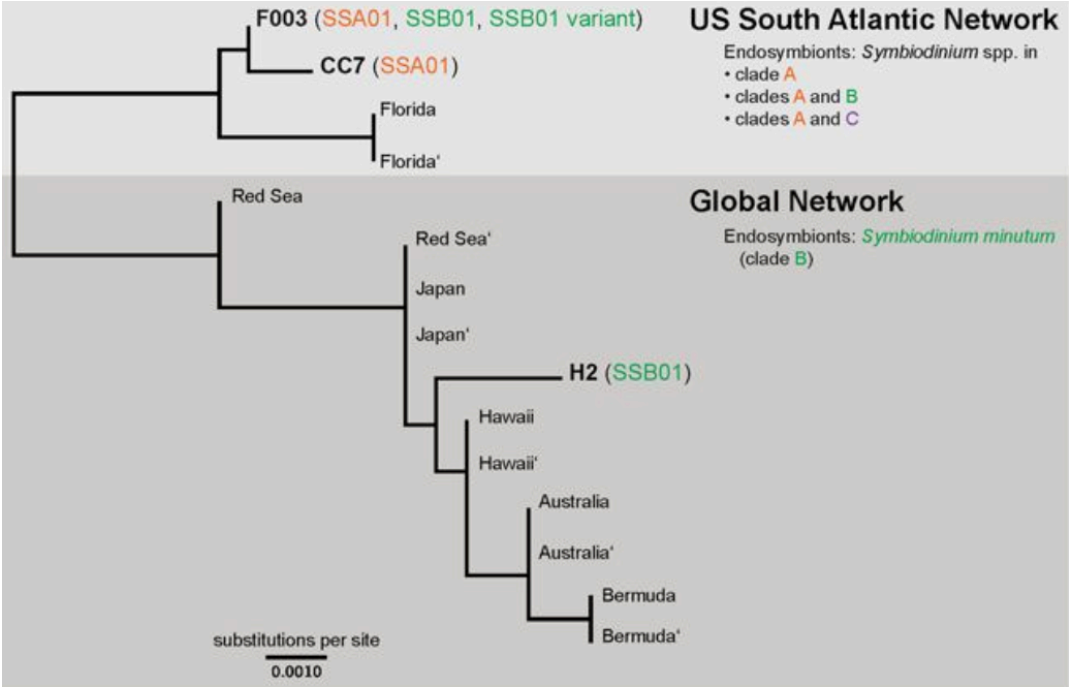


Figure 1-9. Characterization of *Exaiptasia* clonal lines CC7 (male), F003 (female) and H2 (female). Maximum likelihood tree of ~ 2 kb concatenated SCAR genotyping markers showing the relationship of the three clonal lines to the twelve field-sampled Aiptasia sp. individuals grouped into two generic networks (Grawunder et al., 2015).

They are found in shallow tropical marine environments. *Exaiptasia* can reproduce asexually (i.e. pedal laceration) and it is therefore easy to proliferate. Clayton (1985) assessed that asexual reproduction in an intricate process and anemone's culture conditions can affect it. He discovered that the presence of zooxanthellae in *Exaiptasia* could affect the rate of asexual reproduction. His experiments have shown that symbiotic anemone did not significantly enhance lacerate development in comparison with aposymbiotic specimens. The absence of symbionts was negatively received in starved anemones (Clayton, 1985).

Clayton and Lasker (1985) demonstrate that both feeding regime and zooxanthellae affect *E. pallida* in different ways. Zooxanthellae increase individual growth, pedal laceration and population growth when animals are fed once in four weeks. On the contrary, feeding regime has no significant effect on the number of anemones produced asexually by pedal laceration in an eight weeks period. Frequent feeding has a positive increase in individual growth but does not enhance pedal laceration (Clayton & Lasker, 1985).

Experiments conducted on *Exaiptasia pulchella* show the expulsion of their symbionts when subjected at colder temperatures and other studies have focused on a slight increase in temperature, both leading to the same result: bleaching (Muller-Parker & Davy, 2001).

The average range of zooxanthella densities in symbiotic tropical anemones is 0.2 to 0.6 million zooxanthellae per mg protein, whereas *Exaiptasia* spp. have 5 to 11 times more. Specifically, zooxanthellae density in *Exaiptasia pulchella* varies seasonally showing a reduction from summer to autumn (Muller-Parker & Davy, 2001). *E. pallida* is usually kept at 26°C, 65 µmol photons m^{-2} s^{-1}, fed 0, 1 or 3 times a week instead *E. pulchella* has a wider light range of 45-320 µmol photons m^{-2} s^{-1}, 25°C and fed 2 times a week (Clayton & Lasker, 1985; Muller-Parker & Davy, 2001).

Given the lack of nutrients in tropical waters, especially nitrogen, growth in tropical hosts is limited together with pigments of zooxanthellae compared to standard environmental conditions (Muller-Parker & Davy, 2001). A few experiments conducted have shown that *E. pulchella* can take up ammonium from seawater but it is uncapable of retaining nitrate (Wilkerson et al., 1984).

Instead, *E. pallida* kept starved and in nutrient poor seawater conditions showed a nitrogen limitation (Cook et al., 1992).

E. pallida and *E. pulchella* both gain with each generation a new complement of algal symbionts if reproduction was sexual (Figure 1-10) (Clayton, 1985; Grawunder et al., 2015).

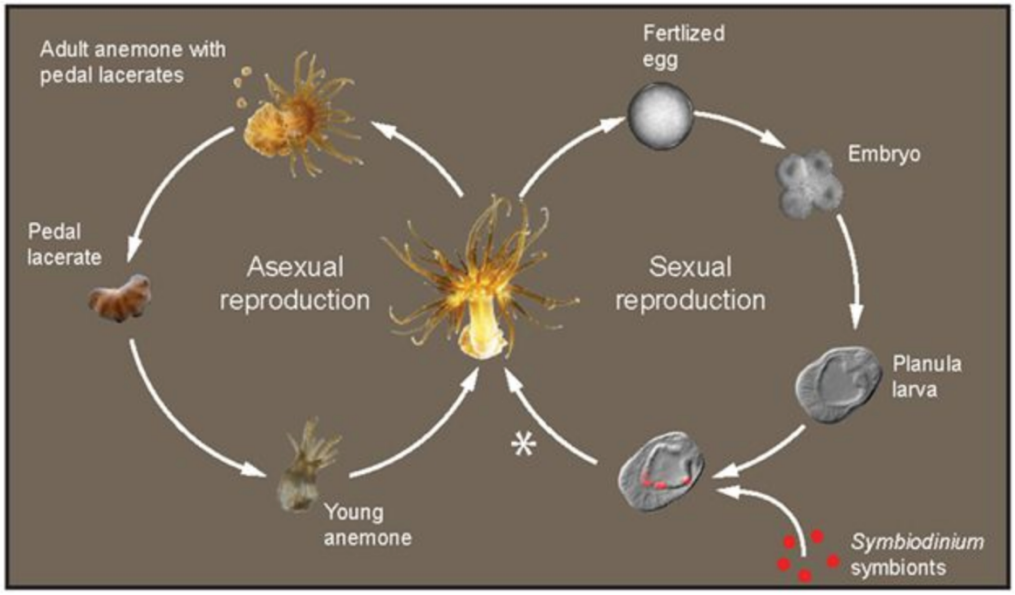


Figure 1-10. Overview of Aiptasia life cycle showing both asexual and sexual reproduction. (*metamorphosis and settlement in the laboratory have not been reported yet, hence it is still an experimental area) (Grawunder et al., 2015).

Early experiment conducted on *Exaiptasia pulchella* showed that the animal response to thermal stress (26°C to 32°C) is the production of superoxide anions (O_2^-) in both aposymbiotic and symbiotic anemones (Nii & Muscatine, 1997).

The most common way to make *Exaiptasia* aposymbiotic is to alternate heat (or cold) shocks with dark treatment and/or using 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which impedes photosynthesis. These methods are slow and require often months of incubation cycles but they do not guarantee the complete removal of symbionts (Matthews et al., 2016). Matthews et al. (2016)

have tested the efficacy of using menthol treatment instead. This technique gives a 97-100% loss of symbionts in only 4 weeks, assuring aposymbiotic hosts long after the treatment if kept at a standard 12h:12h light:dark cycle.

1.8.2 *Exaiptasia* as a coral model

Corals are known to be difficult to work with, they are costly and also costly to keep in mesocosms or aquaria, especially large branching and slow-growing species. Another disadvantage in using corals is the difficulty in applying and interpreting genetic studies, since specimens collected from the wild can have heterogeneous genetic backgrounds (Lehnert et al., 2012). In order to avoid these issues, Lehnert et al. (2012) and others started to develop *Exaiptasia* as a model system to study dinoflagellate-cnidarian symbiosis. *Exaiptasia* belongs to the same phylum (Cnidaria) and class (Anthozoa) as corals, and it also has intracellular symbiotic microalgae that are closely related to those in corals. As opposed to corals, *Exaiptasia* is very hardy, grows and reproduces relatively quickly, especially via asexual reproduction and it also lacks the calcareous skeleton that characterises reef-building corals in the order Scleractinia (stony corals) (Lehnert et al., 2012; Sunagawa et al., 2009). The lack of this skeleton makes *Exaiptasia* unsuitable for this specific aspect of coral biology, e.g. studies dealing with ocean acidification, but it is useful for studies focusing on cell biology and biochemistry. *Exaiptasia* also has the advantage of surviving without its symbiont and, unlike corals, withstands bleaching when provided with sufficient prey for heterotrophy. In addition it can be inoculated, even after bleaching, with different Symbiodiniaceae strains (Belda-Baillie et al., 2002; Sunagawa et al., 2009; Weis et al., 2008).

Thanks to these characteristics, *Exaiptasia* spp. have been used in several studies regarding symbiosis between organism and its host and it proved to be successful using different tools, ranging from microscopy to RNA methods (Perez & Weis, 2006; Sunagawa et al., 2009). Sunagawa et al. (2009), have provided the first large-scale transcriptomic resource for the anemone *Exaiptasia pallida*. This dataset provides the molecular genetic basis for a model organism to explore symbiosis at a molecular, cellular and also genomic level (Sunagawa et al., 2009). *Exaiptasia* can host different variety of Symbiodiniaceae, but not all species (Lehnert et al., 2012). Belda-Baillie et al. (2002) demonstrates in their study that some algal isolates are able to infect aposymbiotic anemones, but others are not, even if there is no competition from other taxa. Moreover, symbiosis between *Exaiptasia* host and micro algae isolates from identical or genetically related anemones appear to be more favourable.

1.9 Overall aims

Considering the major role that DMS can have in both climate regulation and the DMSP-based antioxidant system, there is a need to further examine the fate of demethylated sulphur compounds in coral reef environments under different future climatic scenarios. The aim of the research is to improve knowledge of DMS(P) concentration in *Exaiptasia* (strains CC7 and H2) and their respective symbiotic dinoflagellates (*Symbiodinium linuchae*, clade A4, SSA01 and *Breviolum minutum*, clade B1, SSB01) under temperature-increase. This will help to understand the role of DMS/P as possible antioxidants under future climate change projections and also improve the estimation of DMS reaching the atmosphere, given its importance in climate change. Observing these changes in

symbiotic anemones and in their Symbiodiniaceae cultures alone, will elucidate the possible different antioxidant mechanisms that act when they are in a healthy and damaged (bleached) symbiotic relationship, in contrast to when they are not (Symbiodiniaceae cultures alone). The findings could further help the prediction of the sulphur cycle in a future of climate uncertainties and hopefully improve the current knowledge and understanding of the incredibly complex stress response mechanism in these organisms. Therefore, in order to achieve more knowledge on the subject, Chapter two focuses on the concentration of DMSP in coral *Porites astreoides* on reefs in the Cayman Islands. The concentration of DMSP found in coral will be compared with the one found in macroalgae surrounding the colonies. This will help understand the future concentration of DMSP on coral reefs, given the global trend of decrease coral cover, replaced by algae. Chapter three investigates the effect of higher temperature (max 34°C) on both anemones and Symbiodiniaceae cultures over a 7 days period. This will help understand if both organisms behave the same when alone or in symbiosis. Chapter four focuses instead on the number of Symbiodiniaceae cells that are present in anemones during a higher temperature event (same parameters as chapter 3). A precise count of cells will confirm the importance of Symbiodiniaceae in DMSP production in anemones and add information on the end of the symbiosis between algae and animal during a heat event. Finally, Chapter five aims to enact a real heating event over a three months period. This will elucidate the difference of the effects between a short- and long-term temperature heat experiments on both anemones and symbiotic algae.

Chapter 2 Dimethylsulphoniopropionate (DMSP) concentration in coral *Porites astreoides* and their macroalgal overgrowth in Little Cayman (Cayman Islands)

2.1 Introduction

Dimethylsulphoniopropionate (DMSP) is an organosulphur compound that fulfils multiple roles in ecosystem processes, not solely related to atmosphere and climate (DeBose et al., 2008; Lema et al., 2016; Steinke et al., 2006). DMSP has been suggested to be a multifunctional compound, but the specific physiological role in corals is still unclear (Gardner et al., 2016). Scleractinia corals and their symbiotic dinoflagellates were discovered to produce significant quantities of DMSP (34 – 3841 nmol/cm^2). These high concentrations suggest that coral reefs play a significant role in the sulphur cycle at a local and regional scale (Broadbent et al., 2002). The mucus of healthy coral colonies contains DMSP at concentrations of 1226 – 25443 nM, which is substantially higher than in the surrounding waters (6 – 11 nM) (Broadbent & Jones, 2004; Garren et al., 2014). Lower quantities (about 35% less than in mucus) of DMSP and dimethylsulphide (DMS) have been recorded in the presence of harmful reactive oxygen species (ROS), hence it has been hypothesized that DMSP and DMS may scavenge oxygen radicals and work as antioxidants in cnidarians (Gardner et al., 2016; Krueger et al., 2014; Sunda et al., 2002). DMS plays an important role in global climate, the largest natural flux of sulphur that reaches the atmosphere. DMS could also be the key to future climate predictions since an increase in DMS could

lead to a higher production of cloud-condensation nuclei, possibly enhancing the formation of clouds and therefore cooling as suggested by the CLAW hypothesis (Chapter 1.1, p 12). On the contrary, a decrease could further induce the warming effect by reducing cloud cover.

Another stressor which can lead to a decrease in DMSP in coral reefs is macroalgae overgrowth on corals which leads to coral mortality; it is known that macroalgae generally produce less DMSP per surface area (nmol/cm^2) compared to most corals (Garren et al., 2014). Coral reefs in the Caribbean were dominated by scleractinian corals until the late 1970s to early 1980s, before a shift to an alternative stable state of algal dominance. A change that is representative of the global tropics (Bellwood et al., 2004; Done, 1992; Gardner et al., 2003; Goreau, 1992; Graham et al., 2006; Hughes et al., 2003, 2005; Jackson et al., 2014; Jackson et al., 2001). In the Caribbean, this shift is attributed to the disappearance of the sea urchin *Diadema antillarium*, the keystone algal grazer on Caribbean coral reefs, before a host-specific pathogen decimated their population across the region (Lessios et al., 1984). This event was coupled with the outbreak of coral diseases, particularly the white-band disease (Gladfelter, 1982), causing mass mortality of acroporid corals, the dominant species in the region at that time. The shift was further exacerbated by over-fishing of herbivorous fish stocks (Jackson et al., 2014; Jackson et al., 2001; Jackson, 1997), ending the capacity of coral reefs to function ecologically and instead creating a trophic imbalance (Jennings & Lock, 1996; McClanahan & Mangi, 2000; Micheli et al., 2014).

Corals that are exposed to macroalgae have reduced growth rates by an average of 36.8% due to direct competition but there are considerable intraspecific differences (Vega Thurber et al., 2012).

The following research was conducted in the Cayman Islands (Little Cayman), an archipelago among the most isolated, located in the Northwest Caribbean with low human-population density (Little Cayman counts 328 residents in an area of about 25.6 km^2 = 13 per km^2; Cayman Islands economic and statistics Office, 2016). Furthermore, it is characterized by a lack of riverine systems which minimises possible disturbance from sediment run-off (Burton, 1994). A strict marine no-take legislation has protected the reefs of these islands since 1978, and, since 1986, they have been further protected by a network of actively enforced Marine Protected Areas (Turner et al., 2013). Sadly, the creation of MPAs has not stopped coral reef decline which can be traced back to two major events: the disappearance of the sea urchin *Diadema antillarium* and coral diseases, in particular the white-band disease outbreak in the 1980s (Gladfelter, 1982; Lessios et al., 1984), that contributed to the recurrent bleaching events (i.e. 1979, 1983, 1987, 1991, 1994, 1995, 1998, 2005, 2009) and resulted in substantial coral decline (Gladfelter, 1982; Lessios et al., 1984). The bleaching event of 1998 alone led to a 38% coral mortality and decreased absolute coral cover from 52% to 32% (46.7% reduction in coral cover) with a concurrent increase in macroalgae in Little Cayman (McCoy, 2019).

This study aims to assess concentrations of DMSP in coral *Porites astreoides* and macroalgae because given the future predictions, corals coverage will diminish, and it is important to understand if macroalgae (that will most likely take over the reefs) will provide the same concentration of DMSP in the water. It also

aims to investigate the local differences in DMSP in coral overgrown by macroalgae. Finally, it quantifies group-specific differences in DMSP concentration in macroalgae. A simplistic model to predict the DMSP contribution of algae and corals on a Caymanian reef is also presented.

2.2 Methodology

2.2.1 Study Sites

Corals and macroalgae samples were collected under a research permit grant obtained from the National Conservation Council, Department of Environment, Cayman Islands Government on 20th January 2020. The Cayman Islands are a British overseas-territory consisting of three islands: Grand Cayman, Little Cayman and Cayman Brac. They are centrally located in the centre of the north-west Carribean Sea, between 19° 15' and 19° 45' N latitude and 79° 44' and 81° 27' W longitude. A total of four survey sites were sampled in Little Cayman (Cayman Islands) in February 2020 (Figure 2-1). The study sites were chosen for their similar geomorphological characteristics; two sites were located within existing MPAs (i.e. no taking of any marine life dead or alive; no anchoring – use of fixed moorings only) and two were located outsite but within a 'replenishment zone' where limited protection is occurring (i.e. no taking of conch or lobster; anchor, chain or line must not touch the corals). I wanted to test if the corals/macroalgae within the MPAs (zones with healthier reef, i.e. higher percentage of corals) have higher concentration of DMSP compared to corals/macroalgae located in sites outside the MPAs.

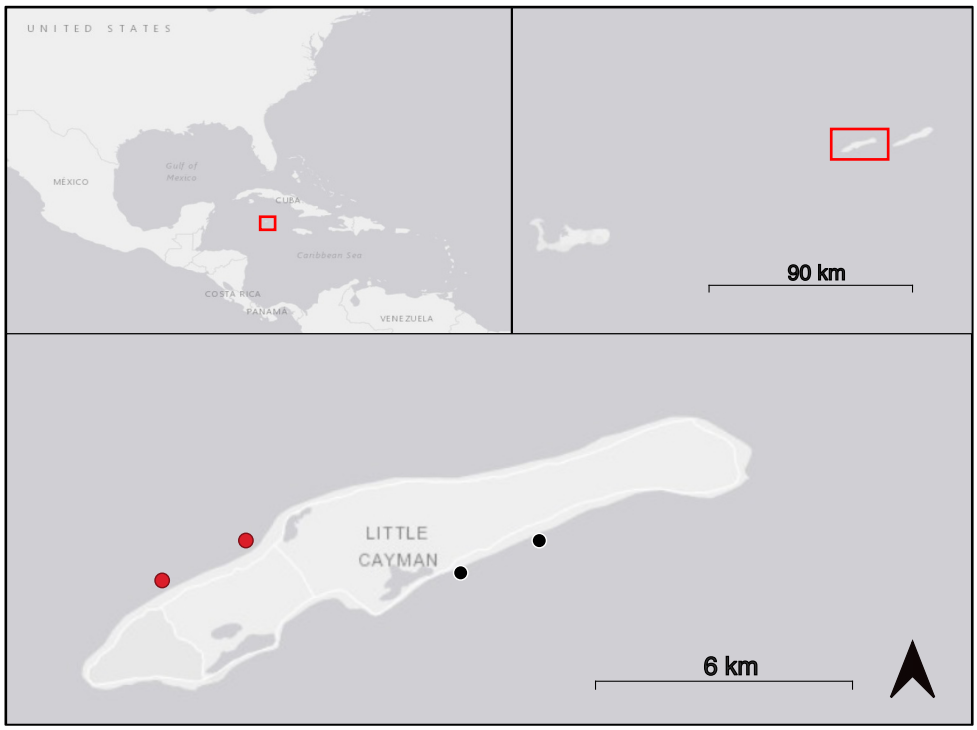


Figure 2-1. Map showing the location of sampling sites. Red dots represent the sites in the Marine Park Zone, while the black dots are the sites in the Replenishment Zone.

Samples were collected at a depth of ~ 12 m at each location, where the water temperature was ~ 28°C and light intensity ~ 6000 lux (approximately 111 µmol m^{-2} s^{-1}) measured by a water-proof temperature and light data logger (Onset HOBO Pendant UA-002-64 deployed at the start of each dive). Dives were conducted on three separate days but with similar environmental conditions, i.e. <5% cloud cover, wind speed of ~18 km h^{-1} easterly wind direction and 1 m waves. The Cayman Islands have weak tideal currents, the average amplitude is 26 cm, therefore mostly wind-driven (Burton, 1994). All dives were conducted between 14:00h and 18:00h local time.

2.2.2 Collection of coral, seawater and macro algal samples

Samples were collected using Self Containing Underwater Breathing Apparatus (SCUBA), the University of Essex health and safety regulations were all followed. Four different colonies of *Porites astreoides* were selected randomly at each site, with a minimum distance of 20 m kept between samples to avoid pseudoreplication. For each of the four chosen colonies, a total of 9 samples were collected: n=3 macroalgae from the edge of the colony (samples were taken randomly, therefore, one to three species of macroalgae were collected), n=3 of coral tissue samples on the edge and finally n=3 coral tissue samples in an area of the coral remote from areas with visibly damaged tissues (Figure 2-2). Four colonies and a total of 9 replicates were sampled due to time restrictions for each dive.

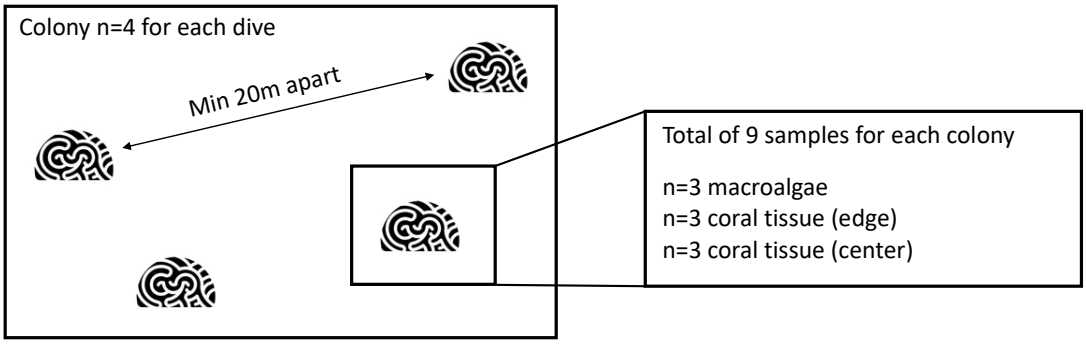


Figure 2-2. Schematic representation of macroalgae and coral tissue samples collection.

Samples were collected as far away as possibile on the same colony whenever possible. Coral samples were taken using a razor blade, scraping an area of approximately 2 cm by 2 cm while the macroalgae were taken from the edge of the live coral (Figure 2-3). Samples were immediately put in pre-labelled zip-lock bags (Figure 2-4). Water samples were collected at each dive site in zip-lock bags at three different depths: 0 m (i.e. surface), 5 m and 10 m (n=3). After each dive and during transport, samples were kept in a chilled cooler, shielded from the sun, and processed within two hours after returning to land. Pictures of macroalgae were taken to assist with later identification.

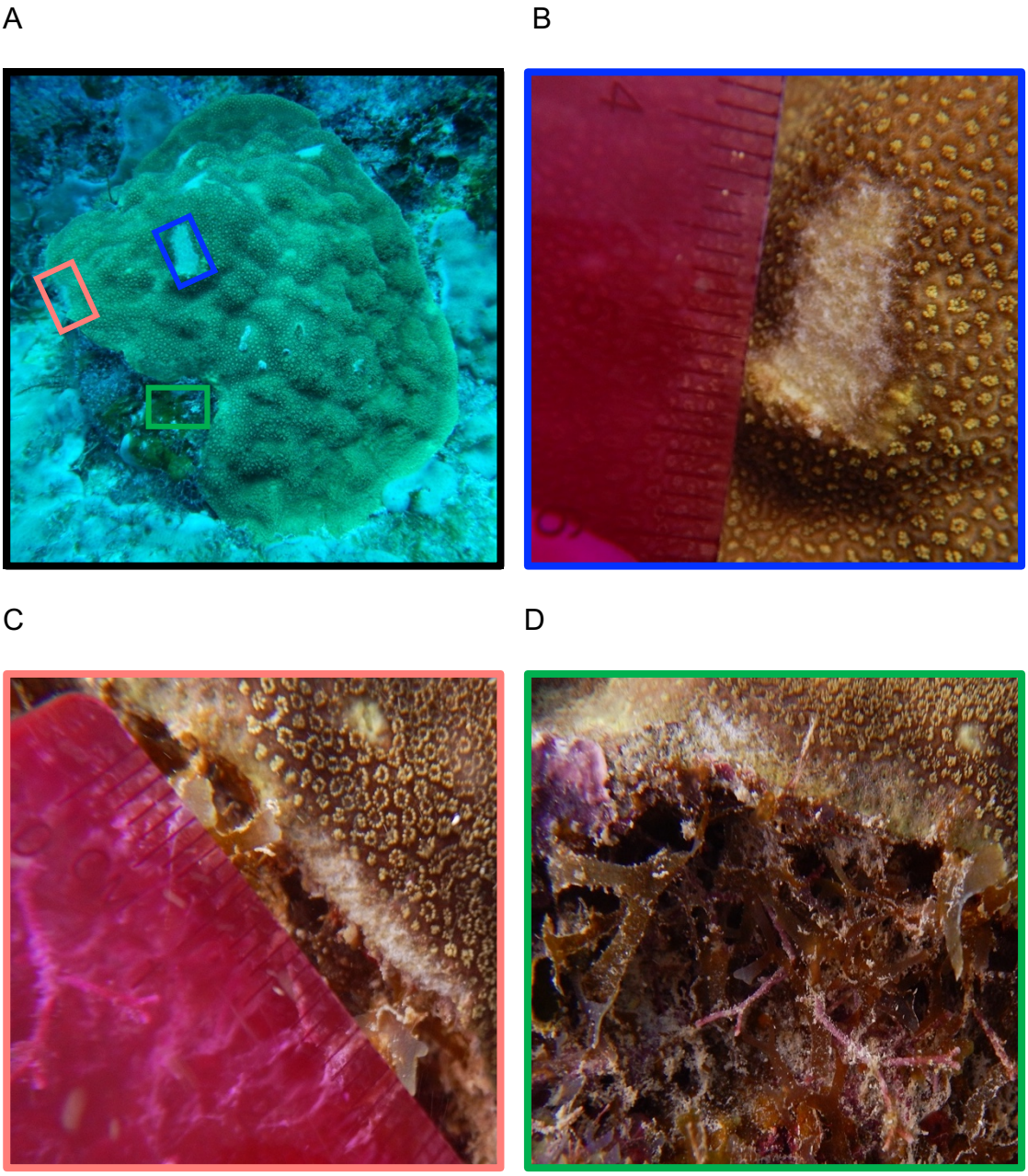


Figure 2-3. Illustration of sample collection. A *Porites astreoides* colony (A) and the location of where the samples were taken from with the respective colours; (B) coral tissue sample at the centre (blue), (C) coral tissue sample at the edge (red) and (D) macroalgae sample growing on the edge of the coral colony (green).

2.2.3 Sample preparation and analysis

Samples were preserved in 3 mL of 0.5 M sodium hydroxide (NaOH), in a 4.95 mL glass vial that was closed gas tightly as soon as the algae/coral was inserted (Figure 2-4). Glass vials were partially filled with the sample, leaving a headspace of 1.92 mL for the analysis of DMS in the gas phase. Water samples consisted of 2850 µL 'whole' seawater and an addition of 150 µL 10 M NaOH (final concentration 0.5 M). Negative controls were prepared with 2850 µL of *f*/2 medium and 150 µL 10 M NaOH upon return to the UK.

Macroalgae were classified into three major groups based on their morphology and colours: brown algae (*Phaeophyceae*), green algae (*Chlorophyceae*), and red algae (*Rhodophyceae*). Macroalgae ID is limited to genus since they were only visually identified from photographs taken during collection. Thanks to the literature available on the most common species present on the Islands, I attempted to also add the most likely species name (Dell et al., 2020; Littler & Littler, 2000; McCoy, 2019; van der Loos, 2021).

Figure 2-4. Example of a coral tissue sample in a Ziplock bag (left) and a coral tissue sample in the process of being preserved in a 4.95 mL vial (right).

DMSP analysis in corals, macroalgae and seawater

DMSP concentration was indirectly quantified after equimolar hydrolytic conversion to DMS in 3 mL of 0.5 M NaOH. All vials were vortexed for 30 s and kept for at least 6 hours at 30°C to allow for equilibration of gases between the aqueous and gas phases in the vial. DMS was then measured using gas chromatography with flame-photometric detection (GC–FPD) as described by Franchini & Steinke (2016). Briefly, a direct headspace injection (200 µL) of the gaseous phase was used to quantify DMS using a gas chromatograph (GC-2010, Shimadzu, Milton Keynes, UK) equipped with a flame photometric detector (FPD) and a 23 m × 0.53 mm × 5 µm HP-1 analytical column (Agilent part number 19095Z-623, Wokingham, UK).

The oven temperature was set at 120°C with a flow rate of 65 cm/min. Carrier gas (He) was supplied at 10.56 mL/min with the flame gases H_2 and air were set at 60 and 70 mL/min, respectively. Injector and detector temperatures were set to 200 and 250°C. Stock solutions with known DMSP concentrations (750 µM, 7.5mM and 75mM) were used to calibrate the instrument. The limit of detection (LOD: 0.9±0.56 nmol) and the limit of quantification (LOQ: 1.2±0.62 nmol) in 3 mL samples were estimated as the DMS concentration corresponding respectively to 3x and 10x the peak height of the chromatogram baseline noise.

2.2.4 DMSP 'model'

Predicting the role of macroalgae and corals in reefs is complex and problematic. Broadbent et al. (2002) attempted to do so by using a simplified procedure whereby the area-normalized concentration of DMSP in corals and macroalgae

was compared to the DMSP in a 15 m deep water column. This model assumes that a square meter's area at the base of the water column is covered 100% by either corals or macroalgae. The model was modified to a 12 m deep water column with the assumption that reefs are degraded with 20% occupied by bare rock/sand/rubble or other organisms different from corals and algae. Different increments were chosen the assess the changes that occur to DMSP in different scenarios.

The values used are based on the samples collected in Little Cayman in this experiment, both MPA and non-MPA. No DMSP concentration value was assigned to 'others', limited literature is available on DMSP concentration in other organisms that are present in a reef, besides corals and macroalgae (e.g. sponges, tunicates, zoanthids) (Van Alstyne et al., 2006). Although more realistic, the model still fails to consider the three dimensional geometry of both organisms. The DMSP concentration is also based only on one species of coral i.e. *Porites astreoides*, which is abundant on these sites but not the only coral present. Given that DMSP concentration varies among species (Broadbent et al., 2002; Van Alstyne et al., 2006), it is important to consider that the model is only using that species' values. In order to have a better accuracy, DMSP concentration of the other most aboundant corals present on Caymanian reefs should be included (e.g. *Montastrea*, *Agaricia* and *Siderastrea*). Such simple calculations only illustrate the possible effect of environmental change on DMSP and, by extension, the DMS produced in future reefs.

2.2.5 Data analysis

Graphical representations and statistical analysis were conducted using R software (R Studio Version 1.4.1717). Linear Mixed-Effect (LME) and General Linear Model (GLM) were chosen instead of a 'classic ANOVA' because they are not limited to two factors with balanced designs. Statistical significance was determined using LME models with random factor (n=3 samples taken within the same coral, algae/coral edge/coral centre) and Tukey HSD post-hoc tests, after the assumptions for each model were verified. The model was run with two factors LOCATION (four levels: Dive1_MPA, Dive2_MPA, Dive1_nonMPA, Dive2_nonMPA), POSITION (three levels: algae, edge, centre) or STATUS (two levels: MPA vs non_MPA). For macroalgae, a GLM was used with COLOR as factors (brown, brown/green, green, red, red/brown, red/brown/green). Due to low replication, factors such as STATUS or LOCATION were not used. The Akaike Information Criterion (AIC) was used to determine the most suitable model to use. Missing samples were classified as 'NA'.

2.3 Results

2.3.1 High concentration of DMSP in coral tissue vs macroalgae

DMSP concentration was significantly different among the sample position (i.e. algae, edge and centre) and the site ($P = 0.012$; Figure 2-5 and Table 2-1). Normalised to surface-area, macroalgae had a significantly lower concentration of DMSP at each site compared to the coral tissue sampled from the edge and the centre of the coral colony. DMSP in algae was on average 12.62 ± 2.18 nmol/cm^2 (range: 9.54 - 16.85 nmol/cm^2), while it was 149.29 ± 12.98 nmol/cm^2 (97.55 - 186.03 ± 23.15 nmol/cm^2) at the coral centre and 128.30 ± 9.42 nmol/cm^2 (99.55 - 156.63 ± 19.17 nmol/cm^2) at the coral edge. There was a significantly higher DMSP concentration in the coral tissue sampled from the centre of the coral colony when compared to the sample taken from the edge at the non-MPA_2 site.

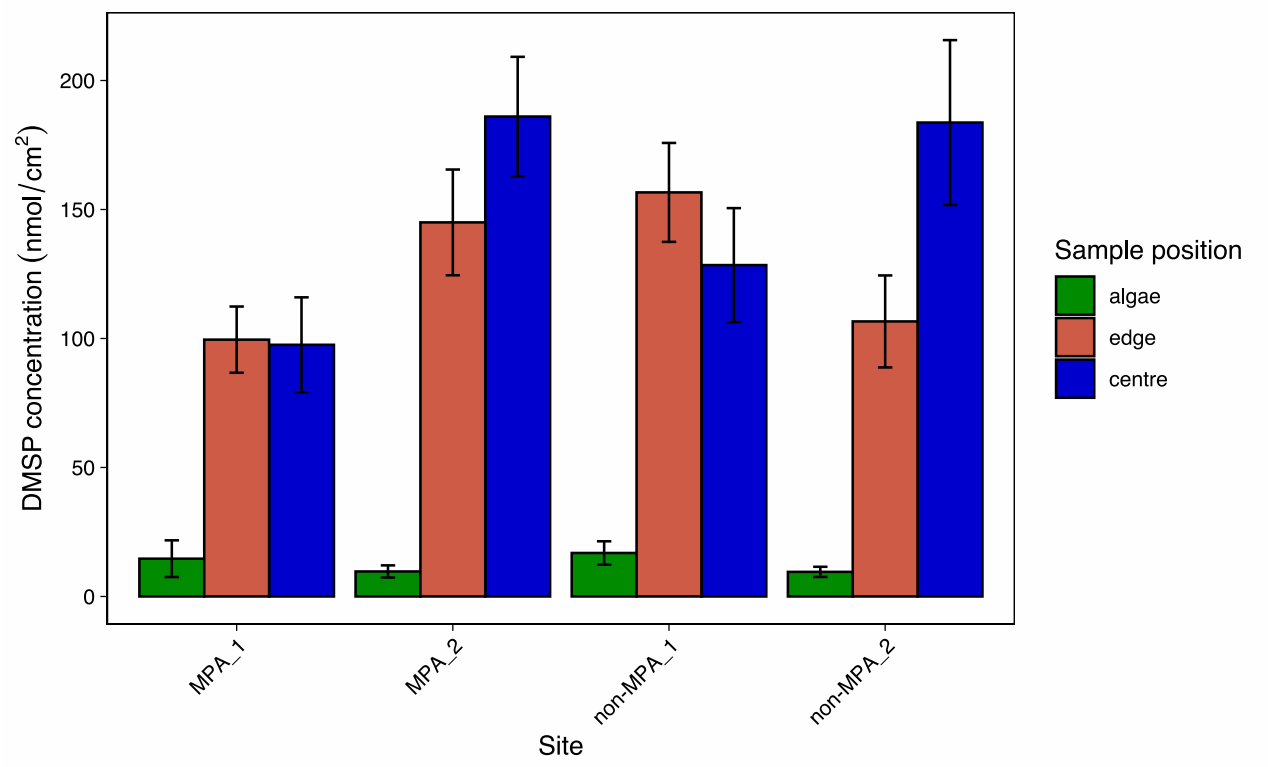


Figure 2-5. DMSP concentration in macroalgae (algae) and coral (edge and centre) at each site sampled. Shown are means±SE (n = 9-12 for each column).

Table 2-1. Summary of significance levels: Post-hoc Tukey's test of the effects of sample position and site of DMSP concentration of macroalgae and coral. The outer layer shows the first variable 'site', the second layer is 'SP' i.e. sample position, where 'A' is macroalgae (green), 'E' is coral edge (pink) and 'C' is coral centre (blue). The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Site		MPA_1			MPA_2			non-MPA_1			non-MPA 2		
	SP	A	E	C	A	E	C	A	E	C	A	E	C
MPA_1	A												
	E	*											
	C	*											
MPA_2	A												
	E	*			***								
	C	**			***								
non-MPA_1	A					*	*						
	E	*			*			***					
	C				*			***					
non-MPA_2	A					*	**		*	*			
	E							*			*		
	C	*			**						**	***	

The location of sampling sites inside or outside of MPAs had no effect on the DMSP concentration and the interaction with the sample position (P = 0.91). DMSP concentration of both MPA and non-MPA sites had a significantly lower concentration of DMSP in macro algae compared to coral tissue, edge and centre (Figure 2-6). Coral tissue at the edge of the colony was not significantly lower in DMSP concentration compared to samples taken in the centre but the trend suggests a decrease in DMSP concentration towards the edge of the coral colony in both MPA and non-MPA sites.

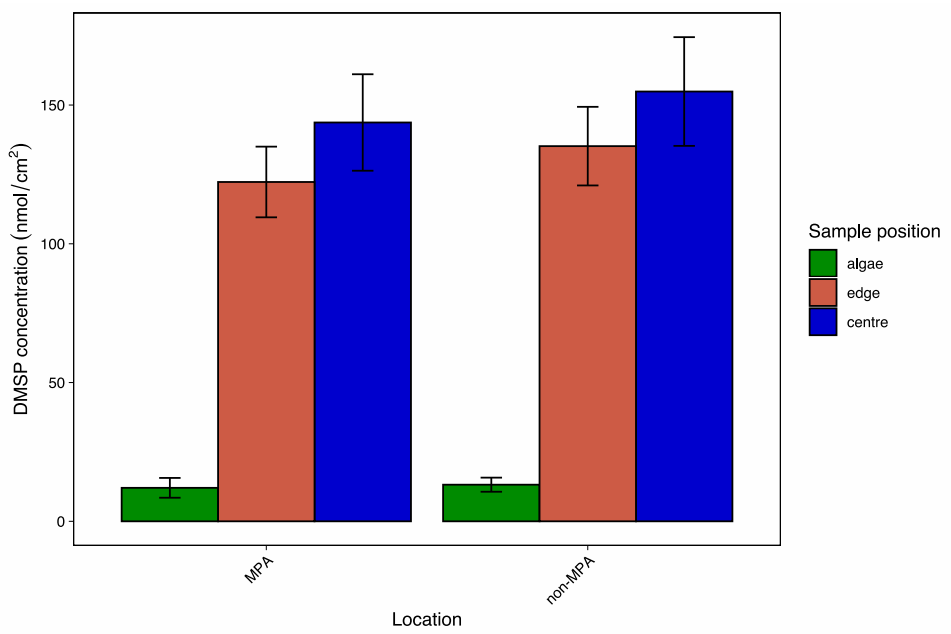


Figure 2-6. DMSP concentration in macroalgae and coral inside and outside of Marine Protected Areas. Shown are means±SE (n = 21-24 for each column).

2.3.2 Lack of DMSP concentration in seawater and scarcity in macroalgae

Seawater DMSP was below the level of detection, 0.9±0.56 nmol.

Where possible, macroalge species names were assigned based on photographic evidence and information from the literature (Kauffmann [web page]; van der Loos, 2021). No further attempts to identify species by, for example, microscopic or molecular techniques were attempted. Based on this approach, a total of five genera were incldued in the analysis (Figure 2-7, Table 2-2):

Table 2-2. Summary of Macroalgae sampled; colour, class, genus and species detailed.

Colour	Class	Genus	Species
Green	*Chlorophyceae*	*Halimeda*	*H. tuna*
Red	*Rhodophyceae*	*Anphiroa*	*A. rigida*
Red	*Rhodophyceae*	*Coelothrix*	*C. irregularis*
Red	*Rhodophyceae*	unknown	unknown
Brown	*Phaeophyceae*	*Dictyota*	unknown
Brown	*Phaeophyceae*	*Lobophora*	*L. variegata*

The majority of samples collected were of *Dictyota* only (n=11), *Halimeda* cf*. tuna* only (n=11) and a mix of red and brown (n=11). Followed by *Lobophora* cf*. variegata* only (n=5), brown and green macroalgae occurred only rarely together (n=3), and the same for a mixture of the three color types (n=3). One sample contained an unidentified red algae (n=1).

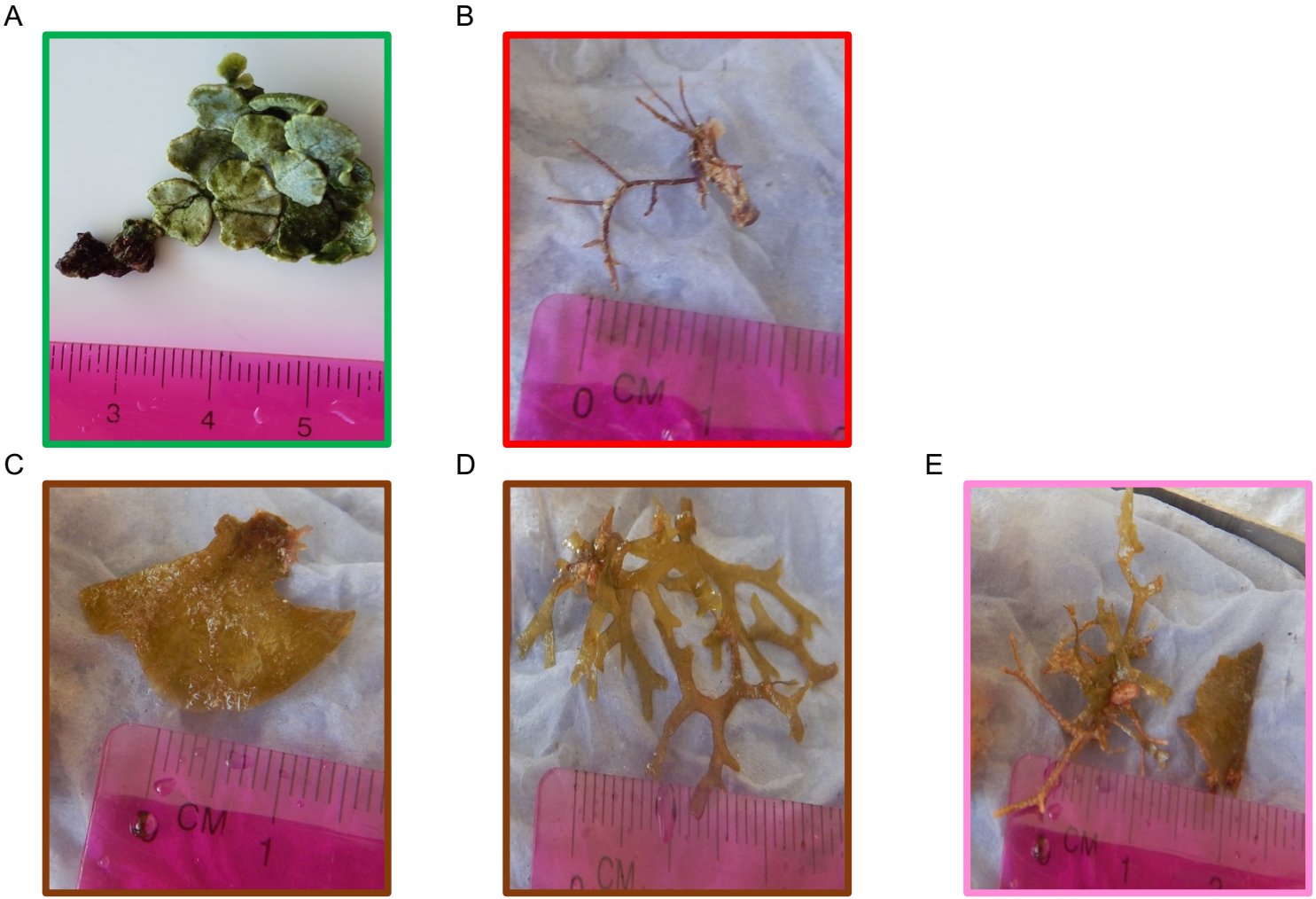


Figure 2-7. Macroalgae samples collected. *Halimeda* cf. *tuna*, the only green algae found (A), *Coelothrix* cf. *irregularis* red algae (B), *Lobophora* cf. *variegate* brown algae (C), *Dictyota* brown algae (D) and a mix of the two brown algae and red algae *Anphiroa* cf. *rigida* (E).

No significant difference in concentration of DMSP was found in the macroalgae sampled based on their color (P = 0.43). The highest concentration of DMSP was found in the red algae *Anphiroa* with 27.12 nmol/cm^2 (n=1), with the lowest being a mix of the three colors with 5.19±3.86 nmol/cm^2 (n=3) (Figure 2-8).

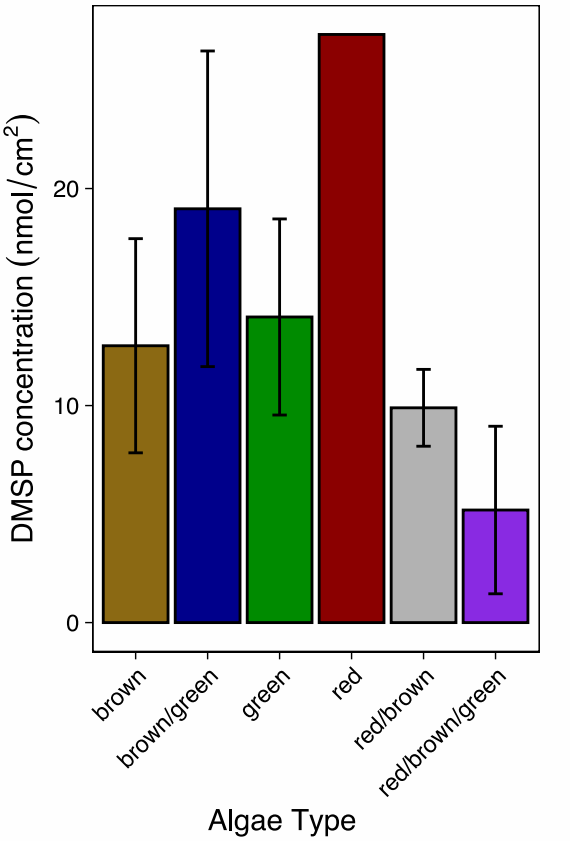


Figure 2-8. DMSP concentration in macroalgae, data are mean (nmol/cm^2±SE), brown n=16, brown/green n=3, green n=11, red n=1, red/brown n=11 and red/brown/green = 3.

When the data from MPA and non-MPA sites was separated, it emerged that non-MPA sites had more of a single species of macro algae (brown only n=6, green only n=6, red only n=1) and only red and brown algae were found together (n=9) (Figure 2-9). Sites within the MPA exhibited a more complex mix of samples (brown and green n=3, red and brown n=2, red/brown/green n=3, brown only n=10, and green only n=5). The DMSP concentration in macroalgae was

statistically the same between MPA and non-MPA, with means (±SE) of 12.06±3.57 and 13.20±2.54 nmol/cm^2, respectively (P = 0.799).

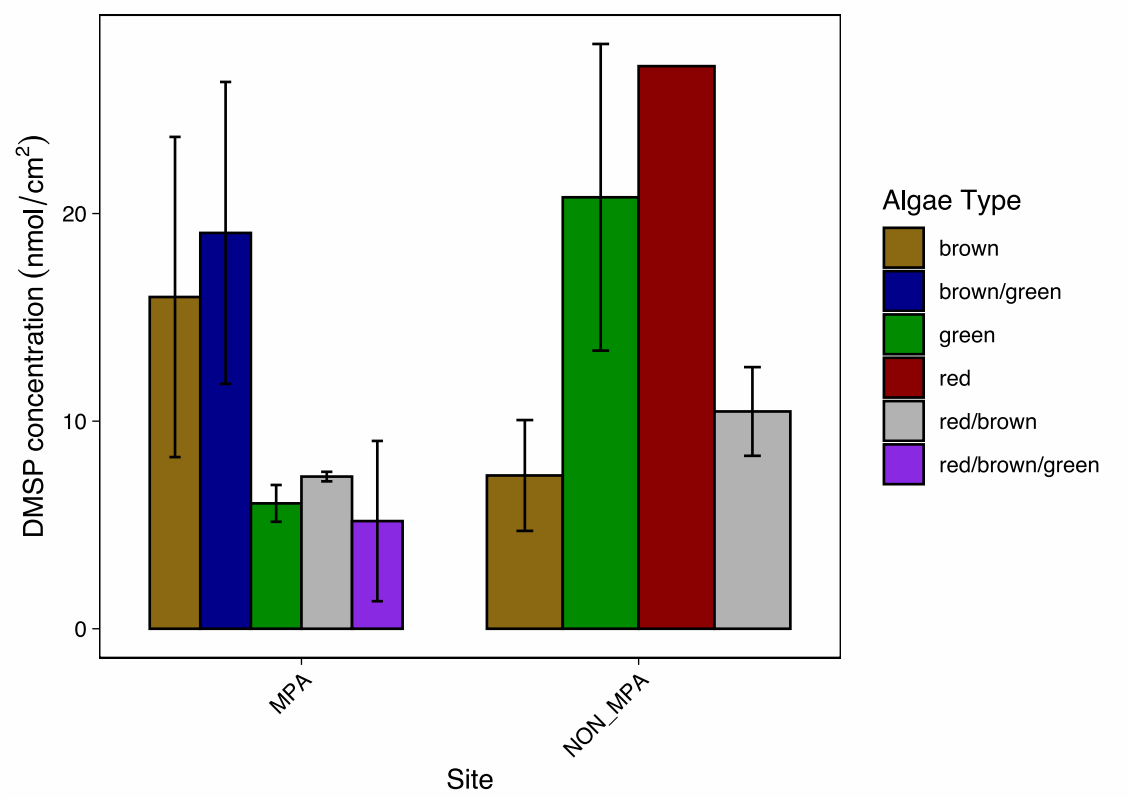


Figure 2-9. DMSP concentration in macroalgae inside and outside of Marine Protected Areas. Shown are means±SE (n = 1-10).

2.3.3 DMSP contribution of macroalgae and corals to Caymanian reef waters

Using data presented here and following the simplistic model, results indicate an estimated contributions of 0 µmol/m^2 from macroalgae and 1112 µmol/m^2 from *P. astreoides* as a best-case scenario, where corals cover 80% of reef, 20% by other organisms and 0% macroalgae present (Table 2-3 and Figure 2-10). More realistically, Caymanian reefs have now a ratio of 20:60:20 (coral:macroalgae:other), which decreases the DMSP contribution to 285.2 µmol/m^2 (calculated using the mean value of coral and macroalgae in this study).

Table 2-3. Comparison of the contribution of macroalgae and corals to the water column of DMSP (µmol/m^2)

Ratio 'corals'	DMSP Coral	Ratio 'algae'	DMSP algae	Ratio 'other'	DMSP 'other'	TOTAL DMSP
80	1112	0	0	20	0	1112
70	973	10	1.2	20	0	974.2
60	834	20	2.4	20	0	836.4
50	695	30	3.6	20	0	698.6
40	556	40	4.8	20	0	560.8
30	417	50	6	20	0	423
20	278	60	7.2	20	0	285.2
10	139	70	8.4	20	0	147.4
0	0	80	9.6	20	0	9.6

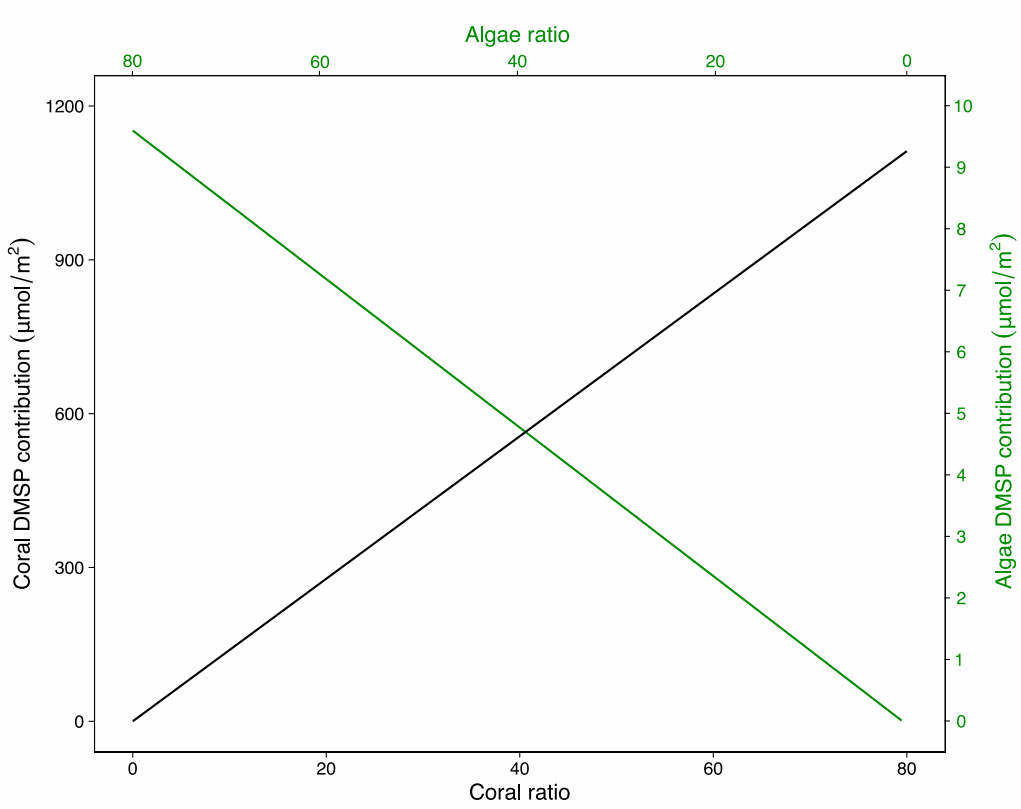


Figure 2-10. Comparison of the contribution of macroalgae and corals to the water column DMSP. DMSP is expressed in (µmol/m^2 ⇔ µmol/12 m^3), axes have different scales (coral in black and macroalgae in green)

2.4 Discussion

DMSP is a source of DMS and this can affect cloud formation and, hence, climate. With ongoing climate and environmental changes including the loss of Caribbean coral reefs and the switch to a macroalgae-dominated alternative stable state, it is important to relate changes in biodiversity and community composition to the potential of DMS production in future coral reef ecosystems.

2.4.1 The importance of healthy corals and reefs for a high DMSP concentration

Contact interactions between corals and macroalgae are common along the margins of coral colonies (Lirman, 2001). In Florida, corals had more than 50% of their basal perimeter in contact with macroalgae during a peak of algal abundance. In the Great Barrier Reef, a staggering 92% of corals surveyed had algal interfaces (Tanner, 1995). Corals can be classified based on their growth form into branching, pillar, table, elkhorn, foliose, encrusting, massive and mushroom corals. Given the higher perimeter-to-area ratio of encrusting, massive and mushroom corals, compared to species with a higher three-dimensionality (e.g. branching or table), they are more affected by macroalgae interference (Hughes, 1989; Tanner, 1995). *Porites astreoides*, an encrusting species, demonstrated a significant decrease in growth rates, three to four times lower, once herbivorous fish were prevented from cropping and controlling algae in the area (Lirman, 2001). A further decrease was noted when additional macroalgae were introduced in the caged treatment of the corals, in order to simulate a higher macroalgae cover (Lirman & Biber, 2000). These studies highlight how macroalgae can affect growth in corals such as *P. astreoides*. Hence, increased macroalgal abundance and consequent decreased growth, can further decrease

coral percentage cover on coral reefs. Removing herbivorous fish, resulting in concomitant increase in macroalgae, will have a deleterious effect on global change. The current increase in sea-surface temperatures, the trend of higher coral mortality, and consequent macroalgae establishment will be concurrent with major changes in the amount of DMSP produced in tropical reefs. This study confirms that macroalgae have a significantly lower concentration of DMSP than coral, thus highlighting the importance of assessing the changes that a shift to macroalgae-dominated reefs have on the sulphur biogeochemistry of tropical coasts. Broadbent (2002) has calculated that the contribution of DMSP to the water column is 15 μmol/m^2 from benthic algae and 2220 μmol/m^2 from corals, supporting the finding in this investigation. This highlights the importance of abundant and healthy corals for a high concentration of DMSP in the waters surrounding coral reefs.

It is recognised that the presence of macroalgae near coral colonies and the resulting competition for resources alters the physical environment and can trigger disastrous changes in coral-associated microbial communities (Vega Thurber et al., 2012). Since microbial processes can be intricately linked with the production of DMSP and/or DMS (Yoch, 2002), such changes could also affect the potential for DMSP production, a poorly explored aspect of coral biology. In view of the well-documented increasing abundance of macroalgae due to climate change, disturbance and pollution, interactions among macroalgae, corals and microbes together with resulting changes in the sulphur biogeochemistry of tropical coasts may be important for shaping the ecology of future coral reefs (Vega Thurber et al., 2012).

2.4.2 Macroalgae are confirmed a small source of DMSP in coral reefs

Dictyota spp. and *Lobophora spp.* were numerically abundant in samples investigated here and are known to be a more prevalent species in the Caribbean (de Ruyter van Steveninck & Bak, 1986; Gaubert et al., 2019; Lesser & Slattery, 2011; Mumby et al., 2005). In April 2017, an assessment conducted in the Cayman Islands reported a coral cover of 9% and 12% with a macroalgal cover between 40% and 53%, Grand Cayman and Little Cayman respectively. In this investigation, the prevalent species were *Dictyota* spp. (> 20%), followed by *Lobophora variegata* (12% to 16%) (Dell et al., 2020). *Halimeda* spp. were recorded as abundant in 2014 in several locations in Little Cayman, where six different species were collected (Peach et al., 2017). *Halimeda* spp. and *Dictyota* spp. were the macroalgae that were most often found being in direct contact with coral colonies, probably due to their almost dominance within the species samples, 77-99% (Lirman, 2001; Lirman & Biber, 2000).

In agreement with previous macroalgae surveys, DMSP was found in all algal species sampled in this study, and concentrations of DMSP were variable among the taxa (Broadbent et al., 2002; Chudek et al., 1987; Dacey et al., 1994; White, 1982). Broadbent et al. (2002) recorded the lowest concentration in *Turbinaria* spp. 1.61 μmol/m^2 and the highest in *L. mollucense* 29.55 μmol/m^2 . The findings by Dacey et al. (1994) range between 0.04 μmol g^{-1} fresh weight in *Caulerpa sertularoides* and 1.4 μmol g^{-1} fresh weight in *Dictyospheria cavernosa*.

This range in DMSP in macroalgae has been explained by different factors, like the abundance of epiphytes (Dacey et al., 1994), physiological conditions (Matrai & Keller, 1994) and the light intensity changes they are subjected to due to shading (Karsten et al., 1991; Levasseur et al., 1994). DMSP concentration in

Halimeda tuna from the Great Barrier Reef was 1.797 nmol/cm^2 (Broadbent et al., 2002) and this is in agreement with the data for *Halimeda* cf*. tuna* reported here (n= 11, 14.08±4.51 nmol/cm^2). Normalised to tissue area, the DMSP concentration in macroalgae was significantly lower compared to coral tissue in this study.

3.4.3 Conclusions

The importance of healthy corals and coral reefs ecosystems is evident for a steady and abundant concentration of DMSP. Events leading to the complete dominance of macroalgae on coral reefs will lead to a further decline in DMSP, and consequent DMS effect. This chapter clearly proves the sharp reduction in DMSP concentration if corals are to be replaced by macroalgae. MPAs do not seem to have a positive influence on the concentration and this should further stress the need to protect corals reefs. Future research should focus on the concentration of DMSP in other corals and macroalgae mostly present in coral reefs. Also, it needs to be taken into account other organisms and substrates that are less popular, for example sandy patches and rubble, especially in degraded reefs. With more research a more accurate model could be produce and therefore a better prediction for future changes.

Chapter 3 The effects of short-term high temperature stress on Symbiodiniaceae and *Exaiptasia*

3.1 Introduction

Climate change has a variety of complex effects on algal communities. Tropical reefs depend on a symbiotic relationship between coral animals and dinoflagellates (several genera in the family Symbiodiniaceae) that live in a symbiotic relationship with their host. Detrimental effects on either host or animal can be crucial for the survival of the whole ecosystem (Hoegh-Guldberg et al., 2007). This symbiosis is important because Symbiodiniaceae provide energy to the host animal through photosynthetic products (e.g. glycerol, glucose, amino acids, fatty acids, lipids), while the host provides inorganic nutrients and shelter to the symbionts (Davy et al., 2012). Their association allows them to live in harmony, allowing coral reefs to continue being an ecological and economical asset. The termination of this favourable symbiotic association is referred to as bleaching (Weis, 2008). Increasing water temperature, irradiance and ocean acidification are recognised as being the main causes for more frequent bleaching events and mortality of reefs globally (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007). Because of increased light and temperature (and other factors like pollution, sedimentation, low tides), Symbiodiniaceae can suffer from oxidative stress when the amount of reactive oxygen species (ROS) is unbalanced and overwhelms existing ROS-removal (antioxidant) processes. Oxidative stress has been identified as one of the major causes of bleaching, and the production of ROS is known to induce the processes that lead to the expulsion of Symbiodiniaceae from their host (Lesser et al., 1990; McLenon & DiTullio,

2012). In comparison to the amount of information on the cause of bleaching, little is known regarding both the molecular and cellular mechanisms that drive the establishment, maintenance and the breakdown of this symbiosis (Weis, 2008; Davy et al., 2012). It is recognised that the symbionts and the host can use the enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) to convert and, hence, scavenge ROS enzymatically (McLenon & DiTullio, 2012). High concentration of DMSP may also function as an antioxidant in the coral/anemone holobiont, by directly reacting with harmful ROS. Moreover, DMSP's enzymatic cleavage produces DMS and acrylate, compounds that are even more effective in scavenging ROS, yielding the DMS-oxidation products dimethylsulphoxide (DMSO) and methane sulphinic acid (MSNA) (Sunda et al., 2002) (Chapter 1.6.2, p. 35). DMS is a well-known and studied gas principally thanks to the CLAW hypothesis (Charlson et al., 1987), that suggests a negative feedback loop from the biogenic production of DMS and its conversion to sulphate aerosols that act as cloud condensation nuclei (CCN). These CCN limit the solar radiation on the Earth's surface and therefore have a role in regulating the climate (Chapter 1.1, p. 12). Coral bleaching caused by heat stress is at least partially due to the photobleaching of photosynthetic pigments in Symbiodiniaceae (Takahashi et al., 2013). It has been suggested that photoprotection by xanthophylls (e.g. chlorophyll *a* and c_2) might be a key factor in preventing coral bleaching caused by high seawater temperature and irradiance (Ambarsari et al., 1997; Brown, 1997). Chlorophylls *a* and c_2 together with peridinin are the major light-harvesting pigments in most dinoflagellate species that bind to two major antennae proteins. Takahashi et al. (2008) suggest that the variation in the photobleaching sensitivity of different Symbiodiniaceae to

thermal stress is strongly influenced by the differences in sensitivity of these antennae proteins to higher temperatures. Photobleaching associated to heat-stress follows severe photoinhibition of the photosystem II (PSII), with the extent of the damage being a result of the imbalance between the rate of photodamage to PSII and the rate of its repair (Takahashi et al., 2008).

Chapter 2 investigated the diversity of DMSP concentrations in corals from the genus *Porites* and associated macroalgae samples at a site in the Caribbean (Cayman Islands). Here, we utilise the availability of distinct clades of the symbionts *Symbiodinium linuchae* (clade A) and *Breviolum minutum* (clade B), and two clonal cultures of *Exaiptasia pallida* (CC7 with a clade A symbiont and H2 with a clade B symbiont) to investigate the possible functioning of DMSP and DMS when coral reef organisms experience temperature-induced stress.

These two clades were chosen because they are the natural symbionts of CC7 and H2, respectively. Another reason was that they have a different thermal resistance, *Symbiodinium linuchae* is thought to be thermotolerant while *Breviolum minutum* is not.

We are testing three main hypotheses within this heat-stress experiment (Figure 3-1):

H1 Under increasing temperature (26°C to 34°C) the growth (cell concentration and cell growth) and concentration of chlorophyll-*a* and chlorophyll-c_2 of Symbiodiniaceae will decrease.

H2 Maximum photochemical efficiency in both Symbiodiniaceae and anemones will decrease in thermally stressed specimens (26°C to 34°C).

H3 DMSP/DMS concentration in thermally stressed (26°C to 34°C) Symbiodiniaceae and anemones will increase.

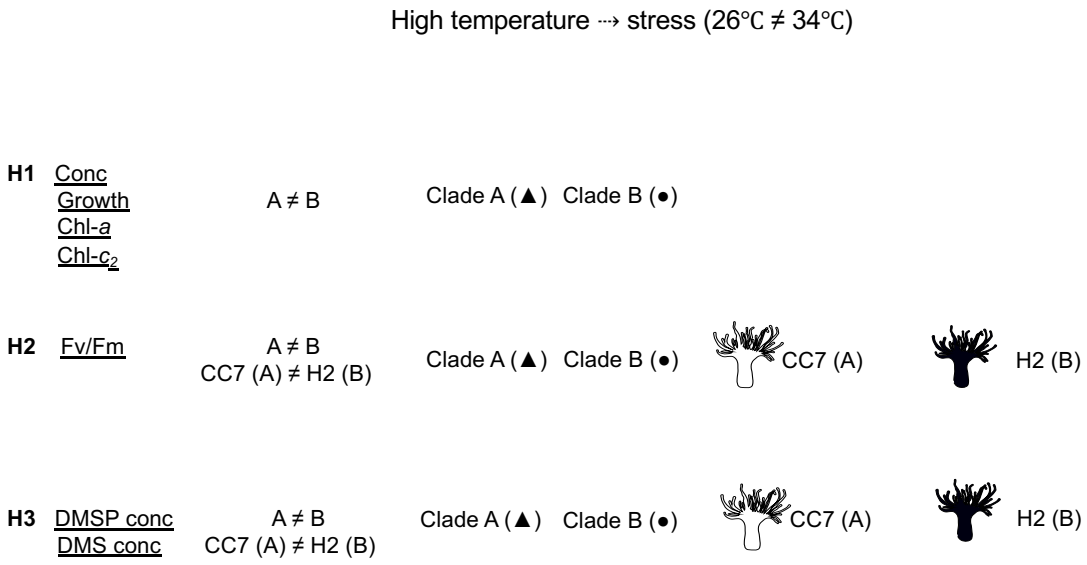


Figure 3-1. Schematic representation of the three main hypotheses. (H = hypothesis, A = Clade A - *Symbiodinium linuchae*, B = Clade B - *Breviolum minutum*, CC7 (A) = *Exaiptasia* CC7 (inoculated with Clade A), H2 (B) = *Exaiptasia* H2 (inoculated with Clade B).

Given the known thermotolerance difference, clade A is expected to survive better at higher temperature than clade B. The same is assumed for the anemones and their respective symbiotic partners (CC7 containing clade A algae will be more thermo-tolerant than anemone H2 containing clade B algae), although differences are expected compared to the Symbiodiniaceae cultures. Anemones could protect the algae from stress or detect it as a hindrance and expel them to avoid excess ROS.

3.2 Methodology

3.2.1 Maintenance of algal stock cultures

Cultures of two different clades of Symbiodiniaceae dinoflagellates were examined: SSA01 *Symbiodinium linuchae*, clade A4, isolated from *Exaiptasia* strain CC7 and suggested to be thermotolerant (i.e. able to tolerate the high temperature, 32°C), host origin: Florida (USA) and SSB01 *Breviolum minutum*, clade B1, isolated from *Exaiptasia* H2 suggested to be less thermotolerant compared to SSA01 (Swain et al., 2017), host origin: Hawaii (USA). Both species were obtained from King Abdullah University of Science and Technology (Saudi Arabia) in February 2019, where they were originally received from the Pringle Lab at Stanford University, USA (Ahmed et al., 2019) and were kept axenic at both institutes by using sterile techniques while being handled but also with the aid of antibiotics. Since the scope of the research is to investigate DMSP/DMS dynamics which are heavily influenced by bacterial activity (Howard et al., 2008), I have discontinued the axenic cultivation of the strains and then confirmed the presence of bacteria before starting the experimentation (DNA analysis confirmed the presence of bacteria). Briefly, both cultures and anemones' DNA

was extracted using 'DNeasy Blood & Tissue Kit' (Qiagen) and universal bacterial primers (27f/1492r) [27f sequence: AGA GTT TGA TCM TGG CTC AG, 1492r sequence: GGT TAC CTT GTT ACG ACT T] were used to assess the presence of bacteria in all samples (n=3 for each culture and anemone strain). The PCR reaction mixtures were incubated at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and 72°C for 2 min, and a final extension of 72°C for 10 min. The cleaned PCR product was sent for sequencing and the obtained sequences were searched using BLAST (Basic Local Alignment Search Tool).

Stock cultures were maintained in *f*/2-si (Medium *f/2* without silica, as in (Guillard & Ryther, 1962)) made with artificial sea water following (Berges et al., 2001). They were kept in 500 mL conical flasks in a controlled-temperature and -light incubator (SANYO MLR-351, Sanyo Electric Co., Osaka, Japan) at 26°C under 12:12h light:dark regime (~90 µmol photons m^{-2} s^{-1}, from a 30 W Osram 'Lumilux Cool Daylight' light). Light measurements were taken in water and using the same vessel that the cultures were also grown in, with a handheld light meter (LI-250A, Li-COR Inc., Biosciences, Lincoln, NE, USA), with a spherical underwater sensor (LI-193, Li-COR Inc., Biosciences, Lincoln, NE, USA).

3.2.2 Experimental design

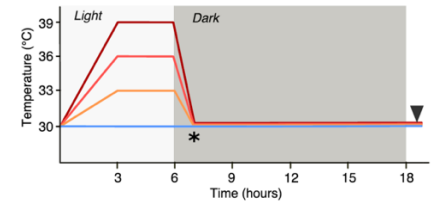
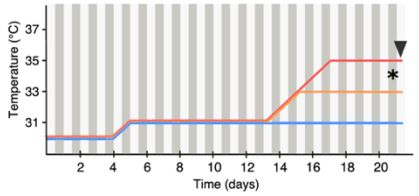
Four separate experiments were carried out at different times but using identical conditions: Experiment #1 took place from the 14th September to the 22nd September 2020 (SSA01 *Symbiodinium linuchae*), experiment #2 was conducted from the 01st October to the 09th October 2020 (SSB01 *Breviolum minutum*) and experiment #3 from the 04th February to the 12th February 2021 (*Exaiptasia*, both

CC7 and H2). An additional experiment (#4) was carried out from the 30th April to the 7th May 2021 (*Exaiptasia*, both CC7 and H2).

Logistical constraints required a staggered protocol for the experimentation: culture samples for the experiments were inoculated with stock cultures at a dilution of 1:10 (50 mL stock culture plus 450 mL medium in 1 L flasks) on the 21st day of growth for controls or on the 22nd day of growth for treatments (i.e. when the stock culture was in stationary growth), and biologically replicated four times (n=4 for control, treatment & *f*/2 control for both control and treatment). Both clades were grown into the onset of exponential growth under control conditions (i.e. 26°C) for 5 days. On day 5 of growth and day 0 of the experiment, experimental treatment flasks were moved to a different incubator (LMS, model 300) set at 27°C with the identical light:dark regime and similar light intensity (light from two 30 W Osram 'Lumilux Cool Daylight', samples were systematically placed in the incubator in order for them to receive ~90 µmol photons m^{-2} s^{-1}) as the control treatment. The temperature was gradually increased to 34°C over the 4 successive days and kept at 34°C until the end of the experiment (Figure 3-2). Heat stress experiments have control temperatures ranging from 25°C to 30°C, treatment temperatures ranging from 31°C to 39°C and they last hours to days, it is clear that there is no standardized method (Table 3-1). I have chosen 26°C as control temperature because it is the temperature both Symbiodiniaceae and *Exaiptasia pallida* were previously acclimated to (their optimal temperature) and 34°C as treatment temperature since it seems the most probable temperature in future decades. The IPCC has recorded total thermal stress for the period 1981-2010, in which the highest monthly sea surface temperature (SST) was used to define the thermal threshold, above which accumulated thermal stress was

calculated as Degrees Heating Month (DHM) as the running total over four consecutive months. The average anomalies range from 1 to 5°C h. SST projections for 2050 are a 1-2°C increase and a further 1-2°C increase for 2010 (IPCC, 2014). Temperatures in Florida and Hawaii, where *Exaiptasia* are originally from, are already reaching 32°C during summer months, I therefore decided to use 34°C. Other studies have also used this maximum temperature, over a different time period.

Table 3-1. Example summary of different temperature profile in heat stress experiments. 'Ctrl' is the control temperature, 'treatment' describes the temperature profile and the maximum temperature reached, 'Time' the hours/days for each temperature increase (or total duration of experiment when 'total' in brackets) and 'organism' the studied organism.

Ctrl	Treatment	Time	Organism	Reference
25°C	32°C and 34°C	1 h (total)	Symbiodiniaceae and anemones	(Goulet et al., 2005)
27°C	33°C	~ 6 days (total)	Symbiodiniaceae	(McLenon & DiTullio, 2012)
27°C	27°C to 34°C (over ~12 h)	4days (total)	anemones	(Tolleter et al., 2013)
28°C	28°C to 31°C (1°C x day) 31°C	4days 3days	coral	(Deschaseaux et al., 2014)
27°C	27°C to 32°C	4h (total)	coral	(Gardner, et al., 2017b)
27°C	27°C to 30°C (1°C x day) 30°C to 32°C (0.5°C x day) 32°C	4days 4days 7days	coral	(Gardner, et al., 2017a)
30°C		18 h (total)	coral	(Voolstra et al., 2020)
30°C		21 days (total)	coral	

Samples were placed randomly on different shelves, swirled daily and moved among the incubator's shelves to ensure that all replicates experienced similar light intensities integrated over the growth period.

In reporting Symbiodiniaceae results below, samples are referred to as 'control' (samples kept at 26°C for the entire growth and experimental period) and 'treatment' (samples exposed to higher temperature during the experimental period). On the 10th February 2021 an unplanned power cut occurred and temperatures in both control and treatment decreased to 15°C overnight (temperature at 09.47 pm was 34.1°C, reached its lowest reading at 08.56 am with 15.4°C and increased back to 34.2°C at 02.22 pm). Because of this unplanned power cut and in an attempt to boost amount of data collected, an additional experiment with *Exaiptasia* (#4) was carried out from the 30th April to 7th May 2021 (given the lack of anemones, data were recorded for day 7 and only for treatment) but once again a power cut occurred on the 3rd May, with temperature decreasing to 23.8°C (temperature at 06.32am was 31.7°C, reached its lowest reading at 10.22 am with 23.8°C and reached back 32.1°C at 11.47 am).

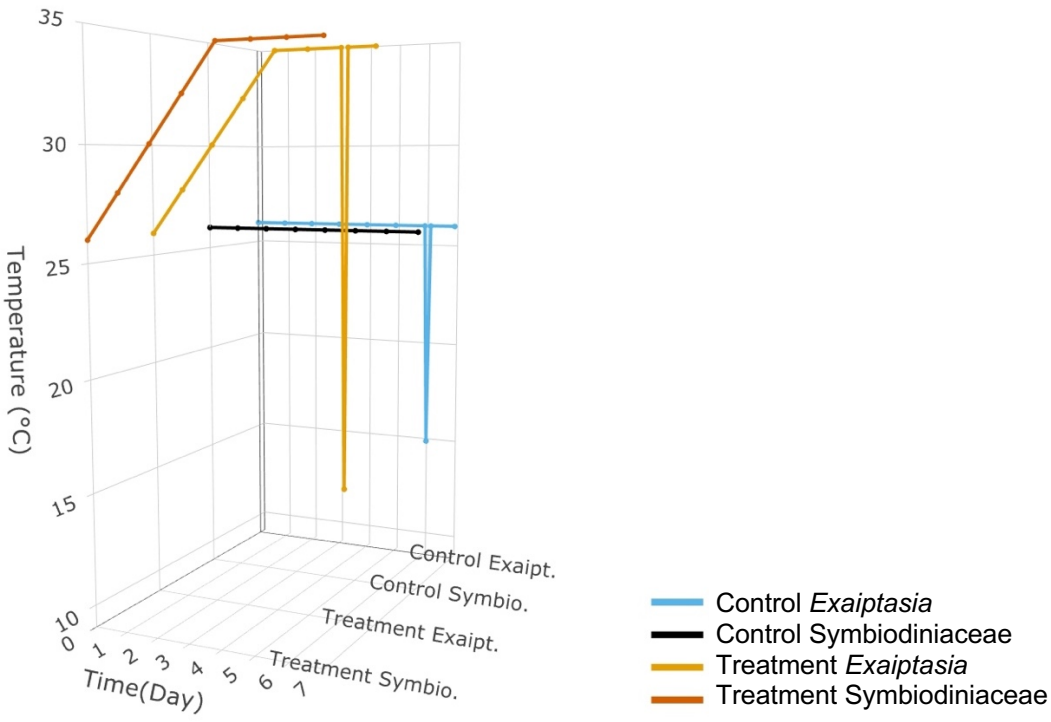


Figure 3-2. Temperature settings during Experiments 1&2 (i.e. Symbiodiniaceae) and 3 (i.e. Exaiptasia). Shown are 'Control Exaiptasia' (blue), Control Symbiodiniaceae (black), 'Treatment Exaiptasia' (yellow) and 'Treatment Symbiodiniaceae (red). Note that on day #5/6 of the Exaiptasia experiment, the temperature in the incubator dropped to 15°C due to power cut in the laboratory overnight, both control and treatment were affected.

3.2.3 Growth and volume of algal cultures

Cell densities of cultured samples of the two Symbiodiniaceae clades were counted microscopically using a haemocytometer (Neubauer Improved). Algae were resuspended by gentle swirling to ensure an even distribution and an aliquot of 500 µL was harvested between 10.00 h and 10.30 h local time and fixed with the addition of 15 µL Lugol's Iodine solution. Cell densities were calculated using six replicate counts from each aliquot, counting 5 squares representing a volume of 0.1 mm^3 each.

Additionally, semi-automated microscopy was performed with a Leica DMI6000B inverted light microscope equipped with a Leica DFC310FX camera and the acquisition and device control were performed by Leica AF6000 Modular Systems (LAS AF) v4.6 (Leica Microsystems CMS GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany). For this purpose, a 96-well plate without the lid was placed on a multi-well plate stage insert, filled with an aliquot of fixed sample (200 µl each) described above. Cells were first examined through the eyepiece under 50× magnification using brightfield illumination in order to have a clear view of the stained specimen. The camera was then switched to a 100× magnification to obtain a live view of the specimen on the LAS AF screen and the focus was adjusted to attain the highest image quality possible. Automated scanning with autofocus was conducted for each of the 96 wells along a 3×5 rectangular pattern to acquire 15 image tiles for each well and the images were acquired with an automated scanning of the pattern with autofocus. All images were saved as bitmap files at highest possible at a resolution of 1392×1040 pixels and kept in a directory with each sample having its own folder. FIJI software was used to process and analyse the microscope image data (Schindelin et al., 2012). The

processing was automated using a script written in ImageJ Macro programming language (Edullantes, 2020). The script was executed one sampling date at a time in the macro interface in FIJI and the spreadsheet data produced in FIJI were processed and analysed in R version 3.6.1 (RStudioTeam, 2020). The total number of cells encountered in each image were used to calculate the growth rate, while the cells' width and the length were used to calculate the volume of the cell, using an ellipsoid formula (Equation 1):

$$V = \frac{4}{3} \pi \, a \, b \, c \qquad \text{(Equation 1)}$$

Where a, b and c are the three radii calculated from the length (a) and the width (b, c) measurements of the cells. The 'Leica method' gave a 2D output, while the formula requires 3D data, therefore these adjustments had to be made.

Growth rates of algal populations were determined from the change in cell number, using the following equation after (Weiler & Eppley, 1979):

$$\mu = \frac{\ln(N)_{t1} - ln(N)_{t0}}{t_1 - t_0} \qquad \text{(Equation 2)}$$

Where μ is the specific growth rate (d^{-1}), and N_{t1} and N_{t0} are the number of cells at times t_1 and t_0 (d).

Although two different techniques were used to count the cells (I was initially unaware of the possibility to use the 'Leica method'), for the main text of the chapter the 'Leica method' method was used, because it proved to be more reliable (amount of sample used for counting was ~63.5 μL with 'Leica method' vs < 5 μL with haemocytometer). Data using the haemocytometer method are reported in the Appendix (Figure 0-2) and demonstrate that the standard error is

smaller when using the Leica method especially for strain SSB01. Using the Leica method also provided the opportunity to explore the cell volume, an important parameter which could not be quantified form haemocytometer counts.

3.2.4 Spectrophotometric quantification of algal chlorophyll-a and -c^2

To quantify algal chlorophylls, the procedures described in Ritchie (2006) were followed. Briefly, 10 mL of culture was filtered onto a glass-microfibre filter (GF/F, 25 mm diameter, 0.7 µm typical particle retention, GE Healthcare Life Science, Buckinghamshire, UK). The filter was folded and placed in a 15 mL centrifuge tube (Thistle Scientific, Glasgow, UK) containing 4 mL of 90% ethanol and stored at -20°C in darkness for up to 4 weeks. On the day of chlorophyll quantification, samples were centrifuged at 4500 × *g RCF* for 10 min at 4°C. Chlorophyll was quantified using spectrophotometric absorbance (A) measurements using 1 mL of the sample supernatant. Absorbance wavelengths were set at 629 and 665 nm, as suggested for dinoflagellates. Chl-*a* was calculated using Equation 3:

$$\text{Chl-a (µg/mL)} = -2.6094 \times (A_{629}) + 12.4380 \times (A_{665}) \times \frac{extraction\ volume\ (ml)}{sample\ volume\ (ml)}$$

(Equation 3)

While Chl-c_2 was calculated using Equation 4:

$$\text{Chl-}c_2\ \text{(µg/mL)} = 29.8208 \times (A_{629}) - 5.6461 \times (A_{665}) \times \frac{\text{extraction volume (ml)}}{\text{sampe volume (ml)}}$$

(Equation 4)

3.2.5 *Exaiptasia* husbandry and biomass estimation

Exaiptasia strain CC7 and H2 (kindly provided by Dr. Nick Aldred, University of Essex, UK) were kept at similar growth conditions as for the Symbiodiniaceae (i.e. at 26°C under 12:12h light:dark regime, ~ 80 µmol photons m^{-2} s^{-1}, from a 37 W fluorescent light), in 1 L plastic containers filled with 800 mL autoclaved artificial seawater (ASW) made by mixing artificial salt (D-D H_2Ocean) with reverse-osmosis water to a salinity of 35, pH 8. Animals were target fed twice a week with 'Cyclops – gamma blister' (Urmston Aquatics, Manchester, UK) that had been previously washed with RO water and autoclaved. Water was changed approximately 5 h after each feed and containers cleaned weekly from algae, with the aid of cotton swabs.

In order to estimate the biomass of the anemones, animals were anaesthetised using a 1:1 mixture of autoclaved ASW and 0.37 M $MgCl_2$ and placed under a dissecting microscope equipped with an eyepiece graticule. A non-linear model was applied to calculate anemone dry weight from oral-disk diameter measurements (Clayton & Lasker, 1985):

$$Y = 0.124\, Z^{1.49} \qquad \text{(Equation 5)}$$

where Y is the dry weight (DW) in mg and Z is the oral disk diameter in mm.

3.2.6 Quantification of DMSP and DMS

DMSP was indirectly quantified after equimolar hydrolytic conversion to DMS using gas-chromatographic analysis. For samples of Symbiodiniaceae, 150 µL of 10 M NaOH (final concentration 0.5 M) was added to an aliquot (2850 µL) of the respective algae culture (clades A and B) in a gas-tight 4.92 mL vial and closed immediately with a Teflon-coated silicone septum. *Exaiptasia* were placed in a vial containing 3 mL of 0.5 M NaOH. Negative controls were prepared with 2850 µL of *f*/2-si medium for Symbiodiniaceae, and 2850 µL of artificial sea water for *Exaiptasia* and NaOH (150 µL of 10 M). All vials were vortexed for 30 s and kept for at least 6 hours at 30°C to allow for equilibration of gases between the aqueous and gas phases in the vial. A different technique was used for DMS concentration 'in-vial purging'. Symbiodiniaceae were filtered (see 3.2.4) and 3 mL of the filtrate was collected in a 4.92 mL vial, *Exaiptasia* was simply kept in the same type of vial with 3 mL of sterile artificial sea water (vials were closed gas-tightly 48 h before analysis was conducted. Given the low concentration of the gas, one needle was injected in the vial and was immersed in the aqueous phase, while the other stayed in the headspace. The aqueous phase was purged with nitrogen gas for 10 min at 30 mL/min and condensed into the cryotrap to be desorbed. DMS was then measured using gas chromatography with flame-photometric detection (GC-FPD) after direct injection of 200 µL of headspace into a gas chromatograph (GC-2010, Shimadzu, Milton Keynes, UK) equipped with a 23 m × 0.53 mm × 5 µm HP-1 column (Agilent part number 19095Z-623, Wokingham, UK) with an oven temperature of 120°C and a flow rate of 65 cm/min. Carrier gas (He) was supplied at 10.56 mL/min and the flame gases H_2 and air are set to 60 and 70 mL/min, respectively. Injector and detector

temperatures were set to 200 and 250°C. Stock solutions with known DMSP concentrations (750 µM, 7.5 mM and 75 mM) were used for calibration of the instrument [details of methodology in (Franchini & Steinke, 2016)].

3.2.7 Fast Repetition Rate fluorometry (FRRf)

For FRRF measurements on Symbiodiniaceae, an aliquot of 2 mL of culture was taken from the flask they were grown in and placed in a clean vial, while *Exaiptasia* were directly measured in a 15 mL glass vial in 3 mL of artificial seawater. Keeping the anemone in the vial used for analysis has prevented the disturbance of the animals; water was changed daily and care was taken that no algae grew on the surface of the vial during the experimental period. For measurements, each vial was inserted into an FRR fluorometer (Chelsea Technologies Group, Molesey, UK). Photosynthetic efficiency (F_v/F_m) was calculated by the FastPro 8 software (version 1.0.55, CTG Ltd.) used to attain and record FRRf measurements using Equation 6.

$$F_v/F_m = (F_m - F_0)/F_m \quad \text{(Equation 6)}$$

With F_0 = minimum fluorescence and F_m = maximum fluorescence yield. Peak excitation was set at 435 nm and fluorescence emission measured at 680 nm (with a 25 nm bandwidth). The measuring protocol was set to 24 sequences per acquisition with a 100 ms sequence interval and a 20 s acquisition pitch. Both algae and animals were dark acclimated for 1 h at their respective temperature before each measurement was taken, as in previous studies.

3.2.8 Statistical analyses

Graphical representations and statistical analyses were conducted using R software (R Studio Version 1.2.5042). Statistical significance was determined using GLMM (General Linear Mixed Models; including negative binomial and Gaussian) and Tukey HSD post-hoc tests, after the assumptions for each model were tested. The model was always run with three factors DAY (four levels: 0, 2, 4, 7), TEMPERATURE (two levels: control, treatment), CLADE (two levels: a, b) or ANEMONE (two levels: CC7 vs H2). If the model with three factors was not significant, the factor 'CLADE'/'ANEMONE' would be removed and the model would be run again, having the two clades/species separate. Akaike Information Criterion (AIC) was used to determine the best model to use.

3.3 Results

3.3.1 Symbiodiniaceae differ under heat stress

Cell concentration using 'Leica method' are shown in Figure 3-3 and Table 3-2. SSA01 started to show a decline at day 4 and ending at day 7 with a significantly lower density of cells compared to control (means ± standard error = 382972 ± 15377 and 531911 ± 22097 cell/mL respectively), while SSB01 concentration declined at day 2 and it kept a stable concentration until the end of the experiment, having a similar cell density compared to control at day 7. The interaction of temperature, strain and day had a significant difference ($GLM_{Day*Temperature*Clade,}$ $P = 0.0005$).

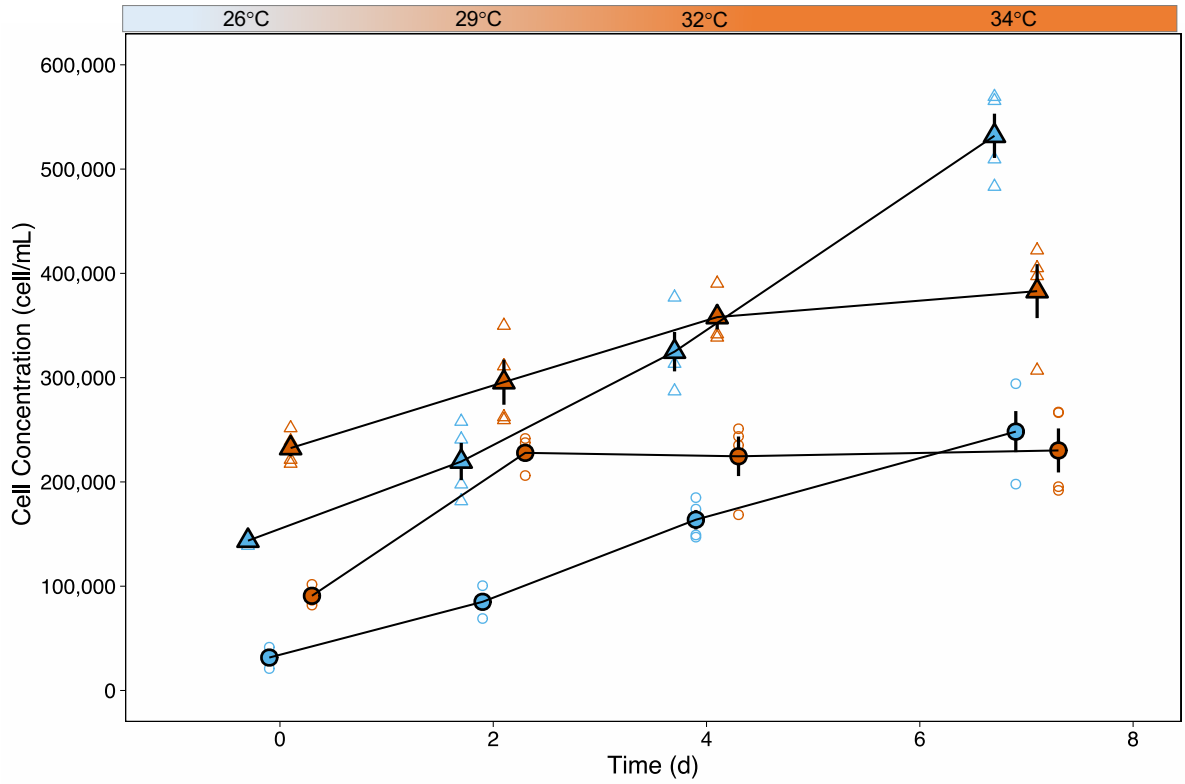


Figure 3-3. Cell counts of *Symbiodinium linuchae* SSA01 (triangles) and *Breviolum minutum* SSB01 (circles), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=4. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-2. Summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade on cell count for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2	***															
		4	***	***														
		7	***	***	***													
	34	0	***		**	***												
		2	***			***	***											
		4	***	**		**	***	*										
		7	***	**		*	***	**										
B	26	0	***	***	***	***	***	***	***	***								
		2		**	***	***	***	***	***	***	**							
		4			***	***	*	**	***	***	***	**						
		7	*			***				*	***	***	*					
	34	0	***	***	***	***	***	***	***	***	***		*	***				
		2	**		*	***			**	**	***	**			***			
		4	*		*	***			**	**	***	**			***			
		7				***			*	*	***	**			***			

Cell growth rate in SSA01 was constant in control cultures at around 0.2 d^{-1} for the entire experiment but in treatment cultures showed a continuous decline from 0.12±0.06 d^{-1} to 0.02±0.07 $d^{-1}$with increasing temperature (if not stated otherwise, reported data represent mean ± standard error). SSB01 had a higher growth rate in control samples compared to SSA01 (0.51±0.11 d^{-1}) and treatment samples exhibited a strong decrease in growth from day 2 to 4 and also at the last day in treatment cultures, ending with no cell growth (Figure 3-4, Table 3-3). Although less pronounced, the growth rate also decreased from 0.5 d^{-1} to 0.14 d^{-1} in the control. The interaction of temperature, strain and day had a significant difference ($LME_{Clade * Temp * Day}$, P = 0.0008).

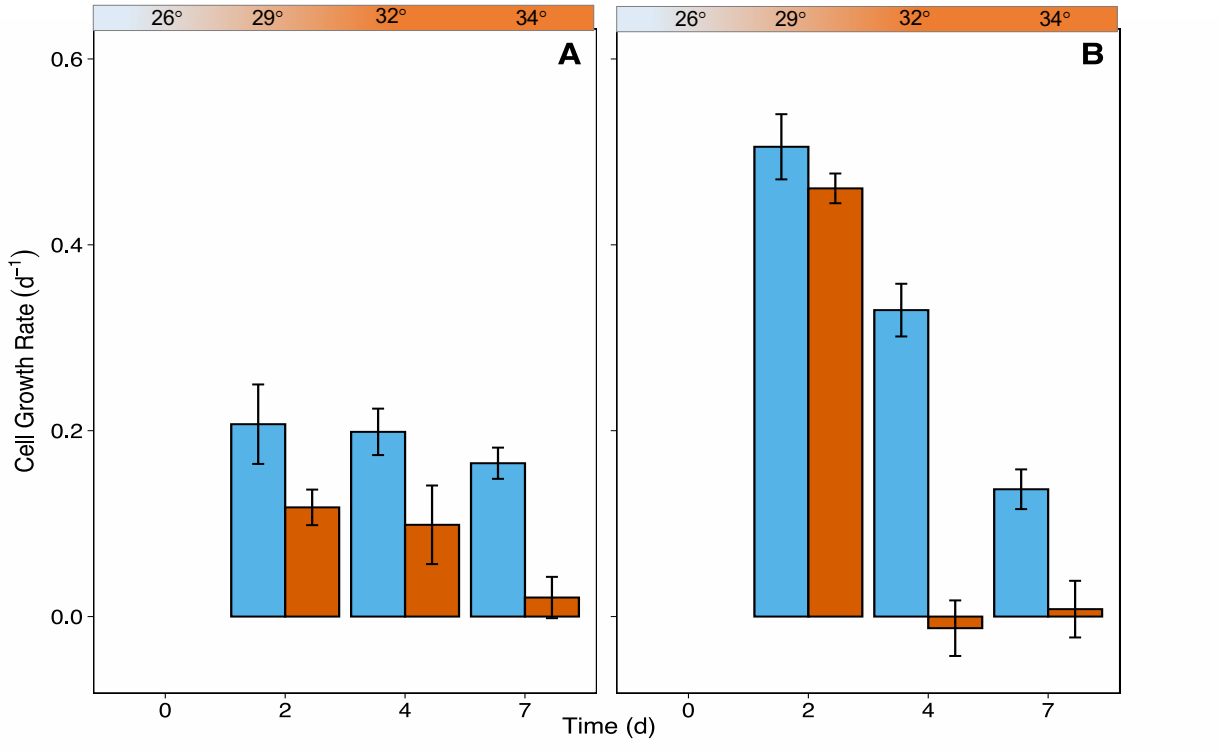


Figure 3-4. Cell growth of Symbiodinium linuchae SSA01 (A) and Breviolum minutum SSB01 (B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red), mean±SE. Cell growth derived from Fig 3-3.

Table 3-3. Summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade on cell count for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2	***															
		4	***															
		7	**															
	34	0		**	**	*												
		2																
		4																
		7		**	*													
B	26	0		**	**	*												
		2	***	***	***	***	***	***	***	***	***							
		4	***	**		*	***	**	**	***	***	***						
		7									*	**	***					
	34	0		**	**	*						***	***					
		2	***	***	***	***	***	***	***	***	***			***	***			
		4		**	**	*						***	***	*		***		
		7		**	**							***	***			***		

Cell volume showed a similar pattern between the two species, whereby treatment recorded a steady decline from day 0 to day 4 but a sharp increase in volume at day 7 (Figure 3-5 and Table 3-4). For both species the interaction between temperature and day had a significant difference, SSA01 ($LME_{Temp * Day}$, $P < 0.0001$) and SSB01 ($LME_{Temp * Day}$, $P < 0.0001$).

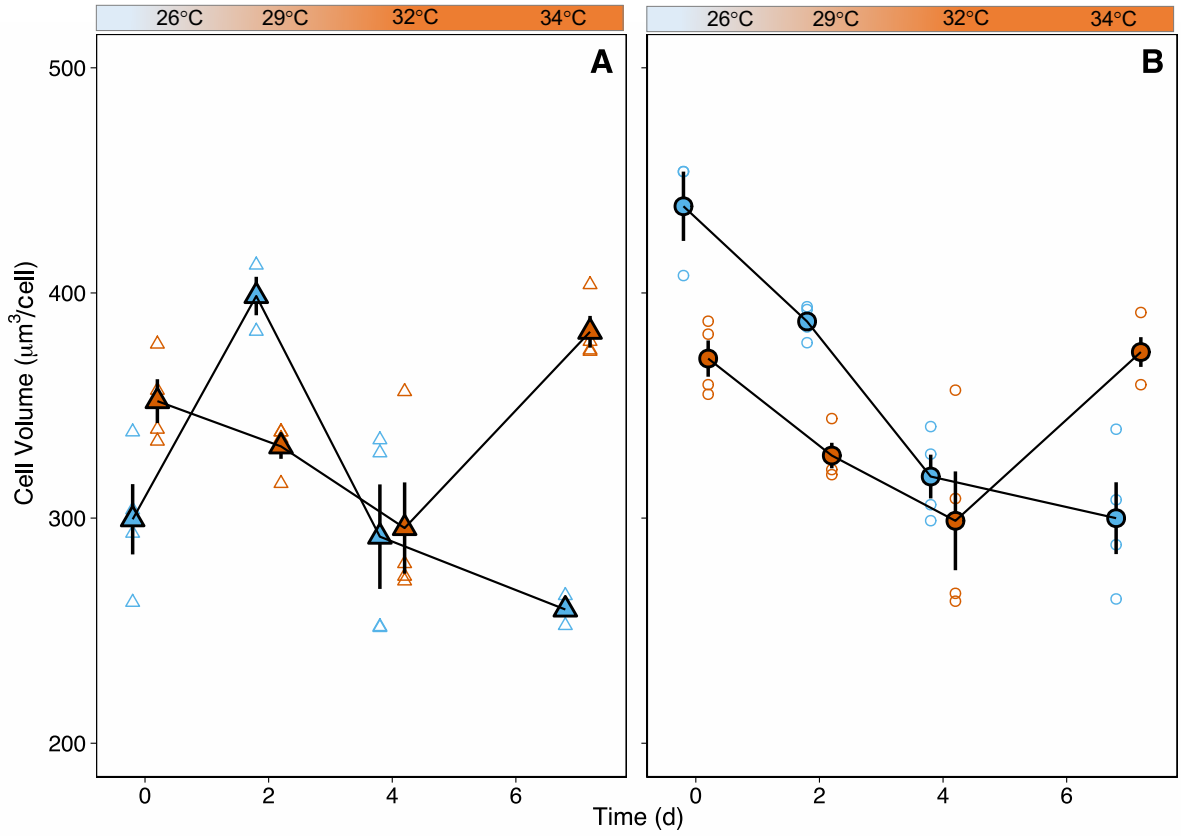


Figure 3-5. Cell volume of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-4. Summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade on cell volume for clades SSA01 (A) and SSB01 (B). The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

A

Clade			A							
	Temp [°C]		26				34			
		Day	0	2	4	7	0	2	4	7
A	26	0								
		2	***							
		4		***						
		7	***							
	34	0				***				
		2				*				
		4								
		7	*			***		***	*	

B

Clade			B							
	Temp [°C]		26				34			
		Day	0	2	4	7	0	2	4	7
B	26	0								
		2								
		4		***						
		7		***						
	34	0								
		2		**						
		4		**						
		7								

Chlorophyll *a* and c_2 had an almost identical pattern within the same clade, the differences were among the two clades. Chlorophyll *a* was significantly different among clade, day and treatment (P = 0.0005), showing very similar values for SSA01 at ~2pg/cell. SSB01 had a higher chlorophyll content from day 0 and slowly decreased to also reach ~2 pg/cell at day 7 (Figure 3-6, Table 3-5 and Table 3-6). The same pattern was observed in Chlorophyll c_2 by both the algae clades (P = 0.0005).

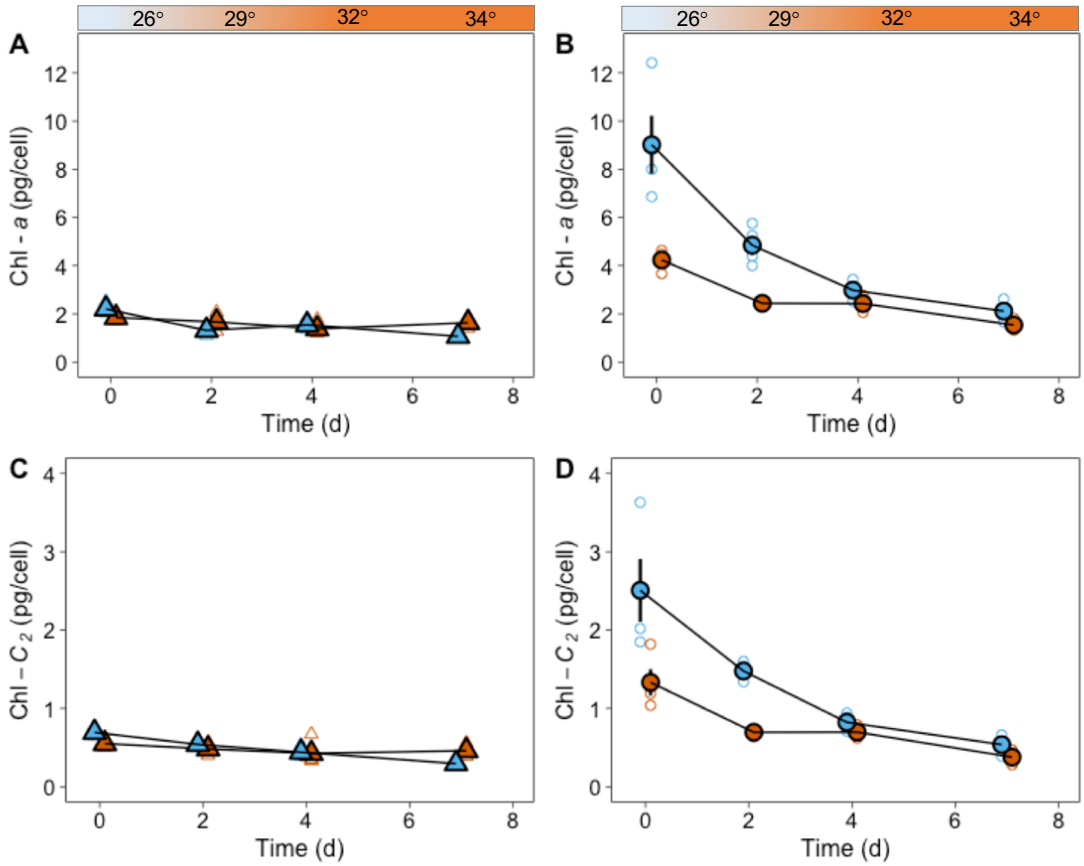


Figure 3-6. Chlorophyll *a* and c_2 of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-5. Chlorophyll *a* summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7																
	34	0																
		2																
		4																
		7																
B	26	0	***	***	***	***	***	***	***	***								
		2		***	***	***	*	***	***	***	***							
		4		*	**	***		*	***	***	***	***						
		7				**					***	***	***					
	34	0				*			*		**							
		2				*					***	***			*			
		4				***			*		***	***						
		7									***	***	***		*		***	

Table 3-6. Chlorophyll c_2 summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '****' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7																
	34	0																
		2																
		4																
		7																
B	26	0	**	***	***	***	**	***	***	***								
		2		***	***	***		***	***	***	**							
		4			*	***		*	*	**	***	***						
		7									***	***	**					
	34	0				*												
		2				***					***	***						
		4				**					***	***						
		7									***	***	**		*	**	**	

Thermal stress treatment samples in SSA01 had a higher F_v/F_m at day 0 and 2, but showing a steady decline throughout the experimental period and treatment was significantly lower than the control by day 7 (Table 3-7). Maximum photochemical efficiency was significantly different across temperature, day and clade ($LME_{day*temperature*clade}$, P = 0.01) (Figure 3-7). In SSB01, the decline in treatment was similar between control and treatment but by day 7, similarly to SSA01, there was a sharp decline and treatment had a significantly lower F_v/F_m. F_v/F_m values for SSA01 were all above or ~0.4, with the exception of treatment at day 7, while for SSB01 values were all below 0.4 and reaching ~0.3 for treatment day 7.

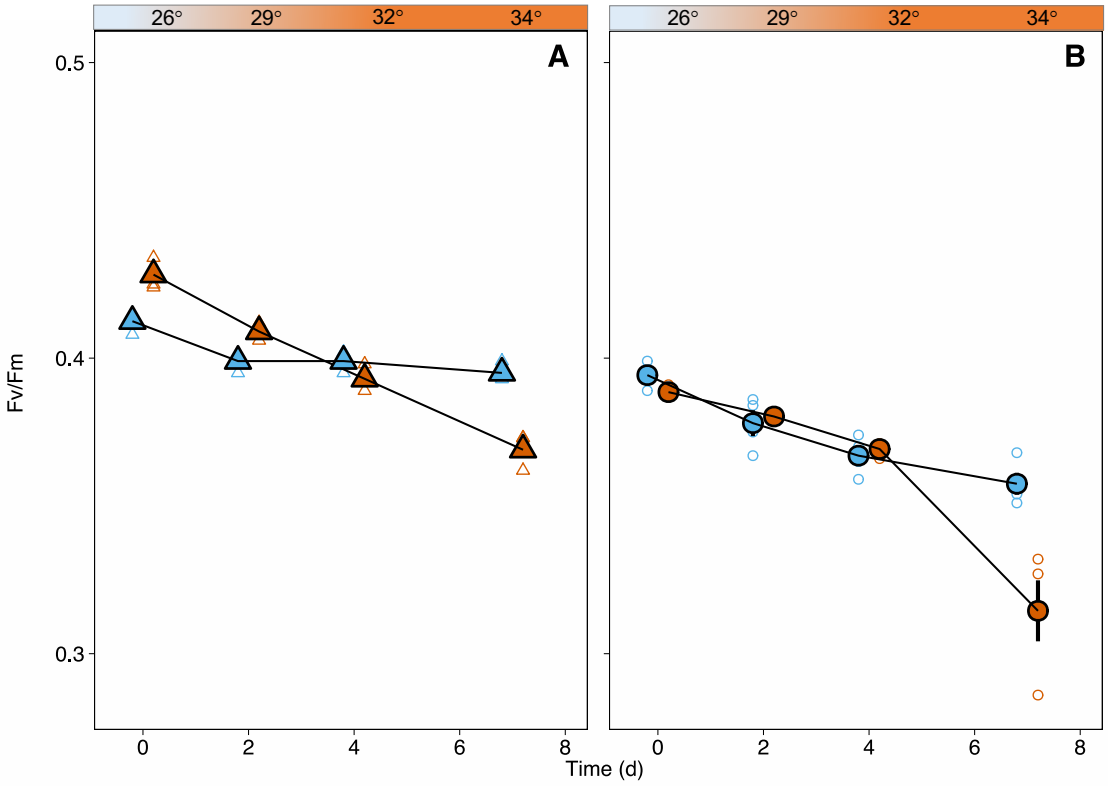


Figure 3-7. Maximum photochemical efficiency of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-7. Maximum photochemical efficiency summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' P ≤ 0.05, '**' P ≤ 0.001, '***' P ≤ 0.0001, empty white cells P ≥ 0.05 and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7	*															
	34	0		**	**	***												
		2					**											
		4					***											
		7	***	**	**	**	***	***	***									
B	26	0					***			**								
		2	***	*	*		***	**										
		4	***	**	**	**	***	***	**		***							
		7	***	***	***	***	***	***	***		***	**						
	34	0	*				***	*					*	**				
		2	**				***	**						*				
		4	***	**	**	**	***	***	*		*				**			
		7	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	

DMSP concentration in both clades was significantly different ($P < 0.0001$), SSA01 had a generally constant concentration below 100 fmol/cell, while SSB01 had a higher concentration (~200-400 fmol/cell) at the beginning of the experiment but the concentration decreased to ~100 fmol/cell by day 7. This pattern is almost identical to the chlorophyll (Figure 3-8 and Table 3-8).

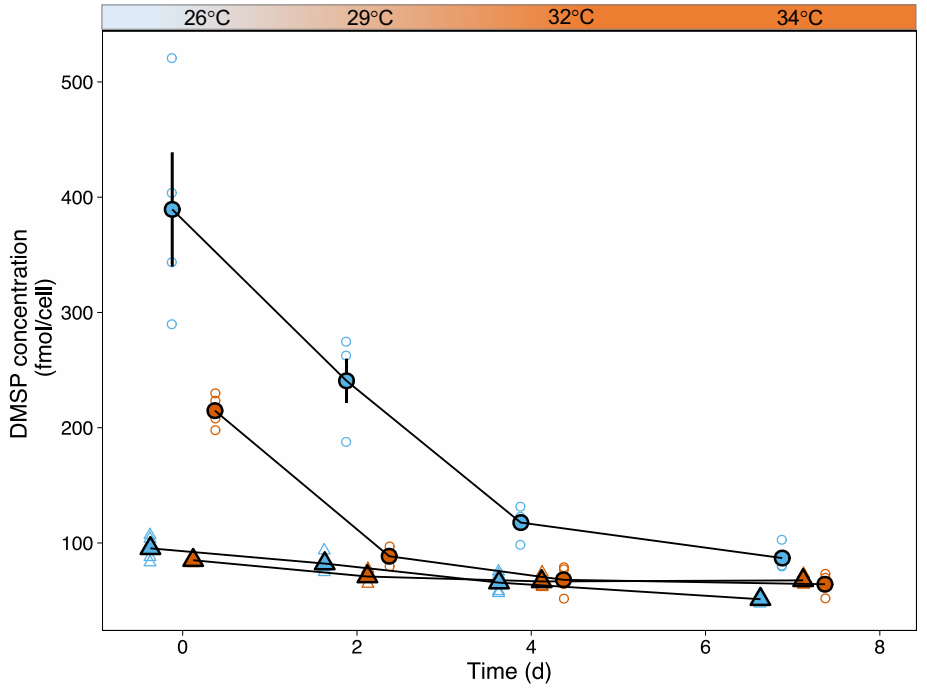


Figure 3-8. DMSP concentration of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-8. DMSP concentration summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7																
	34	0																
		2																
		4																
		7																
B	26	0	***	***	***	***	***	***	***	***								
		2	**	***	***	***	**	***	***	***	***							
		4			**	***			**	***	***	***						
		7				**					***	***	***					
	34	0		*	**	**		**	**	**	*			*				
		2									***	***			***			
		4									***	***	**		***			
		7									***	***	***		***			

Even taking into consideration the cell volume, the concentration of DMSP in both clades was virtually identical to the fmol/cell, with still a statistically significant difference ($P < 0.0001$) (Figure 3-9).

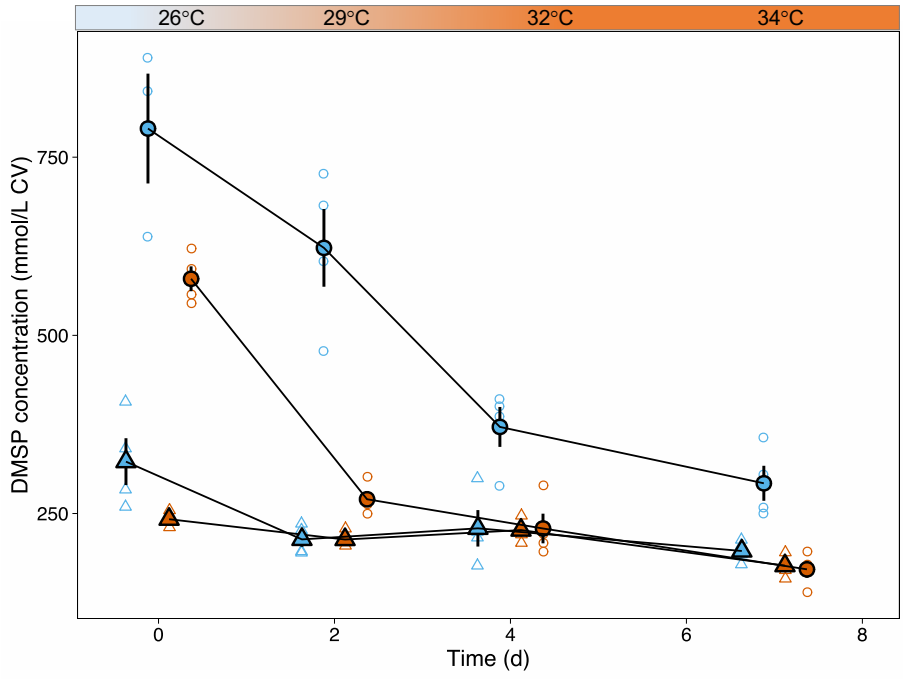


Figure 3-9. DMSP concentration of Symbiodinium linuchae SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red), CV is cell volume. Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

DMSP concentration per chl-*a* content was also significantly different among clades, days and temperature ($P < 0.0001$) but a clearer pattern emerged. SSB01 treatment had a sharp decline from day 0 starting at above 50.97±1.77 nmol/µg · chl-*a* to day 4 reaching 27.89±1.08 nmol/µg · chl-*a*, and a significant increase at day 7 (Figure 3-10 and Table 3-9). Throughout the experiment, treatment SSA01 samples had a mean concentration of 45.09±1.31 and treatment SSB01 samples had 39.45±2.47 nmol/µg · chl-*a*.

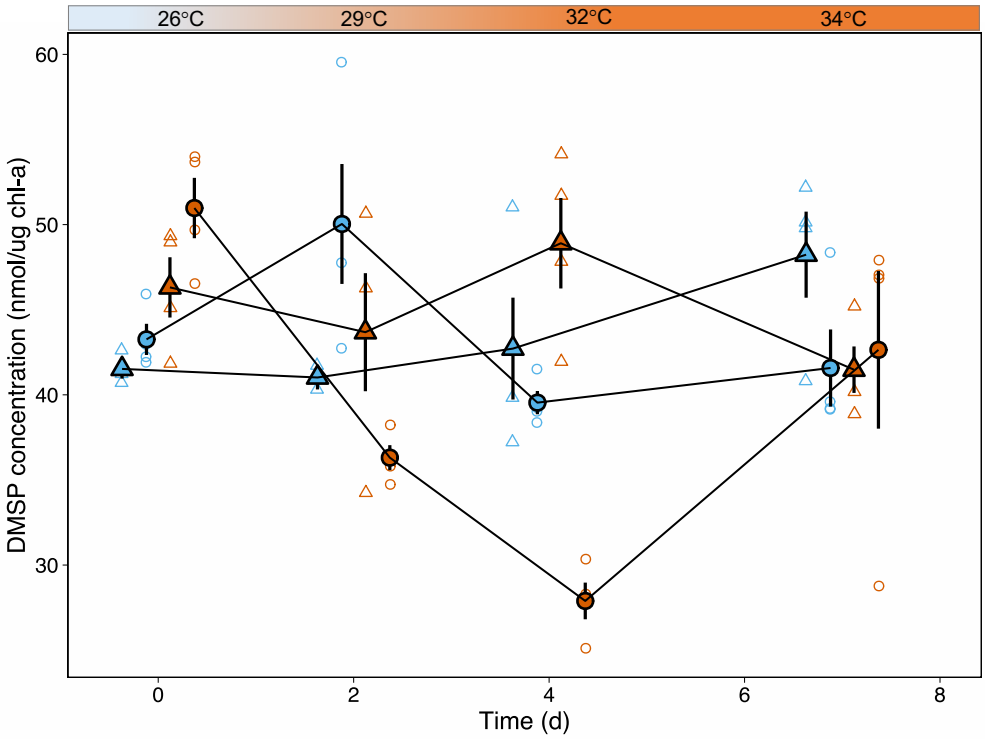


Figure 3-10. DMSP concentration of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-9. DMSP concentration (nmol/µg · chl-a) summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' P ≤ 0.05, '**' P ≤ 0.001, '***' P ≤ 0.0001, empty white cells P ≥ 0.05 and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7																
	34	0																
		2																
		4																
		7																
B	26	0																
		2																
		4										**						
		7																
	34	0											*					
		2													***			
		4	**		*	**	***	*	***		**	**			***			
		7															*	

DMSP concentration in Symbiodiniaceae and chlorophyll-a have a positive correlation, SSA01 has an R^2 of 0.59 ($P < 0.0001$) and SSB01 has an R^2 of 0.95 ($P < 0.0001$) (Figure 3-11).

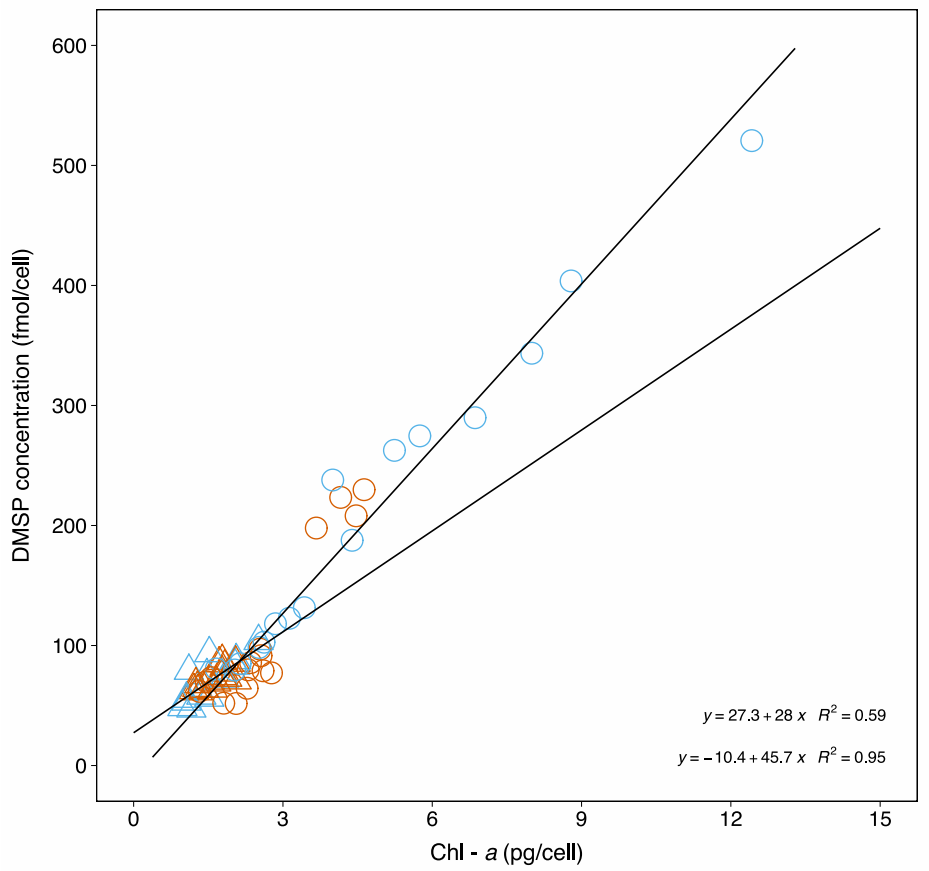


Figure 3-11. Correlation of Chlorophyll-a (x-axis) and DMSP concentration (y-axis). *Symbiodinium linuchae* (SSA01, triangle) and *Breviolum minutum* (SSB01, circle) at control conditions (blue) and treatment conditions (red). Black lines are regression lines of both algae (the respective equation and R^2 are reported, SSA01 top and SSB01 bottom).

DMS concentration in Symbiodiniaceae cultures was also significantly different ($P < 0.0025$). SSB01 control at day 0 had double to concentration of DMS than other samples, 4.18 versus ~1.5 fmol/cell but by day 4, both strains in control condition had a concentration of ~2 fmol/cell, while treatment samples, although not significant, were lower at ~1 fmol/cell (Figure 3-12). Also to note that treatment SSB01 at day 7 showed an increase of DMS compared to the previous measurement but not statistically significant.

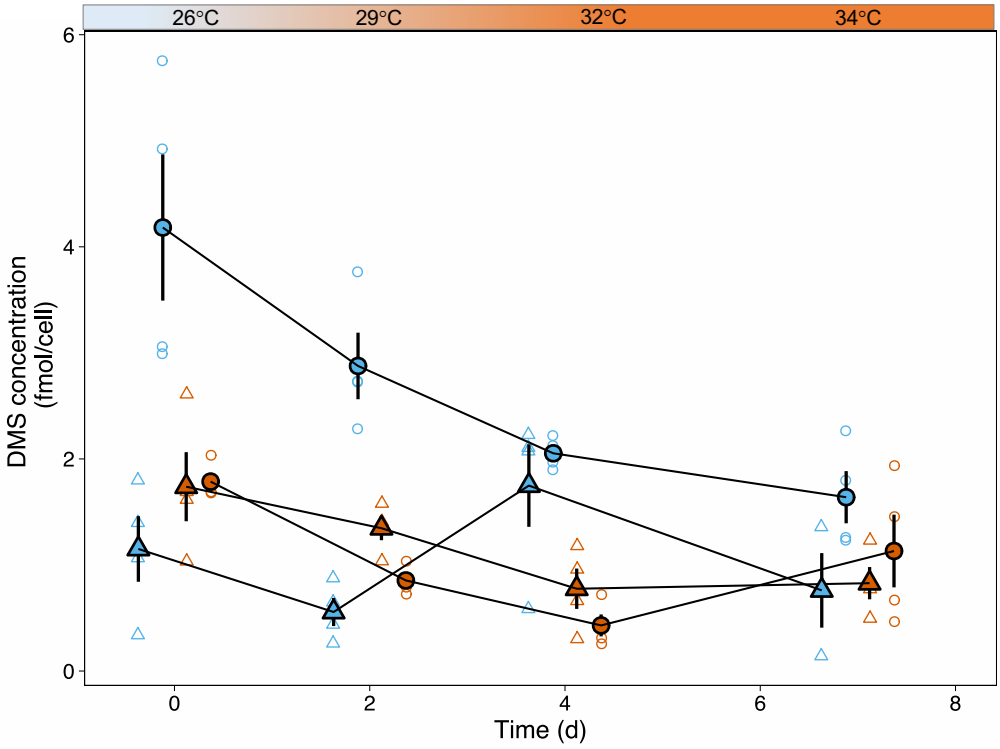


Figure 3-12. DMS concentration of *Symbiodinium linuchae* SSA01 (triangles, A) and *Breviolum minutum* SSB01 (circles, B), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

Table 3-10. DMS concentration summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Clade			A								B							
	Temp [°C]		26				34				26				34			
		Day	0	2	4	7	0	2	4	7	0	2	4	7	0	2	4	7
A	26	0																
		2																
		4																
		7																
	34	0																
		2																
		4																
		7																
B	26	0	***	***	**	***	**	***	***	***								
		2	*	**		*			*	*								
		4									***							
		7									***							
	34	0									**							
		2									***	*						
		4									***	**						
		7									***	*						

3.3.2 The effect of high temperature on *Exaiptasia*

F_v/F_m was not significantly different across temperature, day and strain ($LME_{day*temperature*clade}$, P = 0.107) (Figure 3-13 and Table 3-11). *Exaiptasia* H2, unlike CC7 (P = 0.583), showed a statistically significant difference between days and treatment (P < 0.0001). Thermal stress treatment samples in CC7 (containing SSA01) had a higher F_v/F_m throughout the experiment compared to H2 (containing SSB01), ~ 0.4 versus ~ 0.3. Unlike other variables, F_v/F_m was recorded for a longer period, both before (day -1) and after the experiment was terminated (days 8 to 10). CC7 had a stable maximum photochemical efficiency in both control and treatment, interestingly a very slight increase was recorded at day 7 and 8 but for each other day it was equal. H2 on the other hand, sustained a decline in F_v/F_m in treatment samples on days 6 and 7, however, this was not statistically significant. On day 8, the decline continued but by day 10 the maximum photochemical efficiency went back to ~ 0.3 as in control samples.

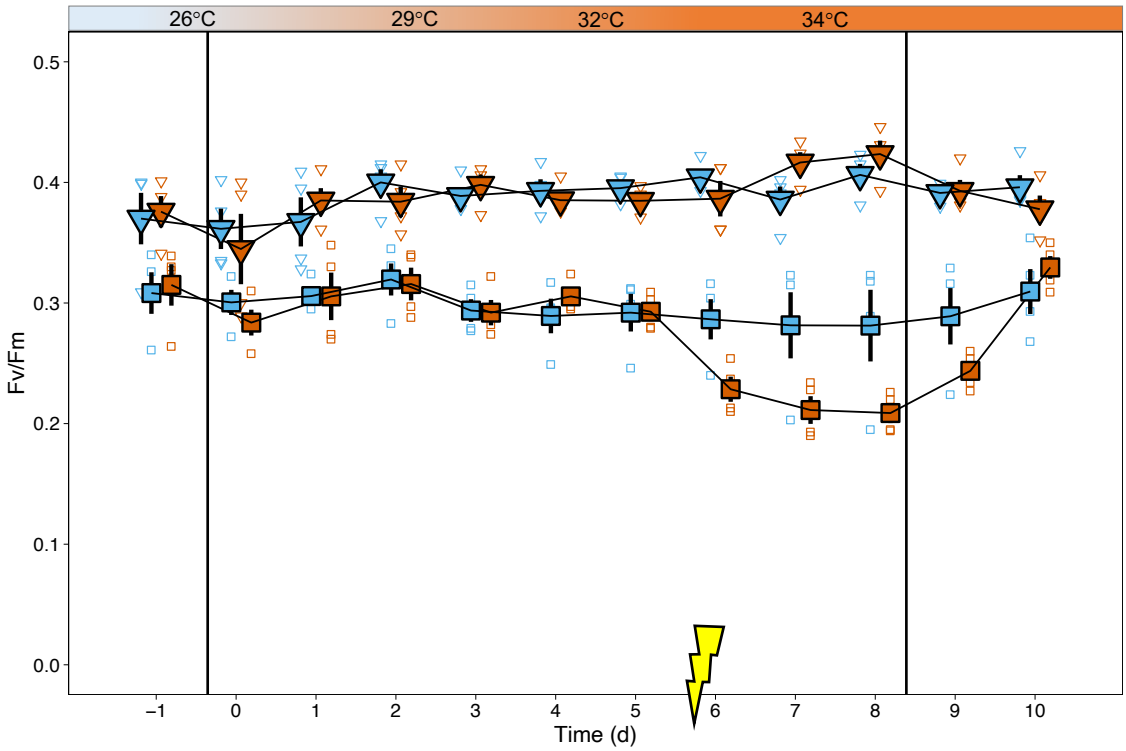


Figure 3-13. Maximum photochemical efficiency of *Exaiptasia* CC7 (in symbiosis with *Symbiodinium linuchae* SSA01) (triangles) and *Exaiptasia* H2 (in symbiosis with *Breviolum minutum* SSB01) (squares), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Flash symbol indicates when the power cut occurred: temperature went down to 15.4°C. Black lines indicate the beginning and end of the experiment (day 0 to 7); extra measurements were taken before and after.

Table 3-11. Maximum photochemical efficiency summary of levels of significance, Post-hoc Tukey's test of the effects of temperature on Exaiptasia pallida H2. The outer layer shows the anemone 'strain', the second layer is 'temperature' 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '***' $P \leq 0.001$, '****' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Strain			H2											
	Temp [°C]		34											
		Day	-1	0	1	2	3	4	5	6	7	8	9	10
H2	34	-1												
		0												
		1												
		2												
		3												
		4												
		5												
		6	***		**	***	*	**	*					
		7	***	**	***	***	***	***	***					
		8	***	**	***	***	***	***	***					
		9	**		*	**		*						
		10								***	***	***	***	

DMSP concentration in *Exaiptasia* CC7 was similar between control and treatment (50.03±2.07 and 43.31±3.44 respectively) and also in H2 (45.38±5.48 and 46.08±5.77 respectively) throughout the experiment. The concentration patterns of DMSP among the two species was not significantly different (P = 0.43), also when *Exaiptasia* were analysed separately (CC7, P = 0.385 and H2, P = 0.929) (Figure 3-14). DMSP generally showed a steady decline from ~ 50 µmol/g at day 0 to ~ 30 µmol/g on day 7. CC7 control had the most stable concentration throughout the experiment, while H2 treatment started with 70.47±13.72 µmol/g at day 0 and had 34.19±1.63 µmol/g at day 7. It is interesting also that when the experiment was repeated, the concentration went down to 11.36±1.98 µmol/g, while the average concentration for CC7 treatment was ~ 30 in both occasions. Also to note that a much greater variability was recorded, at day 7 it was ±3.63 µmol/g, while at day 7 exp #4 it was ±12.1.

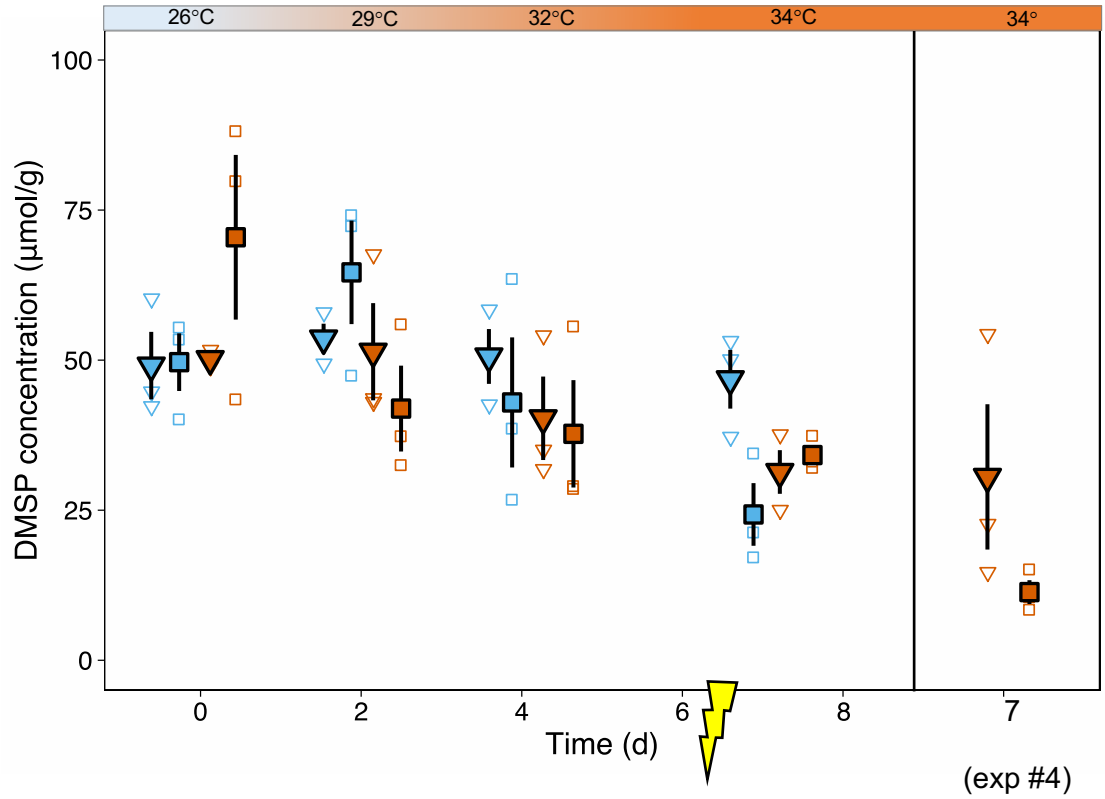


Figure 3-14. DMSP concentration in *Exaiptasia* CC7 (in symbiosis with *Symbiodinium linuchae* SSA01) (triangles) and *Exaiptasia* H2 (in symbiosis with *Breviolum minutum* SSB01) (squares), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Flash symbol indicates when the power cut occurred.

The concentration of DMS in *Exaiptasia* was statistically different among strains, days and treatment ($P < 0.0001$) (Figure 3-15 and Table 3-12). All samples had a constant concentration of DMS at ~ 1000 fmol/mg at day 0. Symbiodiniaceae clade B treatment has a constant increase reaching 22153.1±4016.24 fmol/mg at day 7 compared to the rest of the samples that remained below ~ 5000 fmol/mg throughout the experiment. As for the DMSP concentration, the experiment was repeated (see day 7 (exp #4)) and the second time both strains in treatment had a DMS concentration below ~ 1000 fmol/mg, which is in stark contrast for *Exaiptasia* H2. Although cells were not counted,

Figure **3-16** shows the anemones after the experiment (#4) and the loss of Symbiodiniaceae is visible, especially in H2.

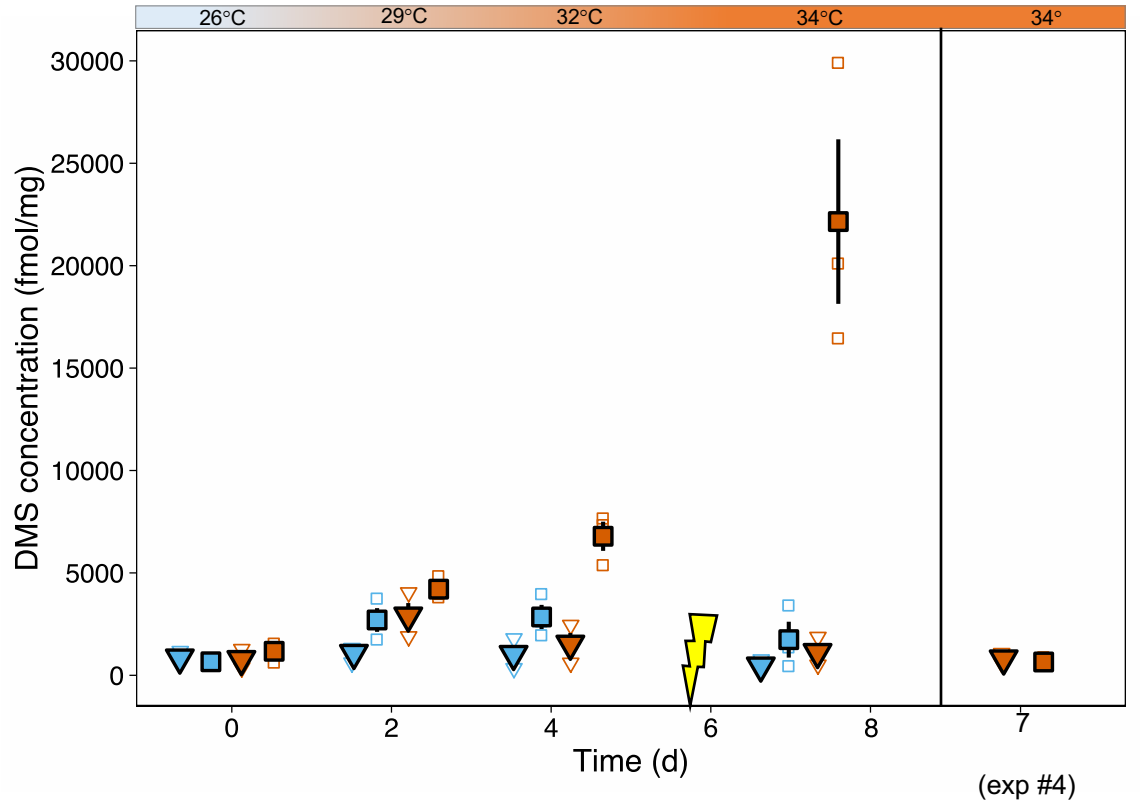


Figure 3-15. DMS concentration of *Exaiptasia* CC7 (in symbiosis with *Symbiodinium linuchae* SSA01) (triangles) and *Exaiptasia* H2 (in symbiosis with *Breviolum minutum* SSB01) (squares), n=4. Shown are control treatments at 26°C (blue) and treatments at increased temperature (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data. Where error bars are not visible, standard error was smaller than the symbol size. Data points are offset by a few hours along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Flash symbol indicates when the power cut occurred.

Table 3-12. DMS concentration summary of levels of significance, Post-hoc Tukey's test of the effects of day, temperature and clade for clades SSA01 and SSB01. The outer layer shows the first variable 'clade' A or B, the second layer is 'temperature', where 26°C is control and 34°C treatment, lastly 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions. Black asterisks refer to the first experiment (exp #3), while in red to the second (exp #4).

Clade			CC7										H2									
	Temp [°C]		26					34					26					34				
		Day	0	2	4	7	8	0	2	4	7	8	0	2	4	7	8	0	2	4	7	8
CC7	26	0																				
		2																				
		4																				
		7																				
		8																				
	34	0																				
		2				*																
		4																				
		7																				
		8																				
H2	26	0																				
		2																				
		4																				
		7																				
		8																				
	34	0																				
		2	**	**	**	***		***		*		***	**			*		**				
		4	* ***	***	***	* ***		* ***	***	***		***	* ***	***	***	***		***	*			
		7	***	***	***	***		***	***	***	***		***	***	***	***		***	***	***		
		8																	***	***		

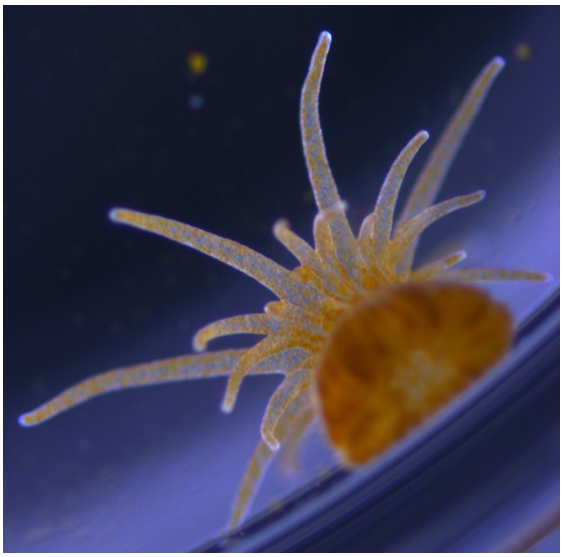
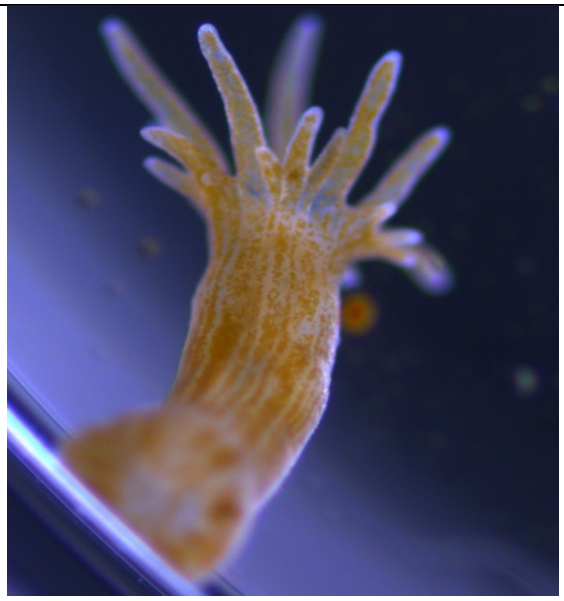
	control	treatment
CC7		
H2		

Figure 3-16. Images showing *Exaiptasia pallida* after the experimental period of seven days (exp #4). Top row shows CC7 and bottom row H2, while the left column represents the treatment they were under (control left and treatment right).

3.4 Discussion

3.4.1 *Symbiodinium linucheae* is more thermo-tolerant than *Breviolum minutum*

Here I have subjected two species of Symbiodiniaceae to increased temperature to confirm relative thermotolerance in SSA01 (*Symbiodinium linuchae*, clade A) and thermosensitivity in SSB01 (*Breviolum minutum*, clade B). Temperature increase from 26°C to 34°C had a negative effect on *Symbiodinium linuchae* growth from when it reached 34°C, while *Breviolum minutum* growth was already affected at 32°C. This supports the hypothesis that SSA01 is thermotolerant as suggested earlier (Díaz-Almeyda et al., 2017; Swain et al., 2017), while SSB01 appears more sensitive to increased temperature (Robison & Warner, 2006; Suggett et al., 2008; Tchernov et al., 2004). Variety in cell growth has previously been observed among several clades, whereby it was similar at 25°C and 30°C and a decrease in cell density started to become evident at temperatures above 33°C, with the exception of clade F in which increased temperature appears to have no effect (Karim et al., 2015).

The growth rate data further demonstrated that SSA01 (average growth rate in control 0.14±0.02 d^{-1} vs 0.02±0.02 d^{-1} treatment on day 7) had a higher tolerance to increased temperature compared to SSB01 (average growth rate in control 0.24±0.05 d^{-1} vs 0.01±0.03 d^{-1} treatment on day 7), which is in agreement with the expectations. Chang et al. (1983) show variability in growth and cell volume depending on light regime and the type of host a particular strain of *Symbiodinium* was isolated from Chang et al. (1983). Similarly, McLenon & DiTullio (2012) observe an average maximum growth rate in *Symbiodinium* clade B, isolated from *A. pulchella* of 0.34±0.02 d^{-1} in the cultures at 27°C, which is in agreement

with our results. Steinke et al. (2011) used *Symbiodinium* isolated from coral (*Symbiodinium* clade A2) and from *Exaiptasia pallida* (clade B1) grown in continuous cultures at 26.8°C at 350 µmol photons m^{-2} s^{-1}, obtaining an average growth rate of 0.33±0.157 d^{-1} and 0.34±0.074 d^{-1}, respectively. They also quantified growth in *Symbiodinium* clade A4 and the growth rate was lower (0.23±0.038 d^{-1}) in comparison to this study. Hennige et al. (2009) also showed substantial variability in growth rates among several Symbiodiniaceae clades at different light intensities, recording roughly half the growth at low light compared to high light (100 and 650 µmol photons m^{-2} s^{-1} respectively). Clade B for example had a growth rate of 0.19±0.03 at low light and 0.39±0.04 at high light. As observed in Grégoire et al. (2017), growth in five different Symbiodiniaceae at 20/27/30/33°C is positive between 20°C and 30°C degrees, but ceases once the temperature reaches 33°C. In fact, SSB01 had a negative growth already at 32°C, while the thermotolerant SSA01 still had a positive growth, although minimal (0.02±0.02 d^{-1}) and just like in the above-mentioned study when the temperature reached more than 33°C (i.e. 34°C) growth was statistically lower compared to control samples (Grégoire et al., 2017).

Cell volume in *Symbiodinium microadriaticum* is also reported having a significantly higher volume on day 5 at a constant temperature of 33°C than control at 27°C (McLenon & DiTullio, 2012). In addition to cell division cessation, another possible explanation for the increase in biovolume in the high temperature treatments is vacuolization and disorganization of cell contents prior to cell death (Franklin & Berges, 2004).

Chlorophylls *a* and c_2 concentration did not show significant differences throughout the experiment period but differed between the two Symbiodiniaceae species. The changes in chlorophylls observed in both clades is in accordance with another study (using HPLC) whereby no changes appear to occur, even when other parameters measured (e.g. F_v/F_m) demonstrated that the dinoflagellate experienced stress (Venn et al., 2006). The trend of *Symbiodinium linuchae* showing a small increase of chlorophyll-*a* was the opposite of what was observed under similar treatment conditions, and, similarly, the decline started only at a temperature of 33°C (Takahashi et al., 2013) as is observed in a variety of coral species (Gardner, et al., 2017a; Gardner, et al., 2017b). The extent of thermal inhibition (decrease in the value of Fv/Fm during high temperature exposure) was drastically enhanced when incubations exceeded temperatures of 34°C for more than 3 days. However, clade A showed a decline also for this parameter once the temperature reached 33°C. This is consistent with previous studies where F_v/F_m decreased in both dinoflagellate and host on day 5, when the temperature reached 33°C in both clades and also in symbiotic anemones (Gardner, et al., 2017a; Karim et al., 2015; Takahashi et al., 2013).

As previously mentioned, all experiments have used slightly different temperature increase profiles, so all comparison are not precise, but it is still possible to draw some main conclusions. This study also concludes that clade alone does not determine the thermotolerance of Symbiodiniaceae, but the species within each clade of Symbiodiniaceae play a major role for a possible adaptation/acclimation to temperature increase in coral reefs (Abrego et al., 2008; Baker, 2003; Frade et al., 2008; Swain et al., 2017). Both *Exaiptasia* and their Symbiodiniaceae host

are negatively affected by high temperature and it is evident that host genetics plays a part in how the symbionts respond to stress (Grégoire et al., 2017). Probably the changes within the holobiont are mainly affected by physiological properties of each Symbiodiniaceae genotype and those of the host are less prominent, but the proportion of either is still unknown (Van Oppen et al., 2011). This study adds information on *Symbiodinium linuchae* and *Breviolum minutum* and their host animal under temperature increase reaching 34°C over a seven days period, compared to other studies. More importantly, it will reflect on the difference of the algae growing alone and in symbiosis with *Exaiptasia*. Future studies should concentrate on different species of Symbiodiniaceae in order to broaden the knowledge and better understand the different ranges of DMSP concentration at higher temperatures. It will also be important to investigate the presence of ROS during the experimental period and further learn the impact that temperature has on this species. This experiment will also benefit with more replication (> n = 3).

3.4.2 DMSP concentration of Symbiodiniaceae in isolation is different to when in symbiosis with *Exaiptasia*

Symbiodiniaceae live in symbiosis with their host and the effect of ROS on the algal symbiont is the first step in the response to increased temperature that finally ends with bleaching by the animal host (Weis, 2008). DMSP concentrations in Symbiodiniaceae controls were different throughout the experimental period in clades A and B (73.57±4.73 and 208.68±33.04 fmol/cell respectively), with the difference between these two clades likely due to the

dissimilar amount of chlorophyll-a present in the cell. In treatment samples, where chlorophyll-a levels were similar between the two strains, the DMSP concentration was more similar (72.53±2.17 and 108.89±16.17 fmol/cell, SSA01 and SSB01 respectively). These values were within the range of data from previous studies, which report high variability across different clades, from as little as 33.8 to a reported maximum of 329 fmol/cell (Broadbent et al., 2002; McLenon & DiTullio, 2012; Steinke, Brading, et al., 2011; Yost & Mitchelmore, 2009). The lack of difference in control and treatment in both clades is in disagreement with previous studies showing that thermal stress and related ROS activity could have influenced the DMSP concentration in the cells, using DMSP as antioxidant and, therefore, producing higher concentrations (Gardner, et al., 2017b; Sunda et al., 2002). A sharp decrease was instead recorded once the DMSP concentration was normalised to chlorophyll-*a*. This is different to the results of Deschaseaux et al. (2014) that demonstrate that DMSP increases at higher temperature compared to control, while the data presented here suggest a significant decrease between days 0 and 4 with a significant increase at the last day of sampling, probably driven by the positive correlation between DMSP and chlorophyll-a.

During Experiment #3, the average DMSP concentrations in *Exaiptasia* in control and treatment conditions were unexpectedly similar in both treatments. Instead, during Experiment #4, the DMSP concentration was lower in H2. The difference recorded on day 7 between experiment #3 and #4 could have been affected by the difference in temperature drop during the blackout. In #3 temperature went down to 15.4°C and this might have actually been positive for the anemones,

allowing them a relief from heat. In #4 instead the temperature was only down to 23.8°C, probably not cooling enough to help H2 strain to cope to last day at 34°C. In fact, although the expectation was to record a higher concentration of DMSP on day 7, the opposite occurred due to the lack of symbionts in the host animal when severe bleaching took place, especially in experiment #4. The lack of DMSP is then in accordance with the theory that DMSP is produced by the algal cell and therefore if less dinoflagellates are present, less DMSP is produced (Van Alstyne et al., 2009). In the flowering plant *Wollastonia biflora*, methionine is converted to S-methylmethionine (SMM), imported into the chloroplast and once there is then converted to dimethylsulphoniopropionaldehyde (DMSP-ald) and DMSP (Trossat et al., 1996). In support of this theory, is the observation that aposymbiotic anemones do not produce enough DMSP to be quantified (Franchini & Steinke, 2016). Another explanation for the general decrease of DMSP in the treatment is that *Exaiptasia* could utilise DMSP as an antioxidant when under stress (Sunda et al., 2002) and converting this to DMSO (Hopkins et al., 2016; Thume et al., 2018). DMSP concentrations in this study (43.31±3.44 to 50.03±2.07 nmol/mg) were similar to those reported in anemones previously (15.09 to 51.82 nmol/mg dry weight, Franchini and Steinke, 2017), but at the lower end when compared to corals (40 to 3831 fmol/cell) (Broadbent et al., 2002; Deschaseaux et al., 2014; Hopkins et al., 2016; Swan et al., 2012). Knowing that asymbiotic anemones have non detectable concentrations of DMSP and that the strains of Symbiodiniaceae used in this study have an average concentration of 73.57±4.73 and 208.68±33.04 fmol/cell respectively, we can assume the concentration when in symbiosis with the host will be similar. Variabilities in

literature could be in part attributed to the sampling method with lower values stemming from samples that were measured directly while the higher concentrations are from materials where tissues were removed using invasive methods and therefore creating extra stress that may have increased the production of DMSP (Caruana & Malin, 2014).

DMSP concentration in Symbiodiniaceae cultures did not show a significant difference on day 7, but *Symbiodinium linuchae* cultures at high temperature had a lower concentration, while *Breviolum minutum* had a higher concentration. Interestingly the exact opposite occurred while in symbiosis with *Exaiptasia*, with CC7 (containing *Symbiodinium linuchae*) showing a lower DMSP concentration compared to control and H2 (with *Breviolum minutum*) had a higher concentration (note that in the repeated experiment also H2 had a lower amount). These findings demonstrate that the dinoflagellates alone might not be able to regulate the concentration of the compound to their advantage when stressed and that the behaviour is affected by the animal host. This difference could also be due to the amount of Symbiodiniaceae expelled, H2 seemed to be more bleached than CC7 and therefore the lack of algae brought a decrease in DMSP concentration.

It is now evident the importance to know the actual amount of Symbiodiniaceae in *Exaiptasia*, in order to have indisputable evidence of the algal input in DMSP concentration when in symbiosis.

3.4.3 *Exaiptasia* also alters the DMS concentration of *Breviolum minutum*

In the experiments with *Breviolum minutum*, both the endosymbiont and the host (H2), are highly affected by elevated temperatures. DMS concentration in the H2 treatment had a steady increase, while, when in isolation as algal culture, the DMS concentration was steady when normalised to cell density and a clear effect was only evident when normalised to chlorophyll-a content. This was also the case in another study (Deschaseaux et al., 2014). *Symbiodinium linuchae* proved once again its thermotolerance. DMS concentration was not significantly different when comparing data from the experiments *in hospite* with data of the alga in isolation. Based on photochemical-efficiency data in the culture alone, there were signs of stress (decrease in Fv/Fm) at the last day of sampling, but this did not occur when in symbiosis with CC7. This proves that in this combination, the host genetics likely play a key role in the symbiont response (Barott et al., 2015; Cunning et al., 2015; Howells et al., 2016).

In conclusion, both Symbiodiniaceae strains were affected by high temperature stress and, as expected, SSA01 had a higher tolerance compared to SSB01. Cell concentration and growth decreased in both strains while Chl-a remained stable. Maximum photochemical efficiency followed the expectations and decreased in Symbiodiniaceae and slightly in anemone H2, while CC7 seemed unaffected by heat. DMSP/DMS did not increase as expected and in the case of *Exaiptasia* this is probably due to the lack of Symbiodiniaceae that were expelled. Overall these findings indicate that at a temperature of 34°C anemones bleach and do not seem able to use DMSP/DMS as an antioxidant. This is not a positive result for the future of coral reefs, but the experiment also has its limitations, no real scenario

will have an increase temperature of 8°C over a week and thus calls for a more realistic approach, with a gradual temperature increase.

3.4.4 Conclusions

This study highlights the impact that a temperature of 34°C degrees will have on Symbiodiniaceae and their animal host. It also shows the difference between the algae growing on their own and in symbiosis with *Exaiptasia*. *Symbiodinium linuchae* is confirmed more thermotolerant than *Breviolum minutum* as pure cultures, but very little difference is shown when in symbiosis with the animal host. The presence of different bacterial assemblages within the holobiont might influence the concentration of DMSP/DMS measured and this should be further investigated. The experiment clearly showed bleaching occurring, which could explain the lack of a sharp increase in DMSP/DMS (i.e. no algae that are the main producer of these compounds). Further research is needed on temperature increasing events, due to the importance of bleaching in these scenarios. Chapter four focuses on the number of cells present in the same organisms studied in chapter three and also using the same temperature profile.

Chapter 4 Bio-Imaging of *Exaiptasia pallida*

4.1 Introduction

Coral reefs have been called ‘rainforests of the sea’ (Davidson, 1998; Knowlton, 2001) and just like land rainforests on land, their importance does not lie necessarily in the diversity of the corals themselves but the millions of species that live in association with them instead. A minimum of 835 species of reef building corals were estimated (Veron, 1995), but a staggering 1-9 million species are believed to form the overall biodiversity of reefs (Reaka-Kudla et al., 1996). Reefs also provide ecosystem services that benefit either directly or indirectly millions of people, like fisheries, cultural services (recreation and tourism) and coastal protection (Moberg & Folke, 1999). Unfortunately, regardless of their invaluable presence, reefs are under the pressure of global and local stressors and are declining worldwide (Hoegh-Guldberg, 1999). Major stressor include marine heatwaves (leading to coral bleaching), ocean acidification, over-fishing, pollution and physical damage, which leads to different responses within the reefs (Hughes et al., 2003; Olguín-López et al., 2018).

Bleaching, the focus point of this chapter, is identified as a stress response to environmental disturbance and commonly occurs due to several different stressors, such as higher solar radiation, higher temperature, salinity changes and increased nutrient/pollutants or diseases. In order to bleach, an increase of 1-2°C for several weeks or 3-4°C for a few days above the summer sea-surface water temperature is sufficient (Brown, 1997; Hoegh-Guldberg, 1999).

In symbiotic cnidarians, including reef-building corals and anemones, the abundance of Symbiodiniaceae and their physiological performance are key

parameters to consider, given their impact on light absorption, assimilations of carbon and nutrients, photosynthetic efficiency and the general response to environmental stress (Nesa & Hidaka, 2009). The assessment of Symbiodiniaceae abundance is especially important when studying bleaching, and several techniques have been established over the years. The most commonly used involves homogenising a tissue sample followed by manual counting of cells released from the tissue using a haemocytometer and normalising the resulting count to a relevant variable (e.g. sample surface area, sample weight) (Nielsen et al., 2018). The main advantage of this method is its affordability but it has several drawbacks due to its inaccuracy as a result of sampling and systematic error (Camacho-Fernández et al., 2018); it is also very time consuming especially since large number of replicates must be counted in order to reach the required precision. Flow cytometers and coulter counters have also been used for this purpose, but they are likely to have the same sampling error and their precision is dependent on preparation of the sample. In addition, these techniques need a minimum cell density in order to be accurate and, obviously, this is a problem when trying to take the measurements on juvenile individuals, small samples or, of course, bleached or partially bleached specimens. Molecular techniques like quantitative polymerase chain reaction (qPCR) (Cunning & Baker, 2013) and fluorescence in situ hybridization (FISH) have been explored (Loram et al., 2007). These approaches are effective because they allow for information on the Symbiodiniaceae clades and, therefore, provide unique results on the structure of their community. Unfortunately, they are not a valid method to determine cell abundance due to the different gene copy number present among different Symbiodiniaceae, which is the cause of

normalisation issues (Cunning & Baker, 2014). The main downside that all of the above-mentioned methods have in common is their destructive nature of the sampling, which also do not allow a re-sampling of the same specimen over a period of time. Also, to note the un-ethical aspect of this kind of research methods, especially when emphasising to the public eye the importance of protecting these species. A non-destructive microscopy-based approach would allow visualisation of the Symbiodiniaceae in their live host, thereby granting the possibility of repeated measurements. In-vivo fluorescence-based microscopy is especially useful in visualising these cells, given the natural fluorescent properties of chlorophyll molecules they possess and therefore not needing to stain the samples with dyes prior to observation.

In chapter three it was understood the importance of the number of cells present in *Exaiptasia* for the concentration of DMSP/DMS and the organisms' health. Here I will investigate further how many cells are present in an anemone at control condition and after an increase in temperature. This has the aim to advance the field of research on the subject, both in microscopy, by exploring new techniques that are ethical for the studied organisms (although these techniques cannot be used easily in the field). But also add knowledge in biology, by having a better understanding on the amount of cells present in the animal host and their loss during bleaching.

I expect to document bleaching occurring in both anemones, as witnessed in the previous chapter. I also aim to focus on identifying the location of the algae within the anemones since very little information is available on this subject. The importance of chlorophyll was also highlighted in chapter three, and in this

context could be used as a fast method to assess the abundance of symbionts present in the anemones.

4.1.1 Experiment hypotheses

I have three main hypotheses within this heat-stress experiment. **H1** under increasing temperature (up to 34°C) the Symbiodiniaceae density in *Exaiptasia pallida* CC7 and H2 will decrease. **H2** The location of the symbiont within the anemones (i.e. body vs tentacles) is not different between species and treatment. **H3** The chlorophyll fluorescence intensity is correlated to the Symbiodiniaceae density of anemones. Additionally, the correlation between dry weigh of anemone and Symbiodiniaceae density is also explored.

4.2 Materials and Methods

4.2.1 *Exaiptasia* husbandry and biomass estimation

Exaiptasia pallida strain CC7 is in symbiosis with SSA01 *Symbiodinium linuchae*, clade A4, known to be thermotolerant (i.e. able to tolerate the high temperature but not thriving in), while H2 contains SSB01 *Breviolum minutum*, clade B1 (not thermotolerant). They were kept at 26°C under 12:12h light:dark regime, ~ 80 μmol photons m^{-2} s^{-1}, from a 37 W fluorescent light, in 1 L plastic containers (filled with 800 mL salt water). Salt water was prepared using autoclaved artificial seawater (ASW) made by mixing commercial synthetic sea salt (H2Ocean Pro+ Reef Salt, Charterhouse Aquatics, UK) with reverse-osmosis water to a salinity of 35, pH 8. Animals were fed twice a week with 'Cyclops – gamma blister' (Urmston Aquatics, Manchester, UK) that had been previously washed with RO

water and autoclaved. Water was changed approximately 5 h after each feed and containers cleaned weekly, with the aid of cotton swabs. For the length of the experiment, anemones were kept singularly in 4.92 mL vials, with 3 mL AWS. Experiments were performed in the daytime, during the light cycle.

In order to estimate the biomass of the anemones, animals were anaesthetised using a 1:1 mixture of autoclaved ASW and 0.37 M $MgCl_2$ and placed under a dissecting microscope equipped with an eyepiece graticule. A non-linear model was applied to calculate anemone dry weight from oral disk diameter measurements (Clayton & Lasker, 1985):

$$Y = 0.124\, Z^{1.49}$$

where Y Is dry weight in mg and Z is the oral disk diameter in mm.

4.2.2 Sample preparation

Each anemone was gently taken out of the vial using a 3 mL Pasteur pipette, and placed in a glass imaging chamber. Anemones were then anaesthetised using the $MgCl_2$ solution described above, a few drops were added on top of the specimen that was later covered by a glass slide. After a few minutes anemones' movement was greatly reduced and anemones were ready for imaging.

4.2.3 Image acquisition

Anemones were observed under an inverted widefield fluorescence microscope (Nikon), the software package NIS-Elements was used to acquire images (Figure 4-1, A-C). A total of six images were taken for each anemone, n=3 for tentacles and n=3 for the body (Figure 4-1, D). To minimize sample exposure, anemones

were observed using the lowest magnification allowing for individual symbiotic cell discrimination (10× objective) and illumination brightness was reduced to 1/16 using a neutral density filter which reduces light intensity without changing the colour balance.

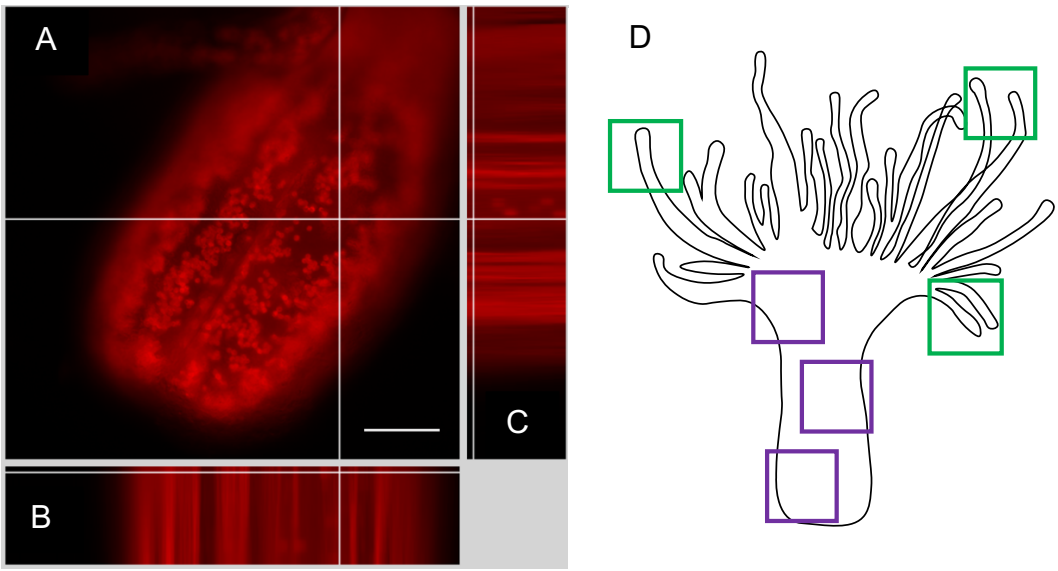


Figure 4-1. (A-C) Portion of the body of *Exaiptasia pallida* imaged by widefield microscopy. 18 slices stack (z-step: 2.65 µm), (A) Maximum intensity projection, (B) and (C) orthogonal projections (xz and yz axes, respectively) displaying symbiotic algae as elongated figures. (D) Example of images acquired for each anemone. N=3 tentacles in green and n=3 body in purple. Scale bar: 200 µm

4.2.4 Image pre-processing

Even though an anaesthetic was used, anemones still made some movements especially when light was directed at the tentacles. This resulted in blurry images and consequently to distorted three-dimensional reconstructions. An additional issue lay in the high background noise level due to the out-of-focus fluorescence inherent to widefield microscopy (WFM). In order to mitigate these matters, relevant plugins in ImageJ software were applied (i.e. Stackreg plugin). Stackreg was created with the scope of aligning slices of an image stack and eliminate the lateral movement artefacts, an unwanted result of separate image acquisition.

This algorithm works by utilising each slide of a stack as a template to align the following slice and continues to do so through until the last image of the whole stack (Thévenaz, 1998). This plugin can use four different methods to align the pictures and we found that 'affine' was the most effective to reduce the moderate motion artefacts in the sampled anemones (Figure 4-2).

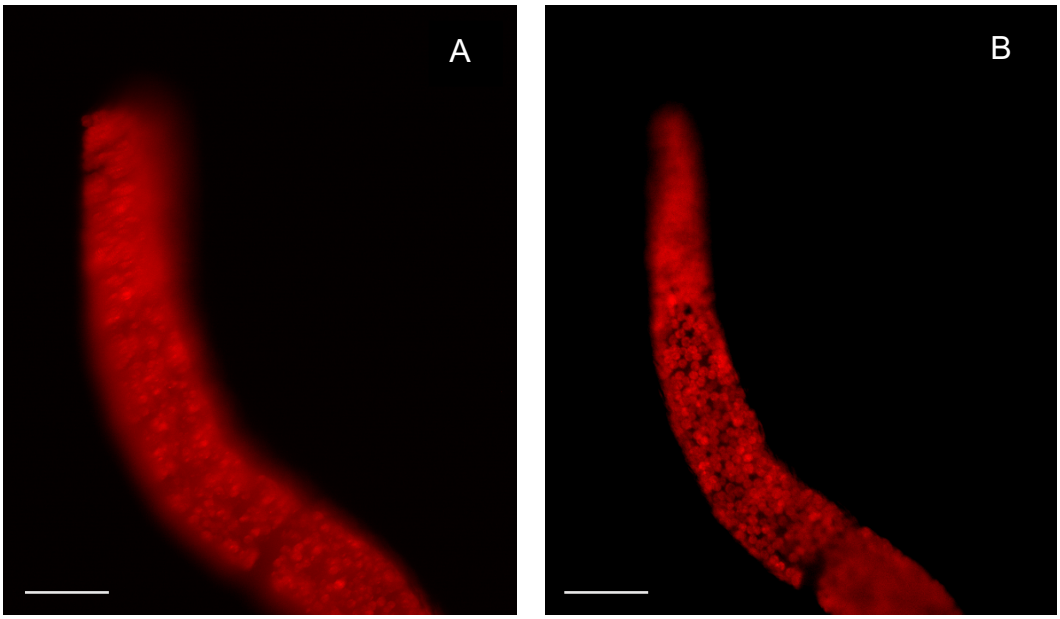


Figure 4-2. Attenuation of motion artefacts in *Exaiptasia pallida* tentacle by image alignment in a Z stack acquisition. (A) Maximum intensity projection before alignment and (B) maximum intensity projection after Stackreg 'affine' alignment. 18 slices stack (z-step: 1.52 µm). Scale bars: 200 µm.

An additional function in ImageJ software was used to reduce the background noise. This function relies on a 'rolling ball' algorithm which works as follows: a background value is assigned for each pixel averaging over a large 'ball' surrounding the pixel, then subtracting this value from the original picture (Figure 4-3). Once the image was on focus, the area of the anemone was measured using the software NIS-Elements.

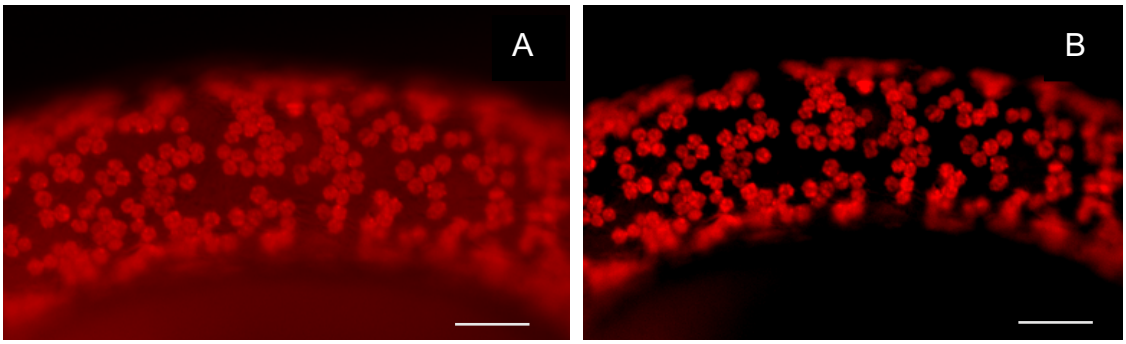


Figure 4-3. Removal of background noise on a portion of *Exaiptasia pallida* tentacle imaged by WFM. (A) Maximum intensity projection before using the function and (B) Maximum intensity projection after background subtraction. 23 slices stack (z-step: 1.12 µm). Scale bars: 50 µm

4.2.5 Algorithm parameters

The algorithm, run with MATLAB, was originally developed to quantify the colocalisation of subcellular particles (called 'blob-like structures) but seemed a great fit also for Symbiodiniaceae (Obara et al., 2013). Briefly, the algorithm works as follows (Figure 4-4): 1) two separate datasets are loaded into the program; 2) a 3D convolution step enhances the fluorescence intensity of the particles. Step 3) defines the location of the particles brighter than the background noise, while during step 4) the position of the Symbiodiniaceae are determined and counted (5).

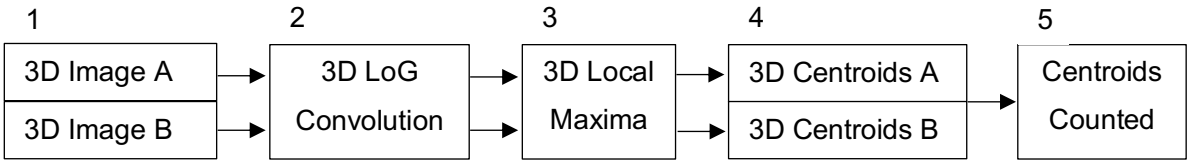


Figure 4-4. Schematic summary of the particles detection procedure.

The three-dimensional algorithm is designed to allow the user to choose the specifics of five different parameters (Figure 4-5, A). For the purpose of this experiment, ‘pixel magnification’, ‘particle radius’ and ‘background noise’ were optimised via a trial-and-error procedure. Several combinations of these parameters were tested, and after the analysis results were checked by visual inspection. The ‘pixel magnification’ X and Y were set-up identical to the original image pixel width and length, while the Z as Z = 2X. ‘Particle radius’ was inversely correlated to the ball-like features detection, with an ideal value of 2000 nm. If the value was set lower it would lead to false positive, while if set higher it would fail to detect the algae. The ‘background noise’ behaves in a similar manner, the optimal value chosen was 0.05 and if set higher it would lead to false negative (e.g. Symbiodiniaceae cells with low fluorescence intensity) and if set lower to false positive (Figure 4-5, B-E).

A

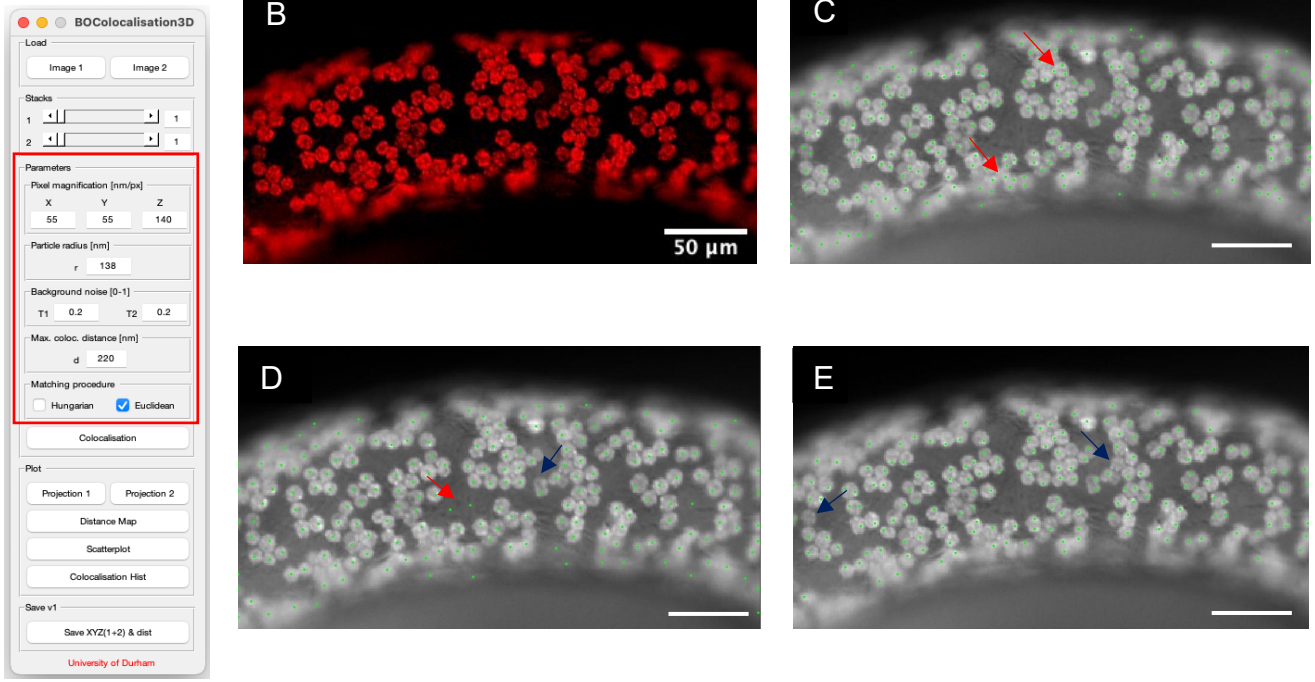


Figure 4-5. (A) Algorithm graphical user interface under MATLAB software allowing the specification of image scale parameters ('pixel magnification'), radius of LoG filter ('particle radius') and detection threshold ('background noise'). (B-E) Examples of results obtained after image analysis by the algorithm, (B) Source file, 17 slices stack, (C) analysis of non-optimal 'particle radius' (r = 1000), (D) analysis using optimal parameters, (E) analysis using non-optimal 'background noise' (T = 0.2). Green dots are detected cells, blue arrows are false negative and red arrows are false positive. Scale bars: 50 µm

4.2.6 Experimental design

Exaiptasia pallida were kept at 26°C under 12:12h light:dark regime, at day 0 half of the anemone required for the experiment (n=3 CC7 and n=3 H2) were moved to the experimental treatment temperature, into a different incubator (LMS, model 300) with the identical light:dark regime and light intensity as the control treatment. The temperature was gradually increased to 34°C over the 7 successive days (Figure 4-6). Samples inside the glass vials were arranged randomly on a tray and water was changed every three days. In reporting results below, samples will be referred to as 'control' (samples kept at 26°C for the entire experimental and recovery period) and 'treatment' (samples exposed to higher temperature during experimental period and kept at 26°C from day 8 to day 21). Data were collected at day 0, 7 and 21 for both control and treatment. The experiment was carried out from the 30th April (day 0) to 7th May 2021 (day 7) and ended on the 6th May (day 21). A power cut occurred on the 3rd May (day 3), with temperature dropping to 15°C overnight and reaching back the respective set temperature the following morning.

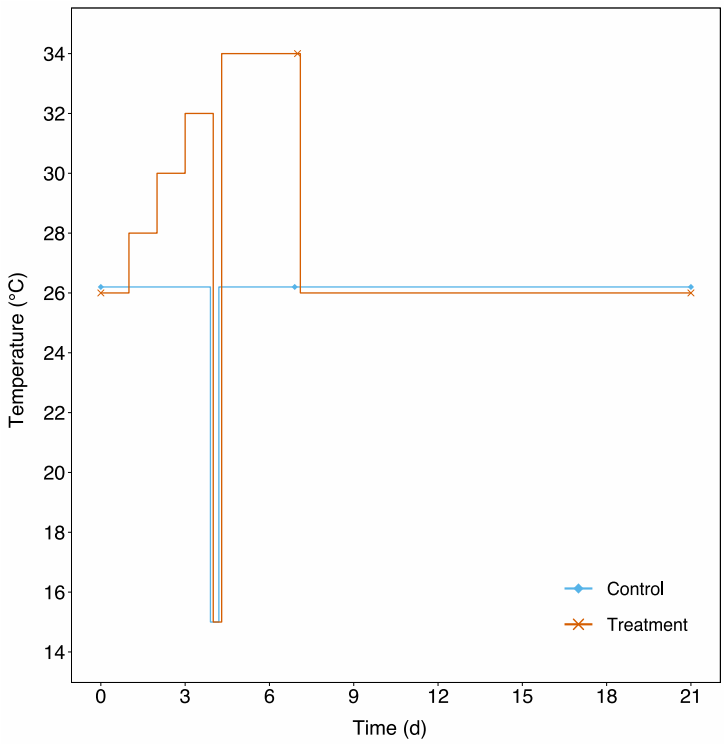


Figure 4-6. Temperature settings during the experimental period. Shown are 'Control' (blue) and 'Treatment '(red). Note that on day # 4 the temperature in the incubator dropped to 15 °C due to power cut in the laboratory overnight, both control and treatment were affected. Diamonds and crosses of each respective colours indicate actual day of sampling (i.e. 0, 7 and 21).

4.2.7 Statistical analysis

Graphical representations and statistical analysis were conducted using R software (R Studio Version 1.2.5042). Statistical significance was determined using LME (Linear Mixed Effect Models), Linear models (correlation) and Tukey HSD post-hoc tests, after the assumptions for each model were tested. LMEs were run with three factors DAY (two or three levels: 0, 7 or 0, 7, 21), TEMPERATURE (two levels: control, treatment) and ANEMONE (two levels: CC7 vs H2) or PART (two levels: tentacle, body). If the model with three factors was not significant, a factor would be removed, and the model would be run again with the two remaining. Model ran with anemones (n=3) as a random effect. Akaike Information Criterion (AIC) was used to determine the best model to use.

4.3 Results

4.3.1 *Exaiptasia pallida* bleach under high temperature, regardless of the strain

No statistically significant difference was found in the interactions among *Exaiptasia*, experimental conditions and day of sampling, ($LME_{Anemone * Temperature * Day}$, P = 0.641) (Figure 4-7). Once the two strains of *Exaiptasia* were removed from the model (factor ANEMONE), there was a statically significant difference in Symbiodiniaceae density between control and treatment on different days ($LME_{Temperature * Day}$, P <0.0001) (Table 4-1). Anemones at day 0 had very similar densities of Symbiodiniaceae between control and treatment but after the temperature increase, treatment samples went from 11,798± 786 to 3,644±550 cell/mm^2 (mean±S.E. between the two strains, CC7 and H2). CC7 lost 42% of its hosts after the temperature increase, compared to day 0 of the experiment, while H2 lost 88% of symbionts. After recovery period (day 21) CC7 lost a further 14% of cells, while H2 gained more than double (Table 4-2)

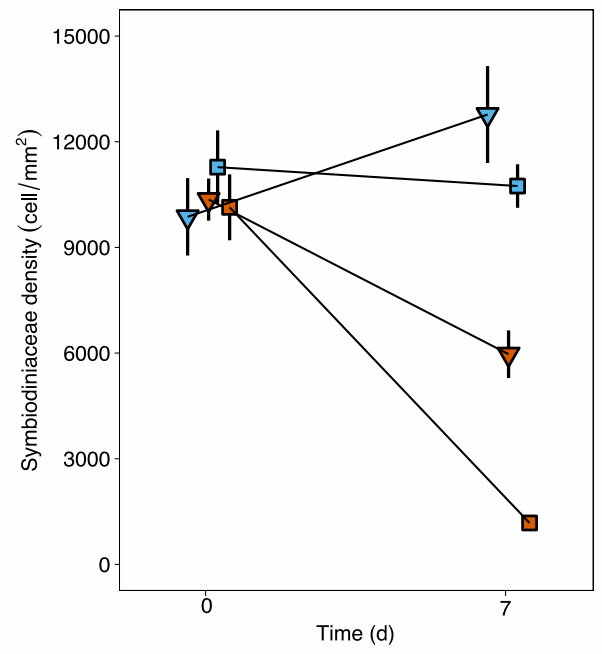


Figure 4-7. Symbiodiniaceae density in Exaiptasia pallida. Strain CC7 is represented by the inverted triangle and H2 by the square. The colour blue represents the control samples kept at 26°C throughout the experiment, while in red the samples that have been subjected to a temperature increase in the first 7 days. The error bar shows standard error when larger than symbol size (n = 3).

Table 4-1. Summary of levels of significance, Post-hoc Tukey's test of the effects of the temperature and day on Symbiodiniaceae density in Exaiptasia pallida. The outer layer shows the first variable 'exp' control or treatment, and 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.01$, '****' $P \leq 0.001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Exp.		ctrl		treat	
	Day	0	7	0	7
ctrl	0				
	7				
treat	0				
	7	***	***	***	

Table 4-2. Summary of Symbiodiniaceae density (cells/mm^2) in *Exaiptasia pallida*. From the top, both 'strain' are CC7 and H2, 'exp' are experimental conditions (control vs treatment), 'day' is day of sampling (0, 7 or 21), Mean and Standard Error follow. % is the percentage increase (green) or loss (red) of Symbiodiniaceae compared to the previous measurement.

Strain	CC7					
Exp.	ctrl			treat		
Day	0	7	21	0	7	21
Mean	9868	12773	7623	10358	5968	5082
S.E.	1098	1375	2226	597	676	901
%		29.44	40.32		42.38	14.85

Strain	H2					
Exp.	ctrl			treat		
Day	0	7	21	0	7	21
Mean	11279	10743	8442	10138	1177	2645
S.E.	1044	618	991	936	160	589
%		4.75	21.42		88.39	124.72

Exaiptasia symbionts density in CC7 and H2 differ significantly among different days ($LME_{Anemone * Day}$, $P = 0.035$) (Table 4-3), but not between experimental conditions ($LME_{Anemone * Exp}$, $P = 0.144$). The lack of symbionts in treatment samples was very obvious while handling the white animals in preparation for the data collection (Figure 4-8).

Table 4-3. Summary of levels of significance, Post-hoc Tukey's test of the effects of the temperature and day on Symbiodiniaceae density in *Exaiptasia pallida*. The outer layer shows the first variable '*Exaipt.*' strain CC7 or H2, and 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.01$, '***' $P \leq 0.001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

	Exaipt.	CC7			H2		
	Day	0	7	21	0	7	21
CC7	0						
	7						
	21						
H2	0			*			
	7	**	*		***		
	21	*	*		**		

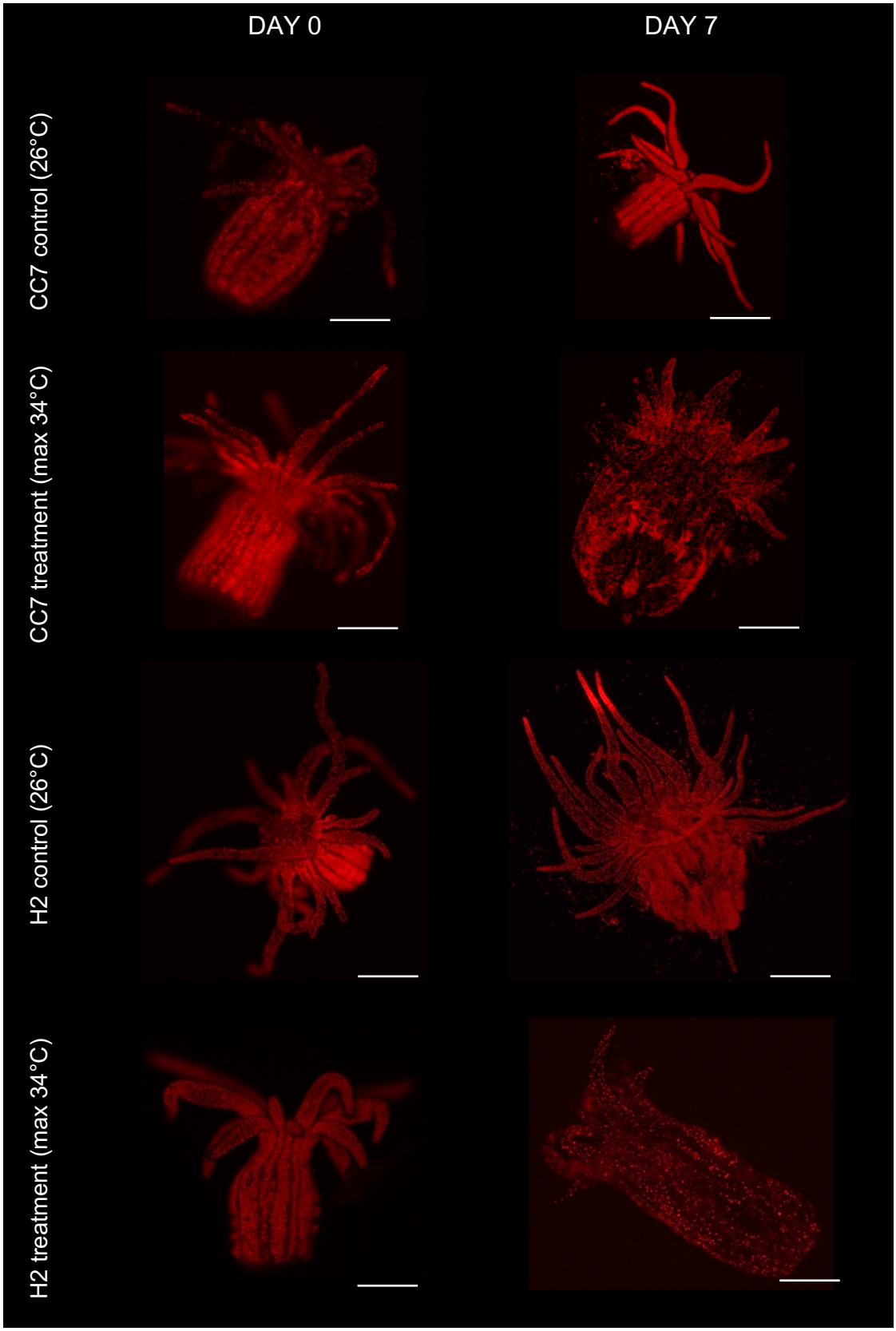


Figure 4-8. Images showing *Exaiptasia pallida* during the experimental period. Top row shows that day of sampling, while the left the anemones strains (CC7 and H2) together with the treatment they were under (control, kept always at 26°C vs treatment, going from 26 to 34°C). Scale bars 400 µm

4.3.2 Symbiodiniaceae location within *Exaiptasia pallida* is not affected by temperature

The Symbiodiniaceae density in *Exaiptasia pallida* CC7 was not statistically significant when analysed with the location of the algae symbiont, the day of sampling and the experimental conditions ($LME_{Part * Exp * Day}$, P = 0.921), same as H2 (P = 0.611) (Figure 4-9 and Table 4-4). Strain H2, unlike CC7 (P = 0.475) had instead a significantly different number of Symbiodiniaceae in its body/tentacles on different day of sampling ($LME_{Part * Day}$, P = 0.007). At day 0, the density of algae was higher in body than in the tentacles, 13,910±1485 vs 9432±926 respectively. Images show the significant difference in symbionts present in control vs treatment for CC7 (Figure 4-10) and for H2 also the higher count in body vs tentacles at day 0 (Figure 4-11).

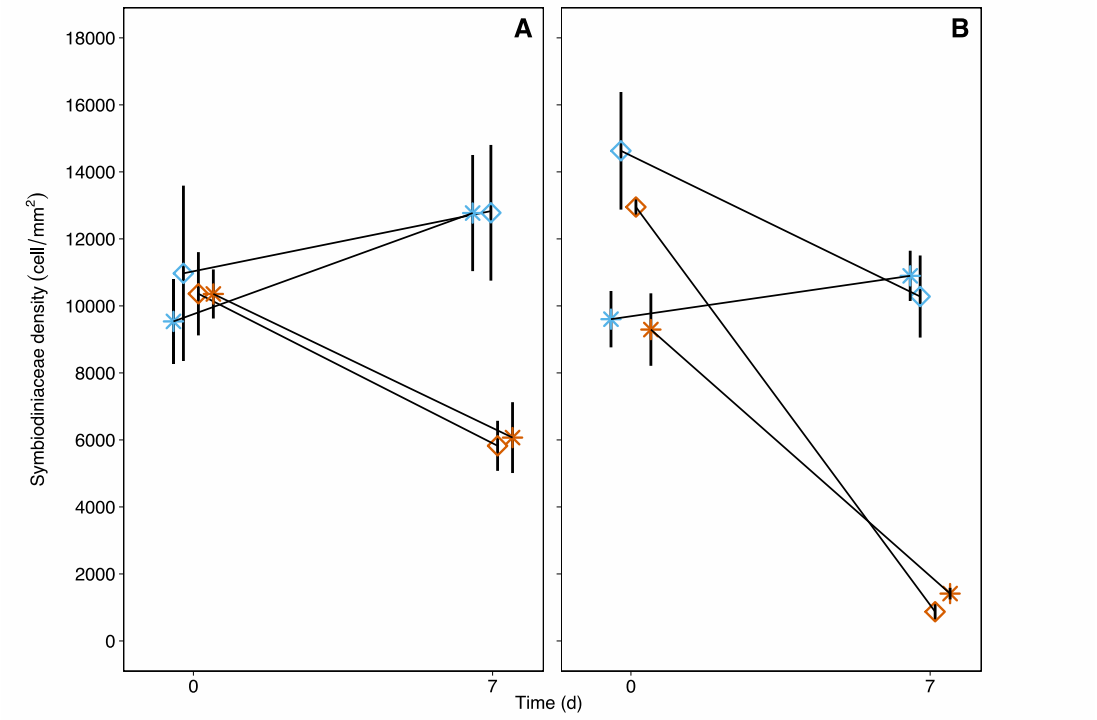


Figure 4-9. Location of Symbiodiniaceae density in *Exaiptasia pallida* strain CC7 (A) and strain H2 (B). Body is represented by the diamond and tentacles by the asterisk. The colour blue represents the control samples kept at 26°C throughout the experiment, while in red the samples that have been subjected to a temperature increase in the first 7 days. Error bar shows standard error (n=3).

Table 4-4. Summary of levels of significance, Post-hoc Tukey's test of the effects of the Symbiodiniaceae density location and day on Symbiodiniaceae density in *Exaiptasia pallida* strain H2. The outer layer shows the first variable 'Part' indicating whether the algae where found in the 'body' or in the 'tentacles' and 'day' indicates when the sample was taken. The centre of the table indicates the levels of significance: '*' $P \leq 0.05$, '**' $P \leq 0.001$, '***' $P \leq 0.0001$, empty white cells $P \geq 0.05$ and grey cells are duplicate interactions.

Part		body		tentacles	
	Day	0	7	0	7
body	0				
	7	***			
tentacles	0		**		
	7	**			

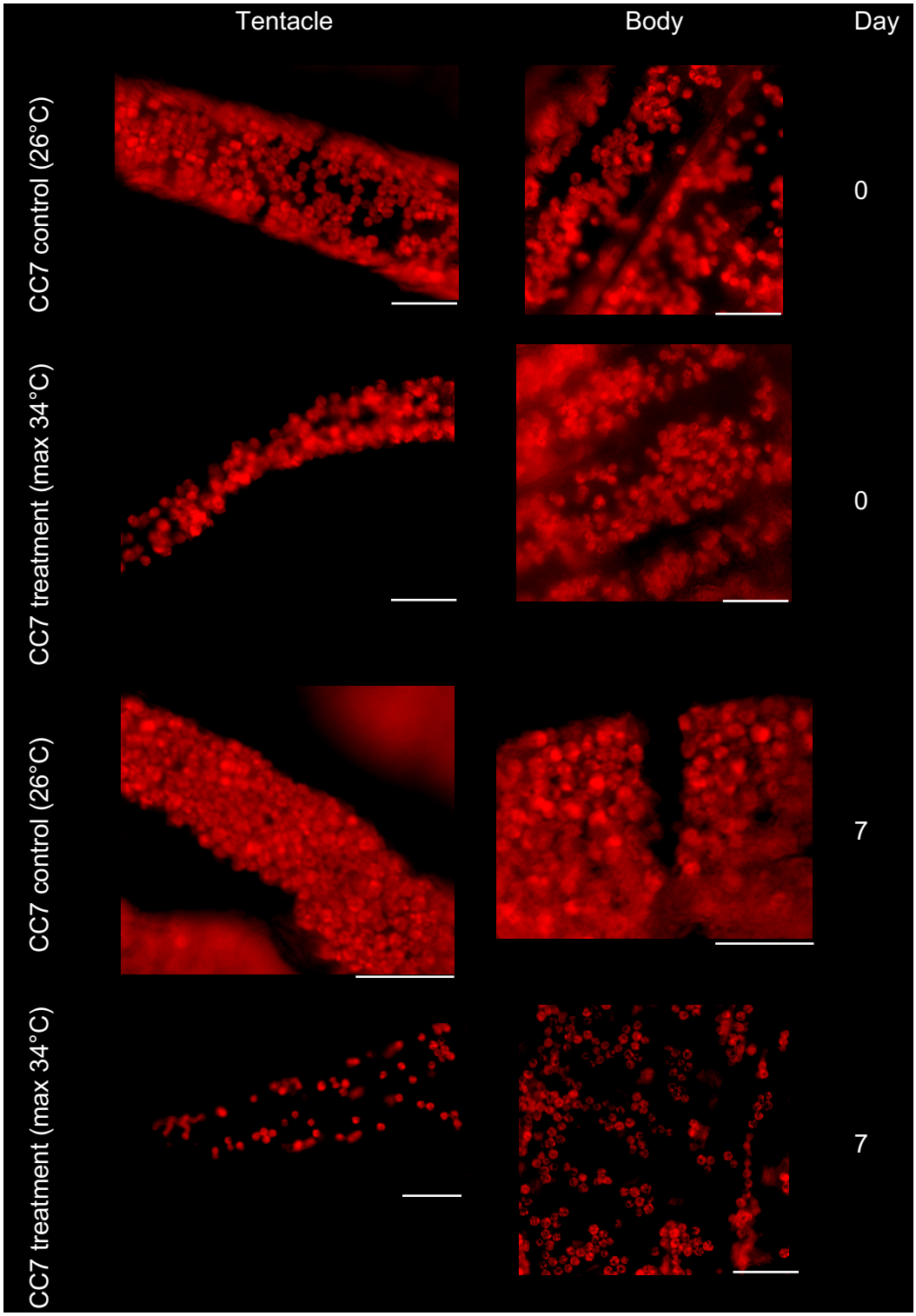


Figure 4-10. The distribution of *Symbiodinium linuchae in Exaiptasia pallida* CC7 during the experimental period. Columns show the part of the body (tentacle or body) and day of sampling (0 or 7), while the rows the treatment they were under (control vs treatment). Scale bars: 200 μm

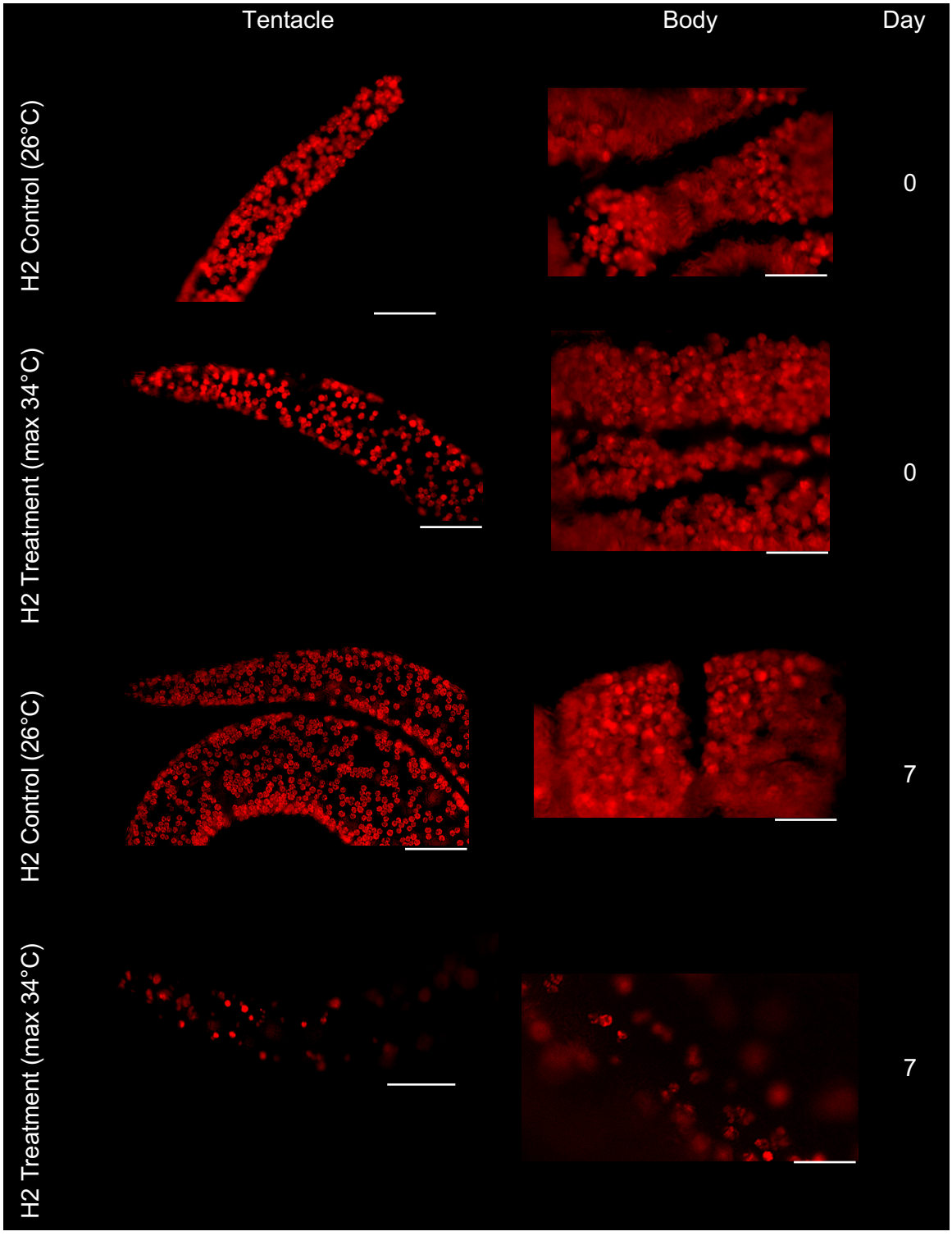


Figure 4-11. The distribution of *Breviolum minutum in Exaiptasia pallida* H2 during the experimental period. Columns show the part of the body (tentacle or body) and day of sampling (0 or 7), while the rows the treatment they were under (control vs treatment). Scale bars: 200 μm

4.3.3 Symbiodiniaceae density is positively correlated with chlorophyll fluorescence intensity but not with anemone weight

There was a significantly positive relationship between Symbiodiniaceae density and chlorophyll fluorescence intensity ($P < 0.0001$, $y=584 + 0.042 x$, $R^2 = 0.39$). When the values were divided by the day of sampling, at day 0 the relationship was no longer significant ($P = 0.378$, $y = 822 + 0.028 x$, $R^2 = 0.08$). At day 7 the correlation was significantly positive ($P = 0.002$, $y = 587 + 0.041x$, $R^2 = 0.65$), while at day 21 there was no correlation similarly to day 1 ($P = 0.418$, $y = 786 + -0.014$, $R^2 = 0.08$) (Figure 4-12).

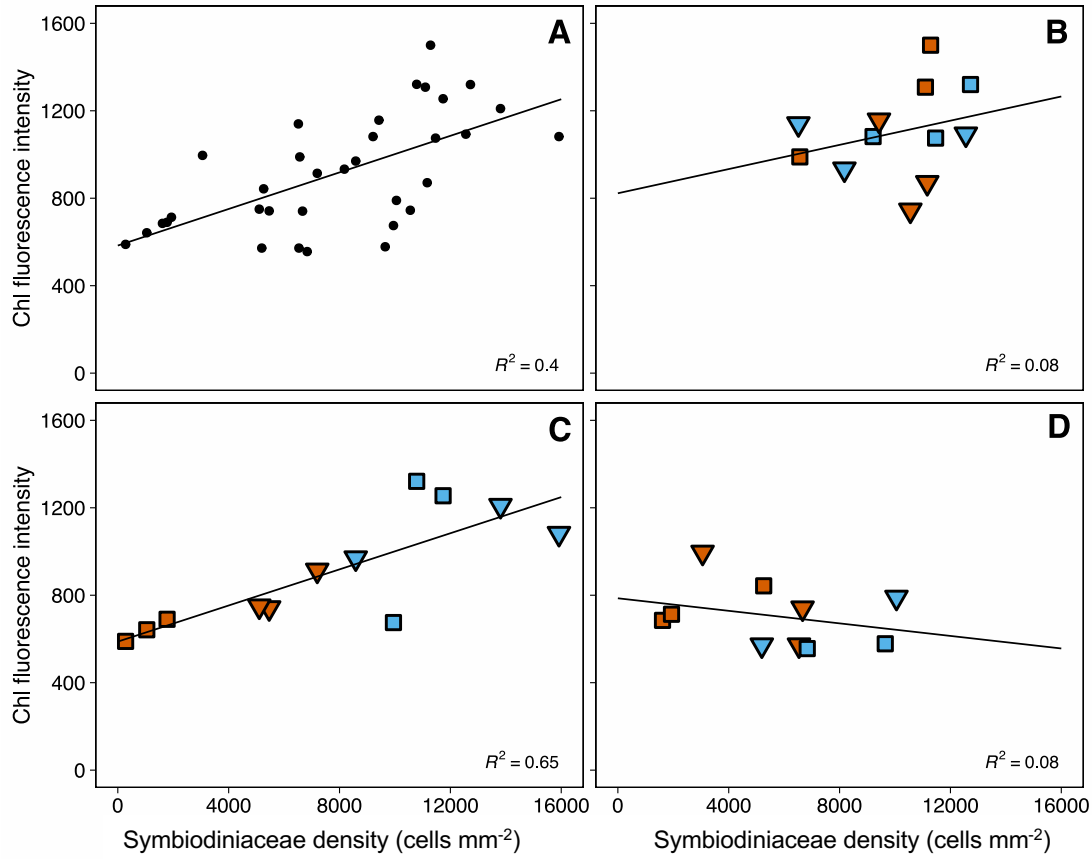


Figure 4-12. Correlations between chlorophyll fluorescence intensity and Symbiodiniaceae density (cells mm^{-2}) in *Exaiptasia pallida*. R-squared values are showing at the bottom of each figure. A, data is represented with no distinctions among strain, experimental conditions or day of sampling. B shows day 1 of sampling, C shows day 7 and D represents day 21. In panels B-D, strain CC7 is represented by the inverted triangle and H2 by the square. The colour blue represents the control samples kept at 26°C throughout the experiment, while in red the samples that have been subjected to a temperature increase in the first 7 days.

There was no statistically significant relationship between Symbiodiniaceae density and anemone weight (P = 0.968, y=7909.31 + 2893.81 x, R^2 = - 0.002). When the values are divided by the day of sampling, at day 0 the relationship was still no significant (P = 0.675, y = 9547.56 – 29764.86 x, R^2 = 0.04). At day 7 the correlation was still absent (P = 0.783, y = 8680.42 – 45563.69 x, R^2 = 0.02), and at day 21 there was no correlation similarly to day 1 (P = 0.709, y = 4831.90 + 36166.27, R^2 = - 0.001) (Figure 4-13).

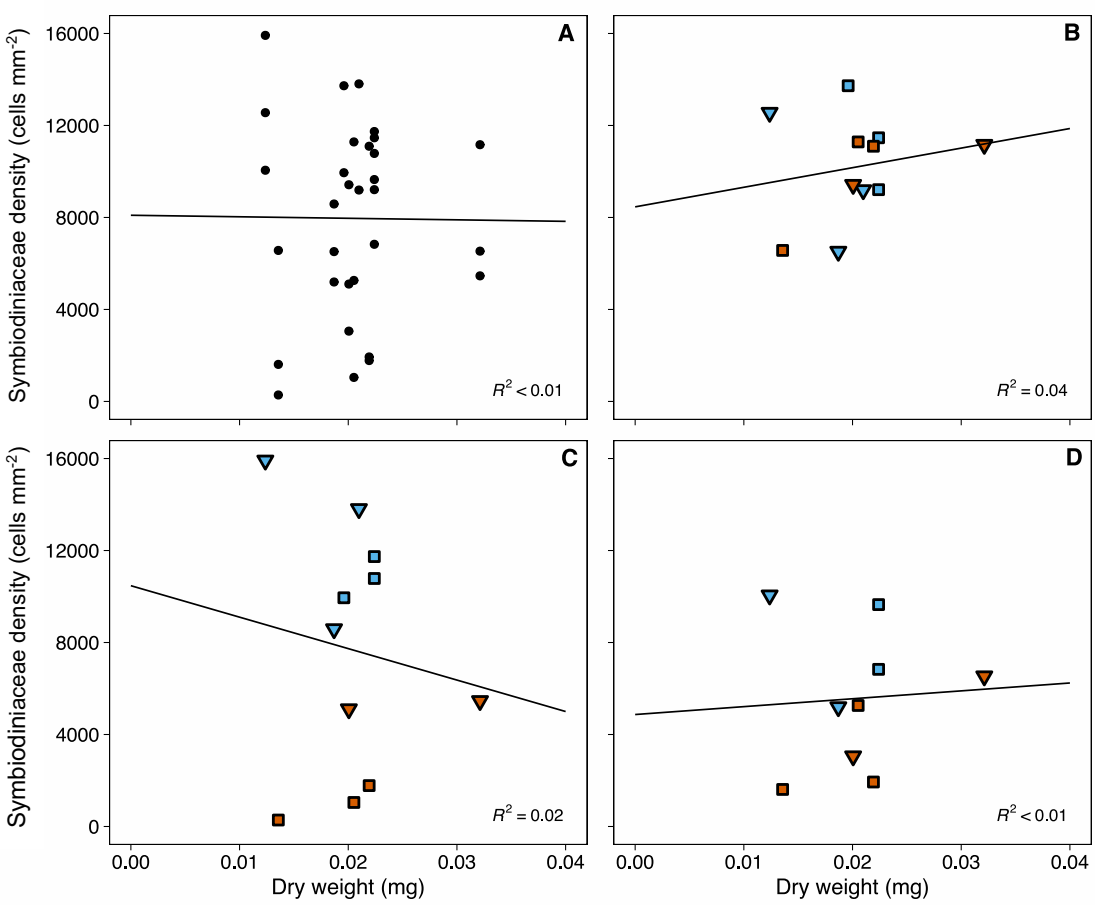


Figure 4-13. Correlation between Symbiodiniaceae density (cells mm^{-2}) and dry weight of *Exaiptasia pallida*, R squared values are showing at the bottom of each figure. A, data is represented with no distinctions among strain, experimental conditions or day of sampling. B shows day 1 of sampling, C shows day 7 and finally D represent day 21. In the last three figures, strain CC7 is represented by the inverted triangle and H2 by the square. The colour blue represents the control samples kept at 26°C throughout the experiment, while in red the samples that have been subjected to a temperature increase in the first 7 days.

4.4 Discussion

4.4.1 Bleaching takes place in both *Exaiptasia* strains subjected to heat stress

Short term heat stress of *Exaiptasia pallida* resulted in significant loss of Symbiodiniaceae (i.e. bleaching) in both strains, consistent with previous studies (Dunn et al., 2000). Although not statistically significant, the different percentage of algae loss observed between the two strains at high temperature could be attributed to the thermotolerance of the Symbiodiniaceae hosted in the anemone. CC7 seemed to retain more algae thanks to its thermotolerant symbionts but did not recover them two weeks after the loss. On the contrary, H2 expelled the 88.39% of its algal symbionts but was capable of rehosting some of them, unlike CC7. More experiments and more replication are needed to prove whether CC7 and H2 lose and regain Symbiodiniaceae at a different pace. Another explanation for CC7 having lost less cells is that bleaching was occurring with necrosis and apoptosis-like cell death and cells were simply not expelled but dying (Dunn et al., 2002, 2004). This hypothesis does not reflect the result obtained from measuring the maximum quantum yield of the photosystem II (PSII) in Chapter 3.3.2 (p. 114), on the same strains and temperature profile. H2 showed a decline in F_v/F_m from day 5, while CC7 seemed unaffected by the heat. This could suggest that the cells in CC7 were healthy and not necrotic as previously thought as a possible cause for less severe bleaching, confirming their thermotolerant ability.

A different theory to explain loss of symbionts is the quantity they have to begin with. Cunning and Baker (2013) suggested that excess algal symbionts increase the susceptibility of reef corals to bleaching (Cunning & Baker, 2013), the opposite of what was previously suggested by Stimson et al. (2002). The first

argues that symbiont loss could be seasonal and this is done to prevent excessive symbiont load (Stimson et al., 2002). This phenomenon should not be regarded as bleaching due to stress, but it is instead a regulatory mechanism of the animal in order to reduce future bleaching susceptibility.

Our anemones had roughly an equal density of cells and therefore heat stress is the cause of bleaching in our treatment samples. But the loss of Symbiodiniaceae also in the control animals could be attributed to this regulatory mechanism. Nesa and Hidaka (2009) also showed that high density of Symbiodiniaceae is not beneficial with high-temperature stress, probably due to the higher concentrations of ROS (Nesa & Hidaka, 2009). The difference in bleaching severity could instead be due to the higher production of reactive oxygen species (ROS) from the non-thermotolerant Symbiodiniaceae (Lesser et al., 1990; Nielsen et al., 2018).

The decline of symbionts even in the control samples at day 21 could seem unusual, but it is known that algae undergo degradation at any given time, making it even more difficult to understand this process (Bieri et al., 2016). The reef-building coral *Galaxea fascicularis*, that was similarly exposed to heat stress, lost the ability of controlling Symbiodiniaceae host cell division and therefore resulting in an unexpected increase cells mitotic index (i.e. the ratio between the number of cells in a population undergoing mitosis to the total number of cells in a population) (Bhagooli & Hidaka, 2002). There are still numerous unsolved questions regarding bleaching, one of which is whether bleaching is the result of an adaptive stress response that leads to a selective reduction of symbionts (later followed by a repopulation by stress-adapted Symbiodiniaceae strains) or if it is

simply an injury due to external stress (Buddemeier & Fautin, 1993; Kinzie et al., 2001; Rowan, 2004).

4.4.2 Chlorophyll fluorescence intensity can give a rough estimate of *Exaiptasia* bleaching status

Chlorophyll content in algae is known to be variable due to different environmental conditions and nutritional status of the holobiont (Takahashi et al., 2013). We found nonetheless a positive correlation between fluorescence and Symbiodiniaceae density, as previously seen in corals (Huffmyer et al., 2020). In Figure 4-12 (C) there is a clear division between control and treatment samples, having control samples with higher chlorophyll fluorescence per Symbiodiniaceae density and the opposite for treatment. This could be a fast way to assess the bleaching status of an anemone. Bellis and Denver (2017) also found anemone fluorescence as a highly significant predictor of visual bleaching status (Bellis & Denver, 2017). They have also looked into the fluorescence difference in the brightest part of the anemone compared to the tentacles, finding diversity among the strains exposed to heat. We have instead found difference when analysing the density of cells present in H2, but not in CC7. The unbalanced distribution of algae in H2 was not due to the heat stress ('treatment' variable was removed from analysis) and only occurred at day 0 and day 21, having more algae in the body than in the tentacles. Gabay et al. (2018) observed that Symbiodiniaceae colonise aposymbiotic *Exaiptasia* starting from the oral disk, where they are taken up. Their proliferation continues in a patchy pattern to the tentacles, and it continues through mitotic division and gastrovascular migration. Once the tentacles are fully colonized, Symbiodiniaceae start taking over the upper part of

the column and work their way down, reaching the pedal disk at last (Gabay et al., 2018). The lack of correlation between anemone size (mg dry weight) and algae density was a positive result, suggesting that specimen used were not 'discriminated' by their size.

Further studies in this area will help us better understand the symbiotic relationship between Symbiodiniaceae are the animal host, especially under heat stress. Imaging is definitely a good tool to establish the density of algae inside the same specimen, allowing to repeat measures over time.

4.4.3 DMS/P are dependent on the presence of Symbiodiniaceae

This chapter reproduced the temperature increase of chapter 3, using the same anemones inoculated by the same strains: SSA01 and SSB01. The results described here are in line with what was visually observed in chapter 3: both anemones have bleached and although not significantly H2 lost more symbionts than CC7. This further proves that the lack of DMSP and concentration increase is due to the absence of the Symbiodiniaceae symbionts.

This finding is in disagreement with the research done in coral *Acropora aspera* (Deschaseaux et al., 2014). With elevated temperature they have also recorded a significantly lower amount of cell density in the animal, but also a significant increase of DMS/DMSP/DMSO. They have in fact concluded that this observation was really interesting since it is the alga that normally is the dominant source of these compounds and therefore they were expecting a lower amount with bleaching. On the contrary, Gardner et al. (2017) found that thermal stress caused a significant decline in concentrations of DMSP in coral *Fungia granulosa*, but unfortunately neither bleaching nor cell counts were mentioned in the study.

As far as I am aware, there is a gap in the literature regarding this subject. Most studies focus on the concentration of the different sulphur compounds in different stress scenarios but very few make a direct link with the number of algae present in the animal at that specific point in time.

4.4.4. Conclusions

This study highlights the advance in the field of fluorescence microscopy, by demonstrating that cell counting in anemones can occur on the same specimens in a non-invasive way. In chapter three, bleaching was observed and the importance of knowing the number of cells present in the organism became obvious. This chapter has developed a methodology that works for this purpose. Bleaching was recorded, similarly to the previous chapter and now a clear connection between lack of DMSP and reduced number of symbionts can be made. *Exaiptasia pallida* CC7 lost more cells than H2, but was also able to recover them after a two weeks period. The location of the cells within the anemone (i.e. body vs tentacle) is not significantly different between control and treatment. Chlorophyll fluorescence intensity could give a rough estimate of bleaching in *Exaiptasia*, especially since size does not seem to be a discriminating factor in algal density.

Future studies should focus on bleaching in different species and the actual recovery time of the algal symbionts, also better explore the correlation between DMSP/DMS concentration and bleaching, since there is not an agreement on this subject.

Chapter 5 The importance of a realistic long-term high temperature experiment in understanding the effects in *Exaiptasia pallida* and their symbiotic algae

5.1 Introduction

Coral bleaching caused by ocean warming is one of the major drivers of decline in tropical coral reefs (Hughes et al., 2003). As discussed in Chapter 1.6 (p. 29), bleaching is the breakdown of the mutualistic symbiosis between the animal host and algae of the Symbiodiniaceae family, ultimately leading to death of the animal and possibly the algae, too (Weis et al., 2008). Photosynthesis by symbiotic algae is the main source of energy for Scleractinia corals. Photosynthetically fixed carbon is moved to the host, where it supports growth, respiration and reproduction corals (Davy et al., 2012; Muscatine, 1990). The partial or complete loss of symbiont populations together with the reduced photosynthetic efficiency of remaining symbionts (Lesser, 2006) damage the photosynthetic capacity of the coral colony and can therefore greatly impact on a coral colony's energy balance (Fitt et al., 2000). Although several causes may result in coral bleaching, the importance of the increase in reactive oxygen species (ROS) in disrupting the symbiosis, damaging cell membranes, lipids, proteins and DNA, is now established (Lesser, 2006; McLenon & DiTullio, 2012). Enzymes like superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) assist with scavenging ROS to avoid damage to the organisms. The production of DMSP/DMS and chlorophyll have also been associated with high ROS presence, with a higher amount of these compounds suggesting a response to an increase of ROS in order to prevent the oxidative damage (Chapter 1.6.2, p. 35).

The difference between survival and death is an intricate combination of different factors, which ultimately determine the threshold of the organisms to heat but also how bleaching differs over space and time in different geographical regions plays a vital role (Osman et al., 2018). This is due to three main reasons. First, the coral/anemone holobiont is able to adapt locally to specific thermal regimes by selecting Symbiodiniaceae species that are more tolerant to heat (Hume et al., 2015) and also through 'genetic rescue', which involves the exchange of heat-tolerant genotypes across latitudes, a regulatory mechanisms of the animal host (Dixon et al., 2015) and their associated bacteria (Ziegler et al., 2017). In fact, corals that naturally live in a high-temperature environment produce healthy reef populations with an elevated tolerance to bleaching (D'Angelo et al., 2015; Osman et al., 2018). Second, bleaching severity is variable depending on the duration of the event (Anthony et al., 2007) and the periodicity of the sea-surface temperature (SST) anomalies relative to historical SST patterns (Heron et al., 2016). For example it was demonstrated that reoccurring thermal events can actually increase stress tolerance (Palumbi et al., 2014). Third, thermal stress is also due to other environmental factors such as light intensity, salinity and nutrient concentration (Suggett & Smith, 2011).

Despite the decades of bleaching, we still have a limited understanding on what determines a coral-bleaching threshold due to absence of standardisation across different studies (Voolstra et al., 2020). Long-term surveys in the field would be the most informative in detecting bleaching susceptibility, since the severity, frequency and hypothetical recovery are direct measures of the corals' response to environmental stress. Unfortunately, they have numerous limitations since bleaching events are difficult to predict, and large-scale in situ surveys are

expensive and time consuming. The second-best alternative is long-term laboratory experiments with high-temperature exposure that mimic the intensity and timing of natural thermal-stress events. This chapter aims to use recorded sea-surface temperature data to reproduce a heat-stress event that naturally occurred in the Florida Keys over a period of several months in 2019 (Coral Reef Watch - NOAA). The heat event was followed until the temperature reached 32°C, but we continued to a maximum of 34°C and lowered it back to 32°C. In comparison with data presented in Chapter 3, the results will also elucidate whether short- and long-term heat stress have the same effect on the studied anemone *Exaiptasia pallida* and their symbiotic algae (*Symbiodinium linuchae,* SSA01 and *Breviolum minutum*, SSB01).

In order to make a comparison between chapter three and five, the same variables will be analysed. This study therefore aims to assess if Symbiodiniaceae and *Exaiptasia pallida* behave similarly in a realistic long-term high temperature experiment, focusing on the concentration of DMSP and DMS in both organisms to better understand future trends. It is importance to establish whether DMSP/DMS decreases or increases during a bleaching event, given the lack of agreement in this area of research.

Four main hypotheses were generated for this heat-stress experiment:

H1 The rate of growth and cell concentration of SSA01 and SSB01 will decrease with increasing temperature (from 26°C to 34°C).

H2 Maximum photochemical efficiency in both Symbiodiniaceae and anemones will decrease in samples subjected to increasing temperature (from 26°C to 34°C).

H3 DMSP/DMS and chlorophyll-*a* concentrations in Symbiodiniaceae and anemones subjected to high temperature (from 26°C to 34°C) will increase.

H4 Short- and long-term heat stress have a different effect on the studied anemone *Exaiptasia pallida* and their symbiotic algae.

5.2 Methodology

Unless otherwise stated, methodology (i.e. maintenance of algal stock cultures, growth and volume of algal cultures, spectrophotometric quantification of algal chlorophyll-*a* and -c_2, *Exaiptasia* husbandry and biomass estimation, quantification of DMSP and DMS and Fast Repetition Rate fluorometry) in this chapter is identical to the one in Chapter 3.

5.2.1 Experimental design

Two experiments were carried out at separate times but following similar procedures: Experiment #1 focused on the algae and took place from 16th October 2020 to 29th January 2021 with SSA01 *Symbiodinium linuchae* and SSB01 *Breviolum minutum*. Experiment #2 focused on the host animals and was

conducted from 18th March 2021 to 18th May 2021 with *Exaiptasia pallida* CC7 and H2.

Algal cultures were inoculated from a stock culture at a dilution of 1:10 (22 mL culture plus 198 mL medium in 500 mL flasks) on 16/17 October 2021, the 21st day of growth when cells were in the stationary growth phase. Six treatments were prepared in biological triplicates and included control for both cultures of SSA01 (C-A) and SSB01 (C-B), high temperature for both cultures of SSA01 (HT-A) and SSB01 (HT-B), only f/2-si medium control (M-C) and only f/2-si medium high temperature (M-HT). Cultures and controls were incubated for four days under control conditions (i.e. 26°C) to reach the onset of exponential growth, before samples were first taken. As it was logistically impossible to carry out all measurements for 12 samples on the same day, measurements of control and treatment samples were staggered by one day, hence, start of sampling commenced on 20th October for control and 21st October for high-temperature treatments. On day 5 of growth, 'high-temperature' cultures and controls (HT-A, HT-B and M-HT) were moved to a different incubator (LMS, model 300) set to 27°C with the identical light:dark regime and light intensity as in the control treatment. In this incubator, the temperature was gradually increased to 34°C over four successive months, kept at 34°C for 5 days and lowered to 32°C until the end of the experiment (Figure 5-1). Samples were arranged randomly on different shelves, swirled every other day to resuspend settled material and moved among the incubator's shelves to ensure that all replicates experienced similar light intensities integrated over the growth period.

In reporting Symbiodiniaceae results, samples will be referred to as 'control' (samples kept at 26°C for the entire growth and experimental period = C-A, C-B

and M-C) and 'treatment' (samples exposed to higher temperature during experimental period = HT-A, HT-B and M-HT). All cultures and control were refreshed with new f/2-si medium on day 21 of growth and measurements were repeatedly conducted throughout the experiment on day 5 of growth (log phase) and day 21 of growth (stationary phase). On 30th November, samples for DMSP and DMS concentration in Symbiodiniaceae could not be processed due to GC malfunction. Instead, they were taken on 2nd December (day 8 on growth curve) including cell counts for normalisation.

Experiment #2 focused on *Exaiptasia* strain CC7 and H2 that were kindly provided by Dr Nick Aldred (University of Essex, UK). They were kept at similar growth conditions as the Symbiodiniaceae (i.e. at 26 °C under 12:12 h light:dark regime, ~ 80 μmol photons m^{-2} s^{-1}, from a 37 W fluorescent light), in 1 L plastic containers filled with 800 mL autoclaved artificial seawater (ASW) made by mixing artificial salt (D-D H_2Ocean) with reverse-osmosis water to a salinity of 35, pH 8. Animals were target fed twice a week with 'Cyclops – gamma blister' (Urmston Aquatics, Manchester, UK) that had been previously washed with RO water and autoclaved. Water was changed approximately 5 h after each feed and containers cleaned weekly from algae, with the aid of cotton swabs.

This experiment was affected by a power cut on 3rd May 2021 and the temperature for anemones in both control and treatment decreased below the desired values for seven hours. For example, in the treatment incubator, temperature was 31.5°C at 05:31h, reached the minimum of 18.8°C by 09:31 h when power to the incubator was restored and reached the original temperature again at 12:31h (Figure 5-1).

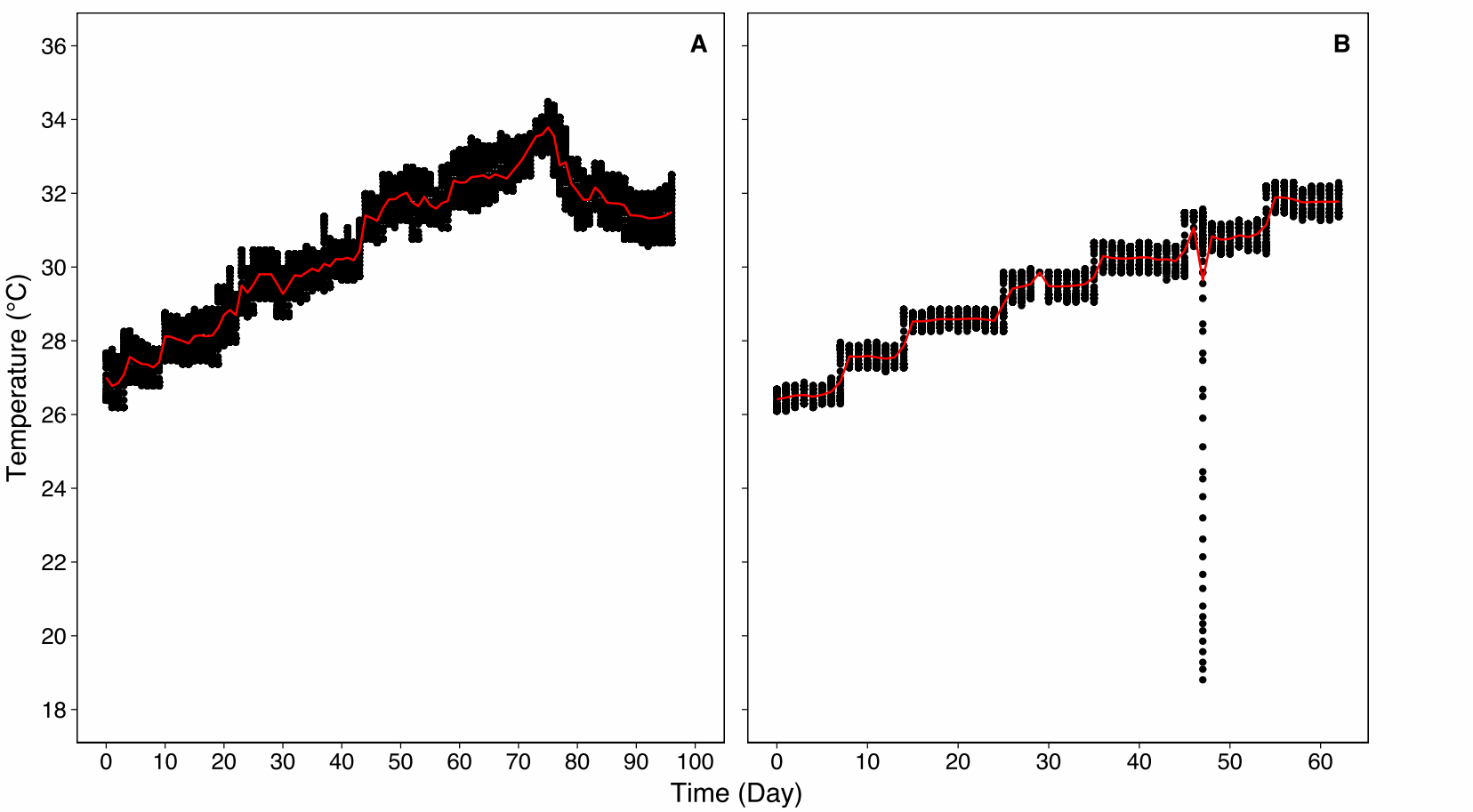


Figure 5-1. (A) Temperature settings during Experiment #1 that focused on Symbiodiniaceae and (B) temperature settings during Experiments #2 on Exaiptasia. Shown are the daily readings (black), red line is the mean temperature recorded daily, temperature recorded with 10 minutes interval between each reading.

5.2.2 Statistical analysis

Graphical representations and statistical analysis were conducted using R software (R Studio Version 1.2.5042). Statistical significance was determined using General Linear Mixed Models (GLMM), Linear Mixed-Effect Models (LME) and Tukey HSD post-hoc tests, after the assumptions for each model were tested. The model was always run with three factors: DAY (ten levels), TEMPERATURE (two levels: control, treatment), STRAIN (two levels: A, B) or ANEMONE STRAIN (two levels: CC7 vs H2). If the model with three factors was not significant, the factor 'STRAIN'/'ANEMONE STRAIN' would be removed and the model would be run again while keeping data for the two clades/species separate. Akaike Information Criterion (AIC) was used to determine the best model to use.

5.3 Results

5.3.1 Symbiodiniaceae differ under heat stress

Cell concentration on day 21 of growth at 28°C was higher in SSB01 than in SSA01, but at 30°C were similar (963,631±46,837 cells/mL and 822162±19,114 cells/mL for SSA01 and SSB01, MEAN±S.E. of both control and treatment). Once the temperature reached 32°C strain SSA01 cell concentration started to significantly decrease in comparison to control samples, while strain SSB01 seemed unaffected by the increase in temperature. Symbiodiniaceae mean cell concentration at 34°C was 606,814±37,006 cells/mL for control and 51,614±13,694 cells/mL for treatment in SSA01, while SSB01 734,037±23,361 cells/mL and 232,447±68,093 cells/mL respectively and they were statistically different compare to their respective control. Although the

temperature was lowered to 32°C, growth in both strains did not recover, having final cell concentrations of 8,454±1,288 and 10,753±1,212 cells/mL, respectively, while cultures at control temperature showed 617,097±7,312 cells/mL for SSA01 and 772,453±77,016 cells/mL for SSB01. Cell concentration in Symbiodiniaceae was statistically significantly different among the two strains, temperature treatment and days ($LME_{Strain * Temp * Day}$, P = 0.0004) (Figure 5-2).

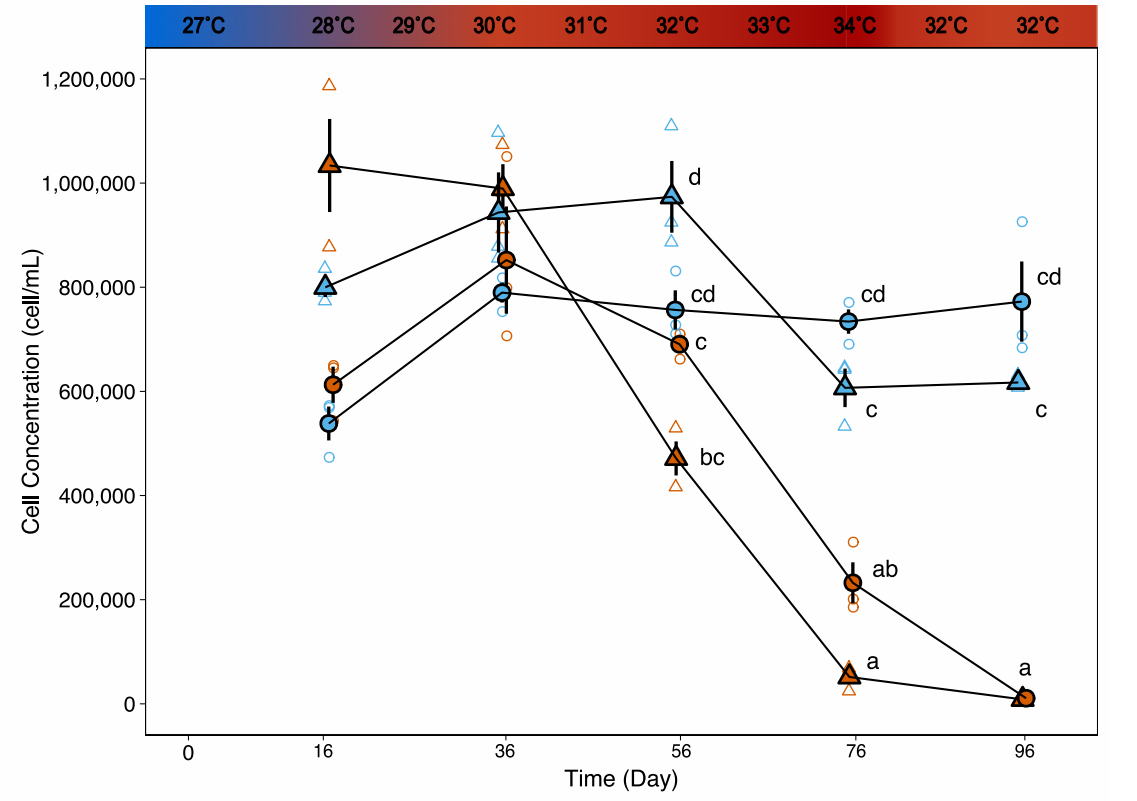


Figure 5-2. A Cell counts of Symbiodinium linuchae SSA01 (triangles) and *Breviolum minutum* SSB01 (circles), displayed only growth at day 21. Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size. Data points for treatment are offset by 0.5 days along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Letters are a short summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance.

The average growth rate of Symbiodiniaceae cells in control samples was 0.162±0.006 d^{-1} in strain SSA01 and 0.209±0.009 d^{-1} in SSB01, while it was 0.095±0.017 d^{-1} and 0.143±0.017 d^{-1}respectively, MEAN±S.E. of day 16, 36, 56, 76 and 96 (Figure 5-3). The two strains were not significantly different between them when analysed by day and treatment (P = 0.115). SSA01 showed a steady decline in growth in treatment samples, and when the temperature reached 34°C the growth was significantly lower than at control temperature. Growth was still significantly lower after a 'heat break' at 32°C with a final growth of 0.01±0.004 d^{-1} while 0.143±0.012 d^{-1} in control samples, ($LME_{Temp * Day}$, P = <.0001). Strain SSB01 at higher temperature had a slower decline compared to SSA01, only at the last sampling date the growth rate was significantly lower than control cultures, 0.18±0.01 d^{-1} and 0.035±0.004 d^{-1} respectively, ($LME_{Temp * Day}$, P = 0.001).

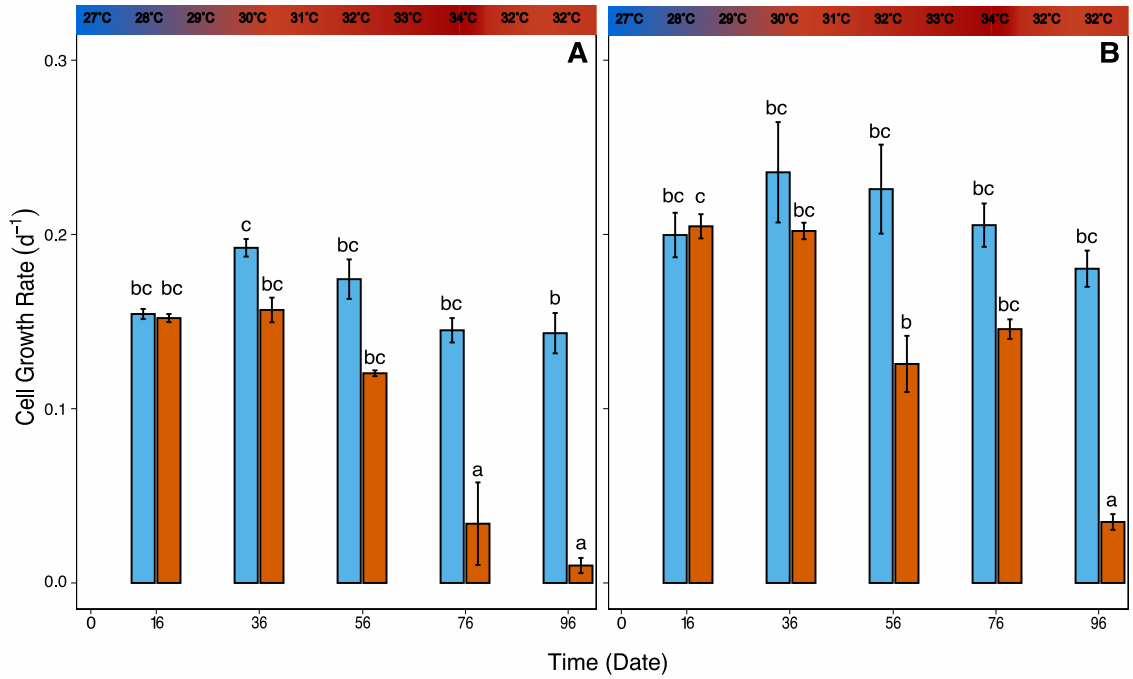


Figure 5-3. Cell Growth Rate (d^{-1}) of SSA01 *Symbiodinium linuchae* (A) and SSB01 *Breviolum minutum* (B), blue for control and red for treatment. Data represents mean±SE, n=3. The bar at the top of the figure shows the temperature at time of sampling for 'treatment'. Letters are representing the significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance.

SSA01 had a larger cell volume (402.5±36.63 μm^3/cell) from the early stage of growth (day 5), while SSB01 reached ~400 μm^3/cell only at day 21. Cell volume between the two strains was significantly different ($LME_{Strain * Temp * Day}$, P = <.0001; Figure 5-4). Cell growth was similar between control at treatment in both strains until when the temperature reached 32°C, SSA01 had a significantly greater cell volume in treatment samples but SSB01 did not. Cell volume in SSA01at high temperature decreased steadily over the following weeks, having a significant lower volume of 164±17.79 μm^3/cell on the last sampling day, compared to 468 μm^3/cell in control samples. Strain SSB01 also showed a decline in treatment samples over time, and on the last day of the experimental

period the difference in cell volume between control and treatment Symbiodiniaceae was not significant, 256 μm^3 and 135 μm^3 respectively.

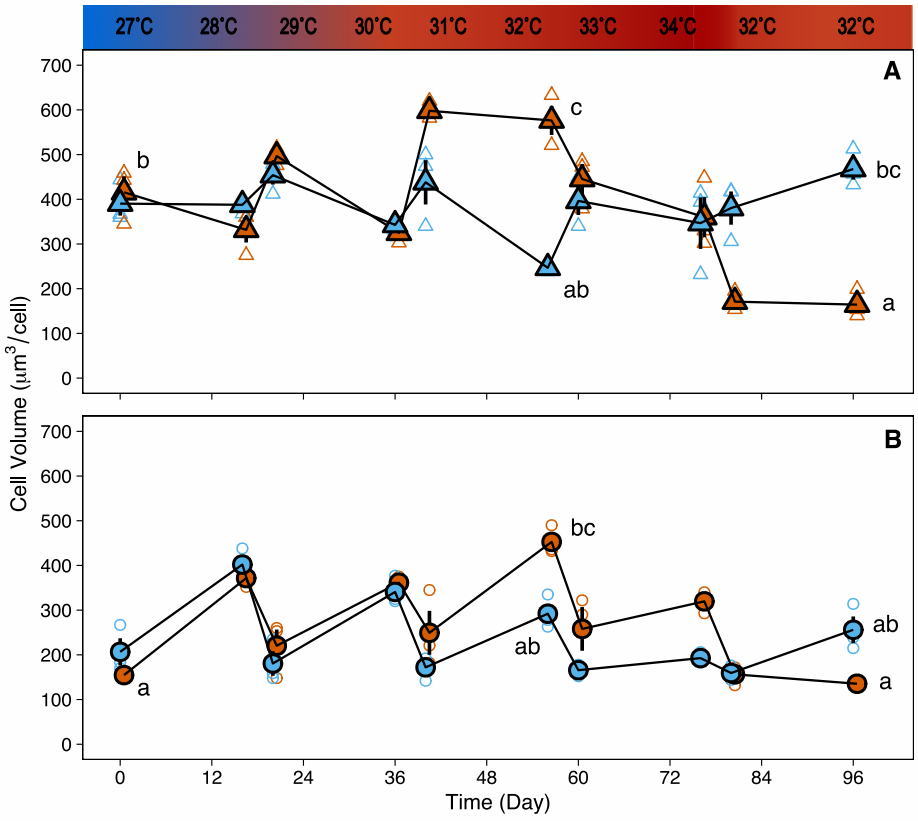


Figure 5-4. Cell volume (μm^3/cell) for *Symbiodinium linuchae* SSA01 (triangles, **A**) and *Breviolum minutum* SSB01 (circles, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Short summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance. Not all significances are shown.

Chlorophyll-*a*, normalised by the total amount of cells present in each sample, was significantly different among strain, day and treatment ($LME_{Strain * Temp * Day}$, P = <.0001). SSA01 had a stable amount of chl-*a* throughout the experimental period, with no significant difference between control and treatment sampled on the same day (Figure 5-5). SSB01 had, instead, a significantly lower amount of chl-*a* once the temperature reached 34°C, having 10.28±0.92 at control and 1.635±0.34 pg/cell at treatment and also when the temperature was lowered to 32°C. Also to note that the amount of chl-*a* present in SSB01 was always higher in control samples at an early growth stage (day 5) of the algae compared to SSA01, with a mean± S.E. of 9.032±0.5 pg/cell and 3.469±0.4 pg/cell respectively. Chlorohyll-c_2 was not significantly different among strain, day and treatment ($LME_{Strain * Temp * Day}$, P = 0.064), and the pattern followed by each strain was similar to chl-*a* (Figure 5-5). Chl-c_2 in strain SSA01 was not significantly different among days and treatment ($LME_{Temp * Day}$, P = 0.319), and also SSB01 ($LME_{Temp * Day}$, P = 0.503).

The amount of chlorophyll-*a* and -c_2 is greater in cultures during their exponential growth (day 5) than stationary phase (day 21), hence the noticeable zig-zag pattern.

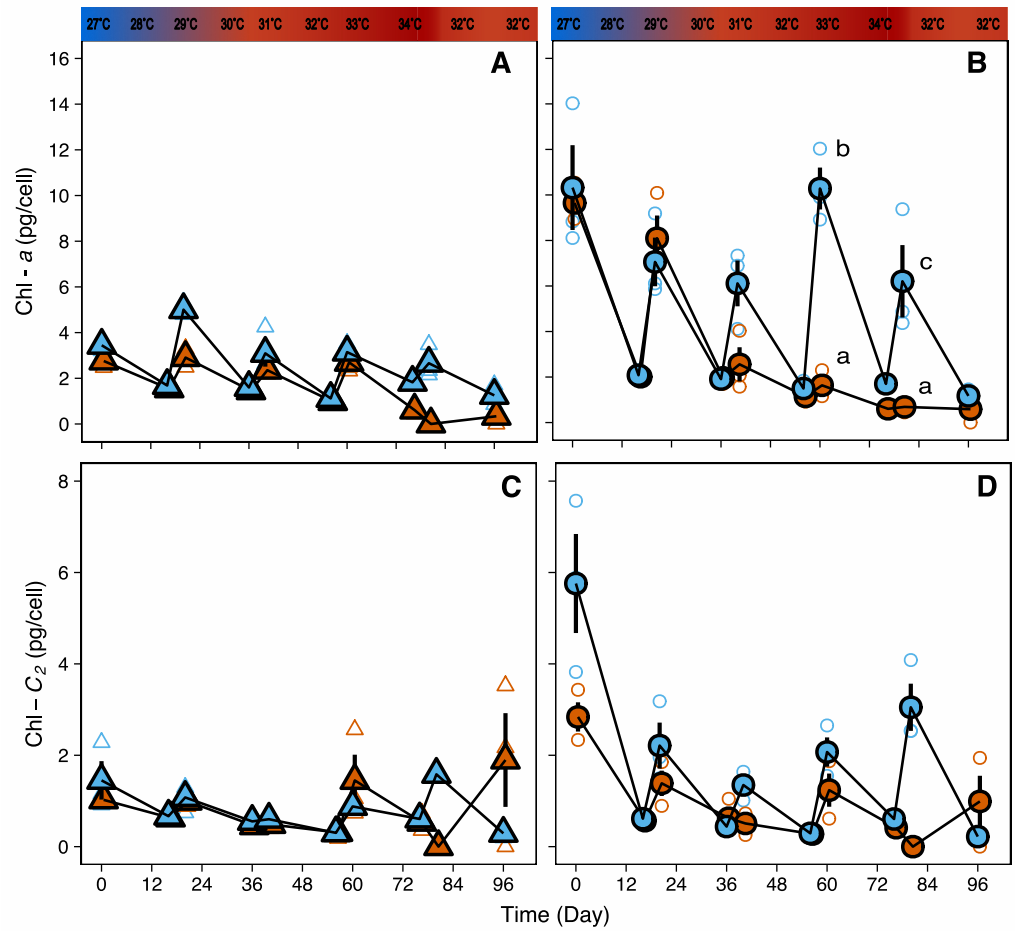


Figure 5-5. Chlorophyll-a (**A** and **B**) and Chlorophyll-c_2 (**C** and **D**) for Symbiodinium linuchae SSA01 (triangles) and Breviolum minutum SSB01 (circles). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. (**B**) Short summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance.

Maximum photochemical efficiency (F_v/F_m) was significantly different across temperature, day and strain ($LME_{Strain * Temp * Day}$, P = <.0001), Figure 5-6. SSA01 had generally a higher value in control samples compared to SSB01, 0.369±0.004 and 0.311±0.009 respectively. The pattern between control and treatment was very similar until the temperature reached 34°C, when both strains had a significant lower F_v/F_m compared to control samples. SSA01 control was 0.343±0.004 and treatment 0.121±0.0107, while SSB01 was 0.253±0.012 and 0.13±0.0126 respectively. At the last sampling day, both strains under heat stress had a lower value compared to control samples and SSA01 recorded the lowest F_v/F_m of the experiment (0.082±0.0257), while SSB01 had an increase to 0.185±0.015.

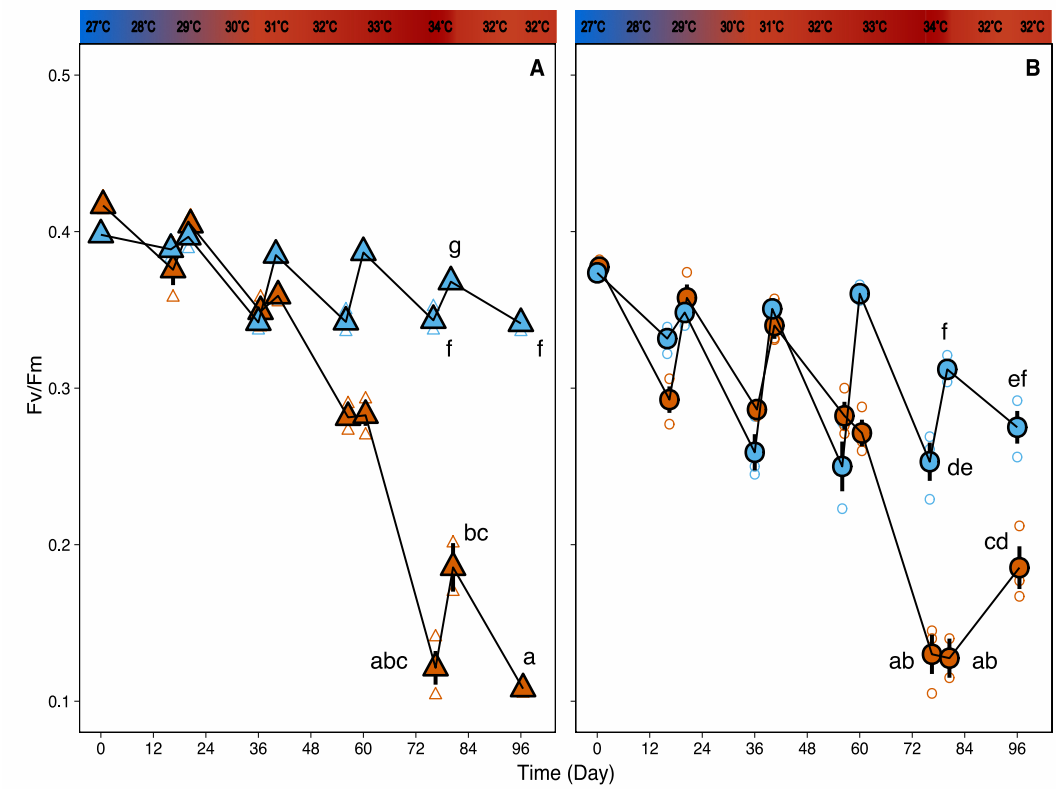


Figure 5-6. Maximum photochemical efficiency (F_v/F_m) in *Symbiodinium linuchae* SSA01 (triangles, **A**) and *Breviolum minutum* SSB01 (circles, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Data points are offset along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Letters are summary of significance by Post-hoc Tukey's test. Means followed by a common letter are not significantly different, 5% level of significance.

The concentration of DMSP (fmol/cell) in Symbiodiniaceae across day, temperature and strain was significantly different ($LME_{Strain * Temp * Day}$, P = 0.006) (Figure 5-7). SSA01 at control had a lower concentration throughout the experimental period compared to SSB01, 92.30±8.3 and 179±27.88 fmol/cell respectively. Strains subjected to heat stress both showed a decline compared to the respective control, of 15.54% (78.99±7.32 fmol/cell) for SSA01 and 43.00% (116.23±15.15 fmol/cell) for SSB01. The pattern of both Symbiodiniaceae was very similar and once the temperature was lowered down to 32°C the

concentration of DMSP was significantly lower than in control samples only in SSB01. DMSP concentration was also normalised for mmol/L · CV and the results were almost identical ($LME_{Strain * Temp * Day}$, P = 0.006).

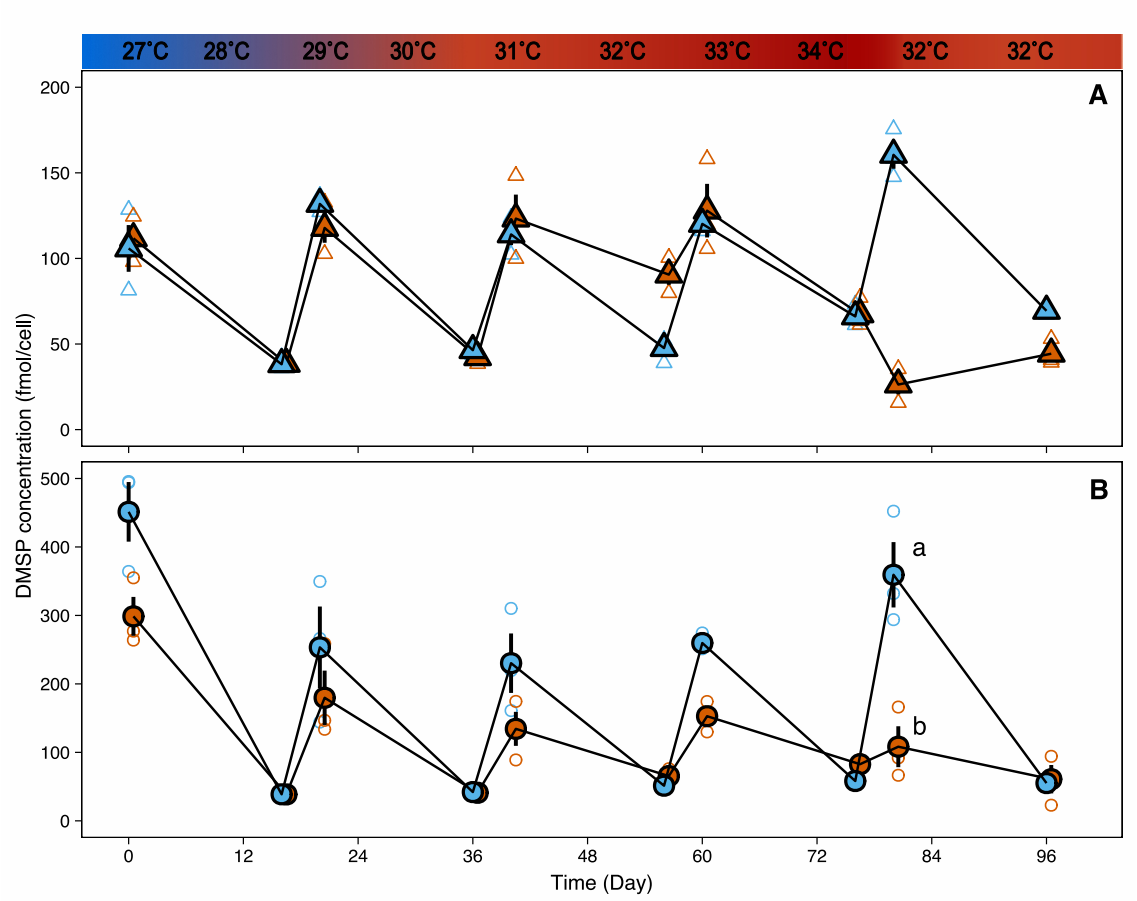


Figure 5-7. DMSP concentration (fmol/cell) in Symbiodinium linuchae SSA01 (triangles, **A**) and Breviolum minutum SSB01 (circles, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red), note y-axes have a different scale. Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSB01, means followed by a common letter are not significantly different, 5% level of significance.

DMSP concentration normalised by chlorophyll-*a* (nmol/µg · chl-*a*) showed a statistically significant different behaviour between Symbiodiniaceae, day and treatment ($LME_{Strain * Temp * Day}$, P = <.0001) (Figure 5-8). With chlorophyll-a normalisation, DMSP concentration was similar in control samples throughout the experiment, 39.20±2.62 nmol/µg · chl-*a* for SSA01 and 37.11±2.97 mmol/µg · chl-*a* for SSB01 and the pattern was virtually identical and stable. Heat-stressed samples, instead started to exhibit a change when the temperature reached 32°C. SSA01 started an increasing-decreasing pattern for the following 3 sample days, while SSB01 started a steady increase followed by a sharp decrease on the very last day. DMSP concentration in SSA01 on last two days went from 0 (non-detected) to 63.99±32.92, instead SSB01 showed an opposite trend with DMSP concentration going from 154.18±18.07 to 59.99±30.89 (nmol/µg · chl-*a*).

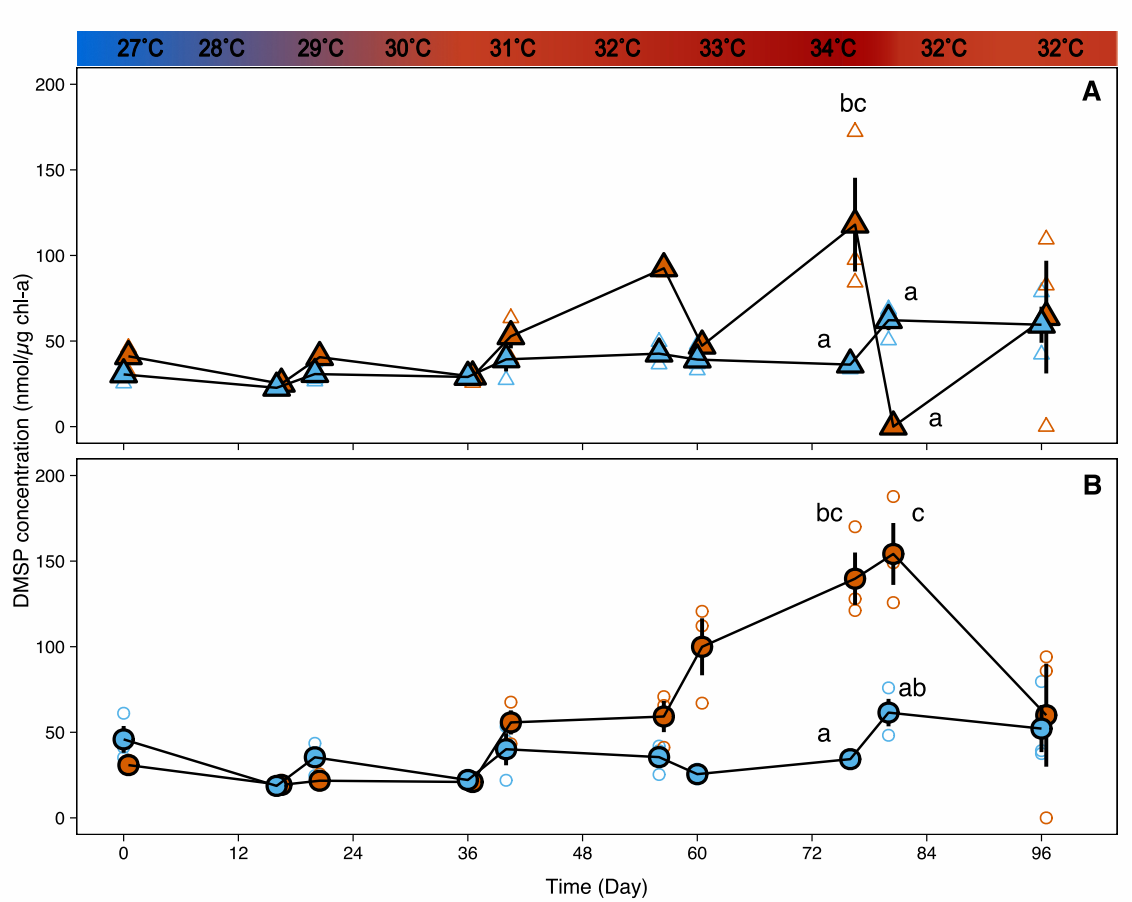


Figure 5-8. DMSP concentration (nmol/µg chl-a) in Symbiodinium linuchae SSA01 (triangles, **A**) and Breviolum minutum SSB01 (circles, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size and data points covered by mean±S.E.. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance. Not all significances shown.

DMS concentration in Symbiodiniaceae was significantly different among strain, day and treatment ($LME_{Strain * Temp * Day}$, P = <.0001) (Figure 5-9). Mean DMS amount in SSA01 at control was similar to SSB01 throughout the experimental period having 1.45±0.27 and 1.03±0.18 fmol/cell. Heat-stressed strain A had a constant concentration until the temperature reached 32°C, thereafter there was a sharp increase to 8.88±0.81 fmol/cell followed by a decrease in the following

three sampling days, ending with 0 fmol/cell DMS. On the contrary, DMS concentration in SSB01 did not seem to be affected by heat until after the temperature was lowered down to 32°C, where concentration jumped to 6.58±0.77 fmol/cell compared to 1.19±0.26 fmol/cell in control samples. On the last day of sampling, SSB01 had yet another significant change, DMS concentration decreased to 3.29±0.41 fmol/cell.

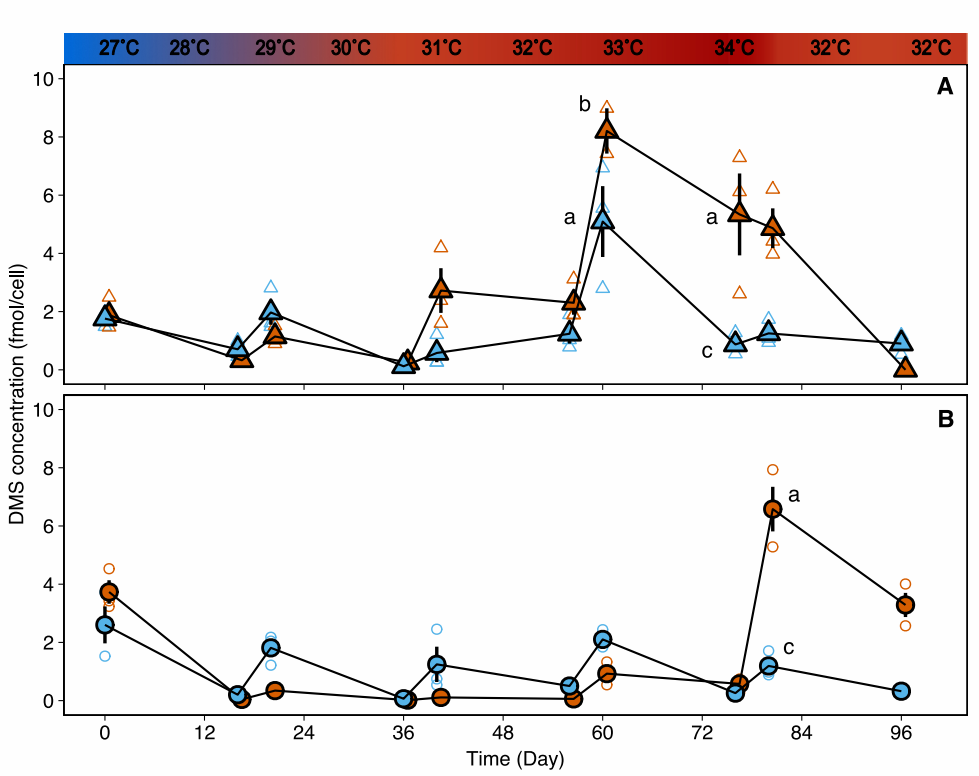


Figure 5-9. DMSP concentration (fmol/cell) in Symbiodinium linuchae SSA01 (triangles, **A**) and Breviolum minutum SSB01 (circles, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 34°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size and data points covered by mean±S.E.. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples. Summary of significance by Post-hoc Tukey's test of the effects of strain, temperature and day on SSA01 and SSB01, means followed by a common letter are not significantly different, 5% level of significance.

5.3.2 The effect of high temperature on *Exaiptasia*

Maximum photochemical efficiency (F_v/F_m) contrary to expectations was virtually the same for anemone strain and also for treatment samples, no significant difference was present when analysed together ($LME_{Strain * Temp * Day}$, P = 0.713) nor when strain was removed from the variables ($LME_{Temp * Day}$, P = 0.084 SSA01 and $LME_{Temp * Day}$, P = 0.159 for SSB01) (Figure 5-10). Exaiptasia CC7 had a slightly higher F_v/F_m then H2 throughout the experimental period at control temperature, 0.378±0.008 and 0.360±0.009 respectively. Treatment samples had instead a F_v/F_m value of 0.378±0.007 for strain CC7 and 0.350±0.009 for H2.

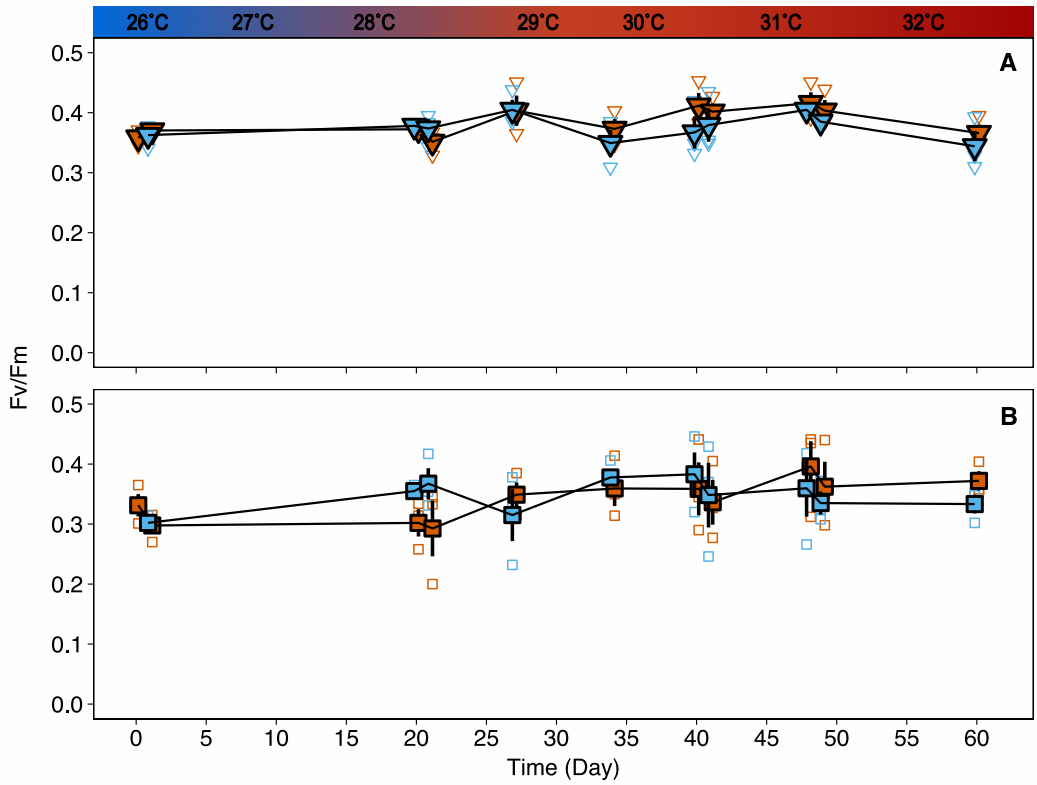


Figure 5-10. Maximum photochemical efficiency (F_v/F_m) in Exaiptasia pallida CC7 inoculated with Symbiodinium linuchae (triangles, **A**) and Exaiptasia pallida H2 inoculated with Breviolum minutum SSB01 (squares, **B**). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 32°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size and data points covered by mean±S.E.. Data points are offset along the x-axis to improve readability. The bar at the top of the figure shows the temperature at time of sampling for 'treatment' samples.

DMSP concentration in *Exaiptasia pallida* was not significantly different among the two strains, treatments and days of sampling ($LME_{Strain * Temp * Day}$, P = 0.283) (Figure 5-11). No significant differences were detected even when anemone strain was removed as a variable ($LME_{Temp * Day}$, P = 0.094 for CC7 and $lme_{\sim Temp * Day}$, P = 0.823 for H2). CC7 at 32°C had a concentration of 11.00±5.61, while at control temperature it was 60.00±5.59 nmol/mg DW. H2 on the contrary had a similar concentration of 33.35±12.45 at control and 39.89±5.99 nmol/mg DW at treatment.

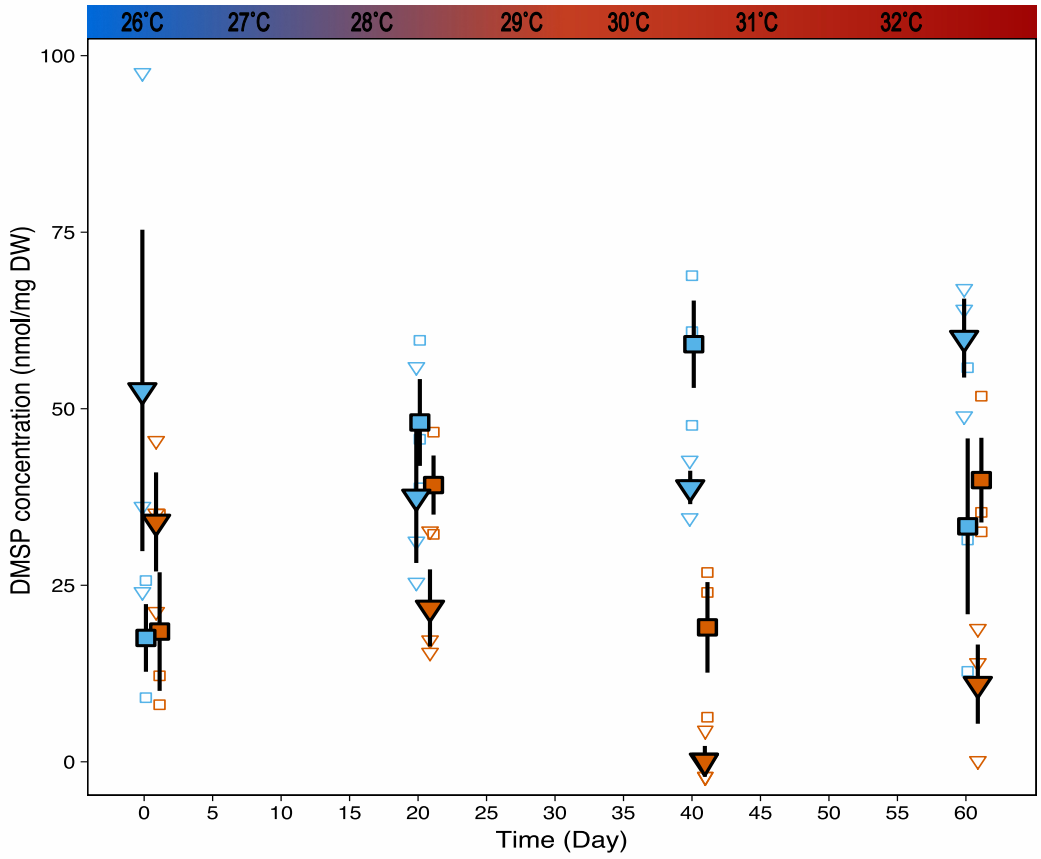


Figure 5-11. DMSP concentration (nmol/mg DW) in Exaiptasia pallida CC7 inoculated with Symbiodinium linuchae (triangles) and Exaiptasia pallida H2 inoculated with Breviolum minutum SSB01 (squares). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 32°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size and data points covered by mean±S.E..

DMS concentration in *Exaiptasia pallida* observed no clear trend and there was no significant difference among strain, treatment or day ($LME_{Strain * Temp * Day}$, P = 0.786) (Figure 5-12). No significant differences were detected even when the strain of the anemone was removed as a variable ($LME_{Temp * Day}$, P = 0.879 for CC7 and $LME_{Temp * Day}$, P = 0.808 for H2). CC7 had a mean total concentration of 47.242±6.11 nmol/mg DW at control and 17.404±4.22 nmol/mg DW for treatment, 63.17% less. H2 had instead a mean concentration of 39.52±5.81 nmol/mg DW in control samples and 29.14±4.17 nmol/mg DW, therefore having only 26.27% less in comparison. Unlike for DMSP concentration, once the temperature reached 32°C the DMS concentration in *Exaiptasia* under heat-stress was similar to control anemones.

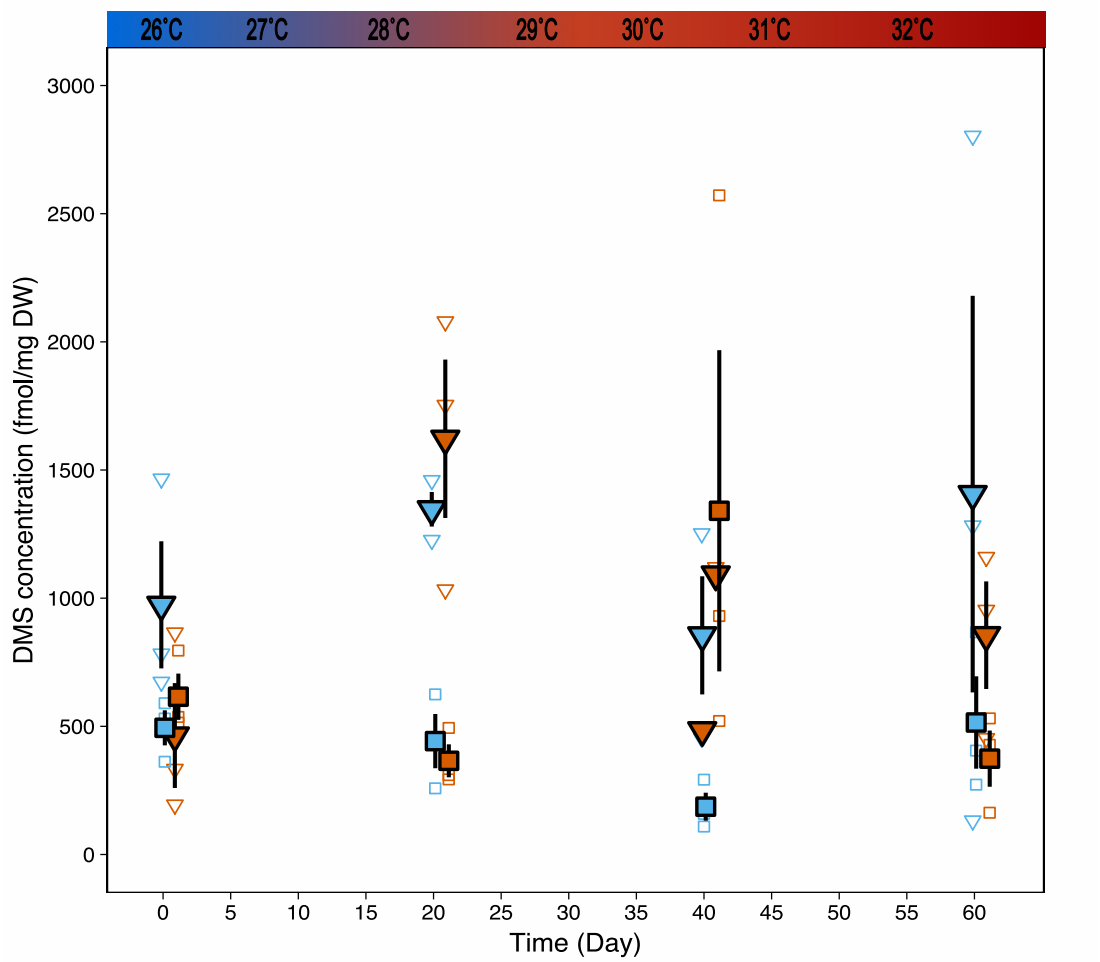


Figure 5-12. DMS concentration (fmol/mg DW) in Exaiptasia pallida CC7 inoculated with Symbiodinium linuchae (triangles) and Exaiptasia pallida H2 inoculated with Breviolum minutum SSB01 (squares). Shown are control treatments at 26°C (blue) and treatments at increased temperatures, reaching a maximum of 32°C (red). Filled symbols indicate the mean with standard error shown by error bars. Open symbols indicate the range of data, n=3. Where error bars are not visible, standard error was smaller than the symbol size and data points covered by mean±S.E..

The experiment ended because not enough anemones survived the next temperature increase. Anemones did not seem to have bleached severely (Figure 5-13).

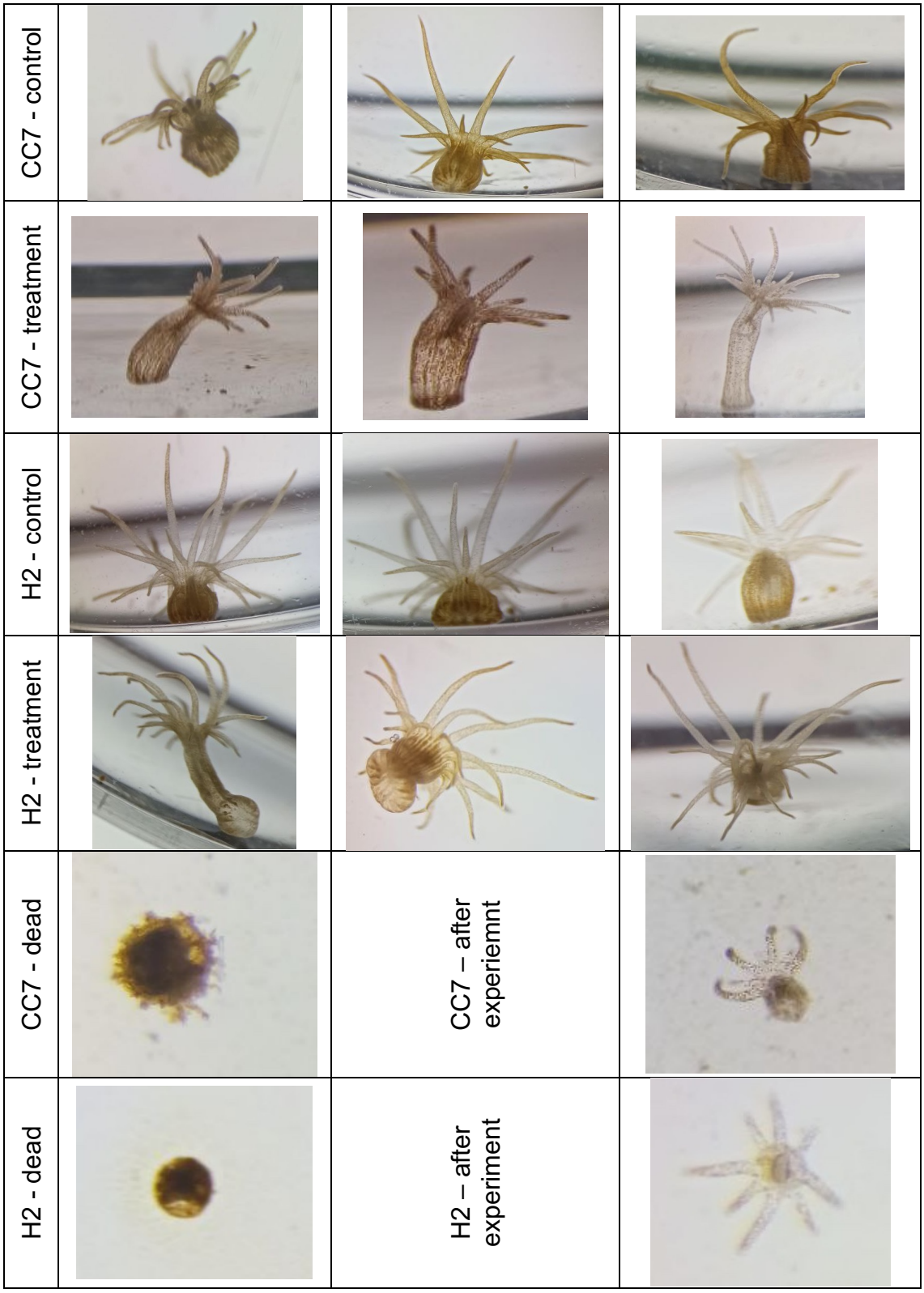


Figure 5-13. Images showing *Exaiptasia pallida* during the experimental period (control and treatment, images taken before DMSP concentration measurements). Last two rows show CC7 and H2, a week after the end of experiment.

5.4 Discussion

5.4.1 *Breviolum minutum* can also sustain prolonged heat stress

Symbiodinium linuchae SSA01 is suggested to be thermotolerant (Díaz-Almeyda et al., 2017), while *Breviolum minutum* SSB01 is suggested to be more thermosensitive (Robison & Warner, 2006; Suggett et al., 2008; Tchernov et al., 2004). In the case of Tchernov et al. (2004), this is based on Symbiodiniaceae cultures and corals grown at 26°C (control temperature) and heated to 32°C (maximum treatment temperature) and maintained at that temperature for 2 months or until they organisms died. They have demonstrated that the thylakoid membrane lipid is a key determinate of thermal-stress sensitivity. By analysing the thylakoid membranes, it was discovered that the threshold temperature which separates sensitive from tolerant species to heat is determined by the saturation of the lipids. Robison and Warner (2006) have instead based their findings on a short-term experiment (10 days) of four species of Symbiodiniaceae coupled with low/high irradiance (100/600 µmol photons m^{-2} s^{-1}) at a temperature of 32°C. They have recorded a significant variation in the sensitivity to heat in the rate of loss in PSII activity and electron transport, PSII reaction centre degradation and also cellular growth.

In contrast to this suggestion, data presented here indicates that SSB01 is more tolerant than previously thought in a long-term experiment (over 90 days).

Cell concentration was significantly lower in SSB01 when temperature reached 34°C, while in SSA01 at 32°C. Karim et al (2015) observed that SSB01 grown at 25°C and 30°C degrees were almost identical, while cultures at 33°C were lower already at day 1. By the end of the experiment at day 14, cultures at 33°C had

~3 log (cell/mL) compared to ~5.5 log (cell/mL). Two strains of clade A were also tested in the experiment, but the species did not correspond to the ones used in this experiment, therefore impossible to compare to SSA01. Symbiodiniaceae thermotolerance is species specific even within a specific clade, for example clade D has the most tolerant species (D_{Ber06}) but also the second most sensitive (D4-5) (Swain et al., 2017).

Cell growth rate (although calculated during both exponential and stationary phases) significantly declined sooner in SSA01 at 34°C than in SSB01. Growth rate in *Breviolum minutum* was also not affected by heat in a short-term experiment at a temperature of 32°C (Robison & Warner, 2006). Karim et al. (2015), that also observed growth rate in SSB01 in stationary phase (day 8 to 14) showed a significant decline of sampled grown at 33°C compared to those at 25°C and 30°C. Cell size has increased significantly by 2.3 folds in strain B grown at 33°C (McLenon & DiTullio, 2012), unlike expectation this behaviour was similar in our strain SSA01 at 32-33°C but not in SSB01. Also photochemical efficiency was not in line with other findings, in this experiment a not significant decline was recorded at 32°C in SSB01, while a drop from ~0.6 F_v/F_m to ~0.4 F_v/F_m was observed by others at the same temperature (Robison & Warner, 2006). Karim et al. (2015) recorded a significant decline in photochemical efficiency in cultures grown at 33°C with a value of ~0.2 (other cultures ~0.6 F_v/F_m), while we saw a decline from ~0.4 to ~0.3 F_v/F_m at 33°C and when the temperature reached 34°C a further and significant decline to ~0.1, as in SSA01 (Karim et al., 2015). These results together suggest that cell concentration decreased in order to avoid energy waste and consequently the growth rate slowed down. The cell volume

was significantly bigger when temperature reached 32°C (in SSA01), suggesting lack of cell division and energy preservation (Goulet et al., 2005).

5.4.2 The unclear role of DMSP, DMS and chlorophyll-*a*

A major difference in the two Symbiodiniaceae species is the variation in response that was observed in these two compounds under heat stress. A significant difference was recorded at the beginning of the experiment SSA01 having a DMSP concentration of 108.79±7.12 while SSB01 374.97±41.29 fmol/cell (means±S.E.) which could be partially attributed to the differences in cladal DMSP production rate (Gardner, et al., 2017a) and the correlation between chl-*a* and DMSP noticed in Chapter 3. In fact, this disparity is absent when DMSP concentration is normalized to chl-*a*, having instead 35.79±3.39 in SSA01 and 38.34±4.89 in SSB01 (mmol/μg · chl-*a*). Furthermore, SSA01 did not show any significant change in DMSP concentration (fmol/cell) between control and treatment samples throughout the whole experimental period. SSB01 instead had an increase in both control and treatment, also with a significant decrease in DMSP coinciding with the decrease in chl-a (at 34°C), once again confirming the correlation between these two compounds. DMSP concentration normalised by chlorophyll-a (mmol/μg · chl-*a*) in SSA01 showed a significant change in treatment cultures after the temperature reached 34°C, a sharp decrease in DMSP followed by a sharp increase. SSB01 has instead displayed a gradual increase from 33°C and recorded a significant decrease on the last day compared to treatment sample the previous day. Important to highlight that despite the sharp increase (SSA01)/decrease (SSB01) of DMSP concentration on the last

sampling day both treatment samples had very similar amount of DMSP on day 96 (63.99±32.93 and 59.99±30.09 mmol/µg · chl-*a*, SSA01 and SSB01 respectively). This could be in the case of SSA01 the beginning of higher DMSP concentration to protect the cell from damage and in SSB01 the inability to produce DMSP due to the prolonged period of lack of chl-*a* or that it was promptly transformed by bacterial cleavage pathways genes to DMS (Raina et al., 2010). As previously explained, DMSP has the potential to scavenge ROS and therefore act as an antioxidant (Sunda et al., 2002). The presence of also higher DMS concentration could confirm the latter hypothesis. However, in the case of SSA01, the behaviour is not clear. DMSP concentration did not change but DMS was significantly higher at 33°C degrees than at control and it slowly declined till the end of the experiment. Previous studies of corals on DMS/P/O at high temperature over a 7-day period show a significant increase in all three compounds (Deschaseaux et al., 2014), but also a decrease over a 4 hrs experiment (Gardner, et al., 2017b). Given that DMSP is the precursor of DMS it is impossible that DMSP was not present, it is more likely that at some point it was produced, and sampling simply did not occur when it happened. Another possibility is a high production of DMSP that was promptly transformed by bacterial cleavage pathways genes to DMS (Raina et al., 2010).

Exaiptasia pallida, contrary to expectations, seemed to be unaffected by heat and no significant difference was recorded in DMS/P. The fact that F_v/F_m was also unchanged between control and treatment might give the impression that anemones were not subjected to enough temperature increase. This was not the case since bleaching occurred in both strains (although seeming less severe

compare to short-term experiment) and that the experiment was terminated earlier than scheduled due to mortality of the animals. One possible explanation for this is that anemones' only defence mechanisms was to expel the Symbiodiniaceae host, regardless of their thermotolerance, since a lower concentration of symbionts means less ROS production (Gardner et al., 2017). Unfortunately, in the current study it is not possible to know whether none of the algal cells experienced appreciable pigment degradation or the algal cells with damaged pigments were eliminated selectively. Another explanation could be due to the sudden changes in temperature that anemones were subjected to. The blackout brought the temperature from 31°C down to 18°C and back up to 31°C, this is obviously a shock they will never have to face in the environment. This event could have led to the death of anemones when the temperature was reaching 34°C.

5.4.3 Differences between short-term and long-term experiment

Mean cell concentration showed that SSA01 had a decline at 34°C in the short-term experiment but a significant decline was recorded at 32°C in the long-term experiment (Table 5-1 and Table 5-2). Surprisingly SSB01 showed a decline at 32°C in the short-term but the mean concentration became significantly lower only when temperature reached 34°C in the long term. Cell growth rate also showed that SSA01 had a higher tolerance in the short term but not in the long term. Cell volume showed a similar pattern for both species in the short-term experiment, while in the long-term was once again SSA01 that showed less tolerance to increased temperature. Chlorophyll-*a* was instead stable in both

experiments and treatments for strain SSA01, while SSB01 was highly affected in the long term as soon as the temperature reached 32°C. Maximum photochemical efficiency (F_v/F_m) showed a similar pattern in both strains between the two experiments, with both strain having a significantly lower F_v/F_m compared to the respective control at 33°C. DMSP concentration (fmol/cell) did not show a clear pattern in the short-term, while in the long term it showed a decline for both strains after the temperature hit 34°C. DMSP concentration (nmol/ug chl-a) had instead the opposite effect on strain SSB01, in the short-term it slowly decreased and had a final rise on the last day of treatment while in the long-term there was a steady increase from when the temperature reached 32°C and a final sharp decline on the last day. SSA01 did not have any significantly different amount between control and treatment samples. DMS in the short term experiment, similarly to DMSP, did not have any particular change while in the long-term both strain showed an increase in DMS concentration at 33°C in SSA01 and for SSB01 when the temperature was lower down to 32°C, both strains experienced a decrease in concentration after that.

In *Exaiptasia* the changes were less evident, F_v/F_m was similar in both strains and experiments, suggesting that Symbiodiniaceae are better protected while in symbiosis and we could speculate that it is the anemones that expels them. DMSP concentration was also not significantly different, although generally SSA01 seemed to have a lower concentration in treatment samples compared to control. DMS concentration followed a similar 'non-significant' pattern in both experiments, with the exception of SSB01 in the short-term experiment, where the heat stressed samples showed a gradual DMS increase. I was instead

originally expecting to see an increase in both sulphur compounds, showing an active defence mechanisms against ROS.

It is important to note that the long-term experiment in *Exaiptasia* did not reach 34°C, but was terminated at 32°C because the majority of the specimens had died. Clearly, even if the F_v/F_m did not show a decline, the anemones could not withstand the temperature they have been subjected to.

These overall differences could be explained by a different response between acute stress (short-term) and chronic stress (long-term) in both organisms. They clearly have different effects on the organisms and it is of vital importance to be able to differentiate among them when predicting future changes. For example, it is misleading to affirm that a Symbiodiniaceae species is thermotolerant and assume the symbiosis relationship will be the best chance for a coral to survive during a bleaching event when no long-term experiment has taken place to confirm it.

Table 5-1. Summary of only the significant differences between treatment and control samples on the same day of sampling. Only the first statistically significant change that occurred throughout the experiment is reported. Arrows indicate if the value at treatment was lower (↓) or higher (↑) in comparison to control or if it was not significantly different (↔).

	short-term			long-term	
Symbiodiniaceae	A	B		A	B
Cell conc. (cell/ml)	↓ 34°C	↔		↓ 32°C	↓ 34°C
Cell growth rate (d^{-1})	↔	↓ 32°C		↓ 33°C	↓ after 34°C
Cell volume (μm^3)	↑ 34°C	↔		↑ 34°C	↔
Chl-a	↔	↓ 26°C		↔	↓ 33°C
Chl-c_2	↔	↔		↔	↓ 34°C
F_v/F_m	↓ 34°C	↓ 34°C		↓ 33°C	↓ 33°C
DMSP (fmol/cell)	↔	↔		↓ after 34°C	↓ after 34°C
DMSP (nmol/ug chl-a)	↔	↔		↔	↑ 33°C
DMS (fmol/cell)	↔	↔		↑ 33°C	↑ after 34°C
	short-term			long-term	
Exaiptasia pallida	CC7 (A)	H2 (B)		CC7 (A)	H2 (B)
F_v/F_m	↔	↔		↔	↔
DMSP (µmol/g)	↔	↔		↔	↔
DMS (fmol/mg)	↔	↑ 34°C		↔	↔

Table 5-2. Summary of the differences only between control and treatment on the last day of sampling. Statistically significant difference in red, non-significant in black, arrows indicate if the value at treatment was lower (↓) or higher (↑) in comparison to control or if it was almost the same (↔). Short-term Exaiptasia pallida has 2 arrows for DMSP/DMS because the experiment was conducted twice.

	short-term			long-term	
Symbiodiniaceae	A	B		A	B
Cell conc. (cell/ml)	↓	↔		↓	↓
Cell growth rate (d^{-1})	↔	↔		↓	↓
Cell volume (μm^3)	↑	↑		↓	↓
Chl-a	↔	↔		↓	↓
Chl-c_2	↔	↔		↑	↑
F_v/F_m	↓	↓		↓	↓
DMSP (fmol/cell)	↔	↓		↔	↔
DMSP (nmol/ug chl-a)	↓	↔		↔	↔
DMS (fmol/cell)	↔	↔		↔	↑
	short-term			long-term	
Exaiptasia pallida	CC7 (A)	H2 (B)		CC7 (A)	H2 (B)
F_v/F_m	↔	↓		↔	↔
DMSP (µmol/g)	↓↓	↑↓		↓	↔
DMS (fmol/mg)	↔↔	↑↔		↔	↔

5.4.4 Understanding short- and long-term stress differences in Symbiodiniaceae and *Exaiptasia pallida*

Bleaching is undoubtedly a very complex mechanism which is still poorly understood. Most times the severity of bleaching seems to be due to the differences in corals/anemones species and also the respective Symbiodiniaceae host (Gardner et al., 2017). The length and severity of the stress is an additional variable that differentiate the response in the same organisms. The studies that infer thermotolerance to SSA01 were done at a maximum temperature of 32°C (Díaz-Almeyda et al., 2017; Robison & Warner, 2006; Tchernov et al., 2004). The fact that both the experiments have instead reached 34°C might explain why SSA01 did not perform as well as expected. Also, to my knowledge, no experiment was conducted for this length of time before. With regards to *Exaiptasia pallida*, thanks to the experiment conducted in chapter four, we know that CC7 had a decrease in Symbiodiniaceae host of 42.38% while H2 of 88.39%. This could imply that the anemone is aware that the host SSA01 within CC7 is trying to fight against the stress. On the contrary H2 could sense that its host SSB01 is unable to contrast the ROS and expels the algae before it gets compromised too. Interestingly, over the long term

This experiment adds new information on the behaviour of these species of animals and symbiotic algae during a non-realistic short-term experiment and a very possible future scenario. Nowadays bleaching is already occurring at 32°C, therefore a rise to 34°C is a realistic scenario and it's worrying given the results of both experiments, especially the long term where both algae and anemones died.

5.4.5. Conclusions

The results presented here demonstrate that there is a clear need to understand the differences between short- and long-term high temperature experiments affecting cultures and anemones. Contrary to expectations, *Breviolum minutum* can also sustain a temperature up to 34°C, while *Symbiodinium linuchae* seems to perform better over a short-term temperature increase. Maximum photochemical efficiency showed a decline in cultures alone, but bleaching was the main response observed in anemones. DMSP/DMS and chlorophyll-*a* once again did not show a clear pattern, but mostly remained unchanged during the experimental period. Anemones died once the temperature reached 32°C for over a 10 days period. The overall comparison between chapter three and five highlights the differences between acute and chronic stress. To my knowledge, this is the first time that an experiment was carried out for this length of time in mesocosm, and compared to a short-term one using the same organisms.

Chapter 6 Conclusions

6.1 What is the role of DMSP/DMS?

Chapter two has clearly showed that if corals die and reefs will become dominated by macroalgae, DMSP concentration will significantly decrease. This will have a cascade effect on the production of DMS and obviously will have a catastrophic outcome for the thousands of species that rely on reefs to survive. DMS contributes to the albedo effect on the planet, a lack of it might lead to even higher temperatures, leading to more bleaching events (Figure 1-1). DMSP and DMS are known to be antioxidant and help with ROS scavenging but the results of chapters three and five do not allow us to confirm such a statement. In chapter three (short-term high temperature experiment with Symbiodiniaceae cultures and anemones), DMSP and DMS concentration in Symbiodiniaceae did not increase in treatment cultures, regardless of the normalisation used, even if their maximum photochemical efficiency was significantly impacted. *Exaiptasia* on the contrary had very little change in F_v/F_m, but similarly to their algal host did not show a higher concentration of DMSP. DMS concentration was instead significantly higher in *Exaiptasia* strain H2 compared with CC7 at the end of the experiment. Nonetheless this behaviour was not confirmed when the experiment was repeated at a later date. This change could be partially attributed to the difference in temperature profile due to the power cut (in both short and long-term experiments), but I think it is mainly due to the amount of Symbiodiniaceae present in the anemones. Both *Exaiptasia* CC7 and H2 sustained such a substantial symbiont loss that probably made it impossible to produce more DMSP/DMS, loss of symbionts confirmed in chapter four. In chapter five, with

sustained high temperature over a long-term period, DMSP did show a different concentration in Symbiodiniaceae, especially in SSB01 by being significantly lower when normalised to cell (fmol/cell) and higher to chlorophyll-*a* (mmol/μg · chl- *a*). DMS concentration (fmol/cell) in SSB01 showed a simultaneous increase which is in agreement with the possibility for these compounds to be used by algae as antioxidants. The behaviour of the same algae when in symbiosis with anemones was different. DMSP concentration in treatment CC7 was lower than in control and DMS was unchanged in both anemones strains. Bleaching was once again observed but it seemed less severe than in the short-term experiment. No clear conclusions can be drawn, but this thesis has provided further knowledge on this particular species. Future work should continue to investigate the relationship between these organisms and DMS/P to understand whether this behaviour is species specific or if it occurs only is specific settings. The relationship between concentration of DMS/P and chlorophyll should also be investigated further, especially since the two strains have behaved differently.

Chapter two gave a clear insight of the lack of DMSP (and therefore DMS) that reefs could face if corals were to be fully replaced by macroalgae. This is obviously a scenario that we must avoid, and action should be taken promptly since the decline has already been recorded. Especially with new evidence from chapter three and five, reefs could already produce less DMSP/DMS under stress, since unlike expectations anemones and their algal symbiont had a lower concentration of DMSP/DMS at high temperature, compared to control samples. On the contrary, if reefs were to produce more DMS, they will help create more cloud condensation nuclei and reduce heat on the Earth.

6.2 The importance of Bio-imaging in understanding bleaching

This thesis shows that the concentrations of DMS/P are tightly related to the number of cells present in the coral/anemone. The methodology described in Chapter four proved to be reliable in assessing the extent of bleaching in *Exaiptasia* and should be implemented in future research. Knowing the amount of Symbiodiniaceae will give a better insight on the concentration of DMS/P and bio-imaging could further examine the health of the algae that were expelled and those still present inside the animal.

6.3 The need of a standardized short/long-term increased temperature experiment methodology

Extensive research has been conducted on temperature increase effects on corals, anemones and Symbiodiniaceae and no single experiment has followed the same temperature increase profile or length of heat at a specific temperature. This has made the comparison with other studies a bit difficult, Voolstra et al. (2020) has in fact highlighted this issue, especially since we still have a limited understanding on what determines the thresholds for corals bleaching and the incompatibility of different methods and the lack of standardization among the studies are partially to blame. Interestingly their paper uses different maximum temperatures between the short term and the long term. This thesis has instead kept a maximum temperature of 34°C for both experiments and tried to replicate a natural heatwave instead of using a hypothetical scenario. I have observed variable results depending on that length of the experiment (short- vs long-term) and this made me question whether all the short-term experiments can be truly

used for predicting future scenarios or if instead, they have been misleading. I believe short-term stress experiments should be used when investigating cell functioning or other processes but might not be the best tool for determining whether a species is better than another one in a climate changing world.

6.4 Is strain SSB01 really thermosensitive?

I chose to use *Breviolum minutum* strain SSB01 at the beginning of my research for two main reasons: it was the natural symbiont for *Exaiptasia pallida* H2 and because it was defined as 'thermosensitive', while SSA01 (symbiont for CC7) was classified as 'thermotolerant'. I was clearly expecting a significant difference in response but it often did not happen and after analysing the data I am no longer confident with this definition. The resistance of SSB01 to long-term heat is positive news for the future but calls for a better understanding of the terms 'thermotolerant' and 'thermosensitive'. It will be important that future work replicates the experiments used to give these definitions over a longer period of time to see if the same behaviour can be sustained or not.

6.5 Concluding remarks

This thesis has evaluated the effects of short- and long-term high temperature effects on two Symbiodiniaceae cultures and the same species in symbiosis with their natural animal host, *Exaiptasia pallida.* This research has significantly improved current understanding in DMSP/DMS concentration in these organisms, the importance of bleaching in this process and the real danger of less sulphur reaching the atmosphere. It has also proposed an effective way to determine symbiont density in a living anemone, and the possibility of repeating measures on the same individual. It ultimately calls for a better distinction between short-term and long-term thermally stressful events and better clarity in the definition of 'thermotolerance'. It further calls for an agreement in experimental design in order to make results more comparable.

Finally, this research provides a stepping stone in gaining a better understanding of the impacts of climate change on DMSP/DMS concentration, which hopefully will lead to further investigation into the effects of higher temperature on reef environments in the future.

References

Abrego, D., Ulstrup, K. E., Willis, B. L., & van Oppen, M. J. H. (2008). Species–specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1648), 2273–2282. https://doi.org/10.1098/rspb.2008.0180

Ahmed, H. I., Herrera, M., Liew, Y. J., & Aranda, M. (2019). Long-term temperature stress in the coral model *Aiptasia* supports the " Anna Karenina Principle " for bacterial microbiomes. *Frontiers in Microbiology*, *10*(975). https://doi.org/10.3389/fmicb.2019.00975

Alcolombri, U., Ben-Dor, S., Feldmesser, E., Levin, Y., Tawfik, D. S., & Vardi, A. (2015). Identification of the algal dimethyl sulfide-releasing enzyme: A missing link in the marine sulfur cycle. *Science*, *348*(6242), 1466–1469. https://doi.org/10.1126/science.aab1586

Ambarsari, I., Brown, B. E., Barlow, R. G., Britton, G., & Cummings, D. (1997). Fluctuations in algal chlorophyll and carotenoid pigments during solar bleaching in the coral *Goniastrea aspera* at Phuket, Thailand. *Marine Ecology Progress Series*, *159*, 303–307. https://doi.org/10.3354/meps159303

Anderson, R. C. (2000). Sea anemones of the north-eastern Pacific: The flowers of the sea. *International Zoo Yearbook*, *37*(1), 321–330. https://doi.org/10.1111/j.1748-1090.2000.tb00737.x

Anthony, K. R. N., Connolly, S. R., & Hoegh-Guldberg, O. (2007). Bleaching, energetics, and coral mortality risk: Effects of temperature, light, and sediment regime. *Limnology and Oceanography*, *52*(2), 716–726. https://doi.org/10.4319/lo.2007.52.2.0716

Baker, A. C. (2003). Flexibility and specificity in coral-algal symbiosis: Diversity, ecology, and biogeography of *Symbiodinium*. *Annual Review of Ecology, Evolution, and Systematics*, *34*(1), 661–689. https://doi.org/10.1146/annurev.ecolsys.34.011802.132417

Barott, K. L., Venn, A. A., Perez, S. O., Tambutté, S., & Tresguerres, M. (2015). Coral host cells acidify symbiotic algal microenvironment to promote photosynthesis. *Proceedings of the National Academy of Sciences*, *112*(2), 607–612. https://doi.org/10.1073/pnas.1413483112

Bates, T. S., Kiene, R. P., Wolfe, G. V., Matrai, P. A., Chavez, F. P., Buck, K. R., Blomquist, B. W., & Cuhel, R. L. (1994). The cycling of sulfur in surface seawater of the northeast Pacific. *Journal of Geophysical Research: Oceans*, *99*(C4), 7835–7843. https://doi.org/10.1029/93JC02782

Belda-Baillie, C. A., Baillie, B. K., & Maruyama, T. (2002). Specificity of a model cnidarian-dinoflagellate symbiosis. *The Biological Bulletin*, *202*(1), 74–85. https://doi.org/10.2307/1543224

Bellis, E. S., & Denver, D. R. (2017). Natural variation in responses to acute heat and cold stress in a sea anemone model system for coral bleaching. *The Biological Bulletin*, *233*(2), 168–181. https://doi.org/10.1086/694890

Bellwood, D. R., Hughes, T. P., Folke, C., & Nyström, M. (2004). Confronting the coral reef crisis. *Nature*, *429*(6994), 827–833. https://doi.org/10.1038/nature02691

Berges, J. A., Franklin, D. J., & Harrison, P. J. (2001). Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. *Journal of Phycology*, *37*(6), 1138–1145. https://doi.org/10.1046/j.1529-8817.2001.01052.x

Bertsch, H. & Johnson, S. (1981). *Hawaiian Nudibranchs*. Oriental Pub Co.

Bhagooli, R., & Hidaka, M. (2002). Physiological responses of the coral *Galaxea fascicularis* and its algal symbiont to elevated temperatures. *Journal of the Japanese Coral Reef Society*, *2002*(4), 33–42. https://doi.org/10.3755/jcrs.2002.33

Bieri, T., Onishi, M., Xiang, T., Grossman, A. R., & Pringle, J. R. (2016). Relative contributions of various cellular mechanisms to loss of algae during Cnidarian bleaching. *PloS One*, *11*(4), e0152693. https://doi.org/10.1371/journal.pone.0152693

Borell, E. M., Pettay, D. T., Steinke, M., Warner, M., & Fine, M. (2016). Symbiosis-specific changes in dimethylsulphoniopropionate concentrations in *Stylophora pistillata* along a depth gradient. *Coral Reefs*, *35*(4), 1383–1392. https://doi.org/10.1007/s00338-016-1475-3

Broadbent, A. D., & Jones, G. B. (2004). DMS and DMSP in mucus ropes, coral mucus, surface films and sediment pore waters from coral reefs in the Great Barrier Reef. *Marine and Freshwater Research*, *55*(8), 849–849. https://doi.org/10.1071/MF04114

Broadbent, A. D., Jones, G. B., & Jones, R. J. (2002). DMSP in Corals and Benthic Algae from the Great Barrier Reef. *Estuarine, Coastal and Shelf Science*, *55*(4), 547–555. https://doi.org/10.1006/ecss.2002.1021

Brown, B. E. (1997). Coral bleaching: Causes and consequences. *Coral Reefs*, *16*(1), S129–S138. https://doi.org/10.1007/s003380050249

Buddemeier, R. W., & Fautin, D. G. (1993). Coral Bleaching as an adaptive mechanism. *BioScience*, *43*(5), 320–326. https://doi.org/10.2307/1312064

Burton, F. J. (1994). Climate and tides of the Cayman Islands. In M. A. Brunt & J. E. Davies (Eds.), *The Cayman Islands: Natural History and Biogeography* (pp. 51–60). Springer Netherlands. https://doi.org/10.1007/978-94-011-0904-8_3

Camacho-Fernández, C., Hervás, D., Rivas-Sendra, A., Marín, M. P., & Seguí-Simarro, J. M. (2018). Comparison of six different methods to calculate cell densities. *Plant Methods*, *14*(1), 30-43. https://doi.org/10.1186/s13007-018-0297-4

Caruana, A. M. N., & Malin, G. (2014). The variability in DMSP content and DMSP lyase activity in marine dinoflagellates. *Progress in Oceanography*, *120*, 410–424. https://doi.org/10.1016/j.pocean.2013.10.014

Chang, S. S., Prézelin, B. B., & Trench, R. K. (1983). Mechanisms of photoadaptation in three strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum*. *Marine Biology*, *76*(3), 219–229. https://doi.org/10.1007/BF00393021

Charlson, R. J., Lovelock, J. E., Andreae, M. O., & Warren, S. G. (1987). Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. *Nature*, *326*(1), 655–661.

Chudek, J. A., Foster, R., Moore, D. J., & Reed, R. H. (1987). Identification and quantification of methylated osmolytes in algae using proton nuclear magnetic resonance spectroscopy. *British Phycological Journal*, *22*(2), 169–173. https://doi.org/10.1080/00071618700650201

Clayton, W. (1985). Pedal laceration by the anemone *Aiptasia pallida*. *Marine Ecology Progress Series*, *21*, 75–80. https://doi.org/10.3354/meps021075

Clayton, W. S., & Lasker, H. R. (1985). Individual and population growth in the asexually reproducing anemone *Aiptasia pallida* Verrill. *Journal of Experimental Marine Biology and Ecology*, *90*(3), 249–258. https://doi.org/10.1016/0022-0981(85)90170-4

Cook, C. B., Muller-Parker, G., & D'Elia, C. F. (1992). Ammonium enhancement of dark carbon fixation and nitrogen limitation in symbiotic zooxanthellae: Effects of feeding and starvation of the sea anemone *Aiptasia pallida*. *Limnology and Oceanography*, *37*(1), 131–139. https://doi.org/10.4319/lo.1992.37.1.0131

Coral Reef Watch - NOAA. (n.d.). *Florida Keys Regional Products 2022-07-19*. Retrieved 21 July 2022, from https://coralreefwatch.noaa.gov/product/vs/gauges/florida_keys.php

Cox, R. A. (1997). Atmospheric sulphur and climate—What have we learned? *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *352*(1), 251–254. https://doi.org/10.1098/rstb.1997.0020

Cunning, R., & Baker, A. C. (2013). Excess algal symbionts increase the susceptibility of reef corals to bleaching. *Nature Climate Change*, *3*(3), 259–262. https://doi.org/10.1038/nclimate1711

Cunning, R., & Baker, A. C. (2014). Not just who, but how many: The importance of partner abundance in reef coral symbioses. *Frontiers in Microbiology*, *5*(1). https://www.frontiersin.org/articles/10.3389/fmicb.2014.00400

Cunning, R., Gillette, P., Capo, T., Galvez, K., & Baker, A. C. (2015). Growth tradeoffs associated with thermotolerant symbionts in the coral *Pocillopora damicornis* are lost in warmer oceans. *Coral Reefs*, *34*(1), 155–160. https://doi.org/10.1007/s00338-014-1216-4

Curson, A. R. J., Liu, J., Bermejo Martínez, A., Green, R. T., Chan, Y., Carrión, O., Williams, B. T., Zhang, S.-H., Yang, G.-P., Bulman Page, P. C., Zhang, X.-H., & Todd, J. D. (2017). Dimethylsulfoniopropionate biosynthesis in marine bacteria and identification of the key gene in this process. *Nature Microbiology*, *2*(1), 17009. https://doi.org/10.1038/nmicrobiol.2017.9

Dacey, J., King, G., & Lobel, P. (1994). Herbivory by reef fishes and the production of dimethyl sulfide and acrylic acid. *Marine Ecology Progress Series*, *112*(2), 67–74. https://doi.org/10.3354/meps112067

D'Angelo, C., Hume, B. C. C., Burt, J., Smith, E. G., Achterberg, E. P., & Wiedenmann, J. (2015). Local adaptation constrains the distribution potential of heat-tolerant Symbiodinium from the Persian/Arabian Gulf. *The ISME Journal*, *9*(12), 2551–2560. https://doi.org/10.1038/ismej.2015.80

Dani, K. G. S., & Loreto, F. (2017). Trade-off between dimethyl sulfide and isoprene emissions from marine phytoplankton. *Trends in Plant Science*, *22*(5), 361–372. https://doi.org/10.1016/j.tplants.2017.01.006

Davidson, O. G. (1998). *The enchanted braid: coming to terms with nature on the coral reef* (1st edition). Wiley.

Davy, S. K., Allemand, D., & Weis, V. M. (2012). Cell biology of Cnidarian-dinoflagellate symbiosis. *Microbiology and Molecular Biology Reviews*, *76*(2), 229–261. https://doi.org/10.1128/MMBR.05014-11

Dawson, R. A., Crombie, A. T., Pichon, P., Steinke, M., McGenity, T. J., & Murrell, J.C. (2021). The microbiology of isoprene cycling in aquatic ecosystems. *Aquatic Microbial Ecology.* https://doi.org/10.3354/ame01972

de Ruyter van Steveninck, E. D., & Bak, R. P. M. (1986). Changes in abundance of coral-reef bottom components related to mass mortality of the sea urchin *Diadema antillarum*. *Marine Ecology Progress Series*, *34*(2), 87–94.

DeBose, J. L., Lema, S. C., & Nevitt, G. A. (2008). Dimethylsulfoniopropionate as a foraging cue for reef fishes. *Science*, *319*(5), 1356–1356. https://doi.org/10.1126/science.1151109

Dell, C. L. A., Longo, G. O., Burkepile, D. E., & Manfrino, C. (2020). Few herbivore species consume dominant macroalgae on a Caribbean coral reef. *Frontiers in Marine Science*, *7*(1). https://www.frontiersin.org/articles/10.3389/fmars.2020.00676

Deschaseaux, E. S. M., Jones, G. B., Deseo, M. A., Shepherd, K. M., Kiene, R. P., Swan, H. B., Harrison, P. L., & Eyre, B. D. (2014). Effects of environmental factors on dimethylated sulfur compounds and their potential role in the antioxidant system of the coral holobiont. *Limnology and Oceanography*, *59*(3), 758–768. https://doi.org/10.4319/lo.2014.59.3.0758

Díaz-Almeyda, E. M., Prada, C., Ohdera, A. H., Moran, H., Civitello, D. J., Iglesias-Prieto, R., Carlo, T. A., LaJeunesse, T. C., & Medina, M. (2017). Intraspecific and interspecific variation in thermotolerance and photoacclimation in Symbiodinium dinoflagellates. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1868), 20171767. https://doi.org/10.1098/rspb.2017.1767

Dixon, G. B., Davies, S. W., Aglyamova, G. V., Meyer, E., Bay, L. K., & Matz, M. V. (2015). Genomic determinants of coral heat tolerance across latitudes. *Science*, *348*(2), 1460–1462. https://doi.org/10.1126/science.1261224

Done, T. J. (1992). Phase shifts in coral reef communities and their ecological significance. *Hydrobiologia*, *247*(1), 121–132. https://doi.org/10.1007/BF00008211

Dunn, S. R., Bythell, J. C., Le Tissier, M. D. A., Burnett, W. J., & Thomason, J. C. (2002). Programmed cell death and cell necrosis activity during hyperthermic stress-induced bleaching of the symbiotic sea anemone *Aiptasia sp*. *Journal of Experimental Marine Biology and Ecology*, *272*(1), 29–53. https://doi.org/10.1016/S0022-0981(02)00036-9

Dunn, S. R., Thomason, J. C., Le Tissier, M. D. A., & Bythell, J. C. (2004). Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration. *Cell Death & Differentiation*, *11*(11), 1213–1222. https://doi.org/10.1038/sj.cdd.4401484

Dunn, S. R., Thomason, J. C., Le Tissier, M. D. A., Bythell, J. C., & Moosa, M. K. (2000). Detection of cell death activity during experimentally induced bleaching of the symbiotic sea anemone *Aiptasia sp*. *Proceedings of the Ninth International Coral Reef Symposium Bali, Indonesia*, *January 2000*, 145–145.

Edullantes, B. (2020). *Thermal responses of marine phytoplankton: Implications to their biogeography in the present and future oceans* [Phd, University of Essex]. http://repository.essex.ac.uk/28764/

Exton, D. A., McGenity, T. J., Steinke, M., Smith, D. J., & Suggett, D. J. (2015). Uncovering the volatile nature of tropical coastal marine ecosystems in a changing world. *Global Change Biology*, *21*(4), 1383–1394. https://doi.org/10.1111/gcb.12764

Exton, D. A., Suggett, D. J., McGenity, T. J., & Steinke, M. (2013). Chlorophyll-normalized isoprene production in laboratory cultures of marine microalgae and implications for global models. *Limnology and Oceanography*, *58*(4), 1301–1311. https://doi.org/10.4319/lo.2013.58.4.1301

Fitt, W. K., McFarland, F. K., Warner, M. E., & Chilcoat, G. C. (2000). Seasonal patterns of tissue biomass and densities of symbiotic dinoflagellates in reef corals and relation to coral bleaching. *Limnology and Oceanography*, *45*(3), 677–685. https://doi.org/10.4319/lo.2000.45.3.0677

Frade, P. R., Englebert, N., Faria, J., Visser, P. M., & Bak, R. P. M. (2008). Distribution and photobiology of Symbiodinium types in different light environments for three colour morphs of the coral *Madracis pharensis*: Is there more to it than total irradiance? *Coral Reefs*, *27*(4), 913–925. https://doi.org/10.1007/s00338-008-0406-3

Franchini, F., & Steinke, M. (2016). Protocols for the quantification of dimethyl sulfide (DMS) and other volatile organic compounds in aquatic environments. In *Hydrocarbon and Lipid Microbiology Protocols* 43(1), 161–177. https://doi.org/10.1007/8623_2016_206

Franchini, F., & Steinke, M. (2017). Quantification of dimethyl sulfide (DMS) production in the sea anemone *Aiptasia sp*. To simulate the sea-to-air flux from coral reefs. *Biogeosciences*, *14*(24), 5765–5774. https://doi.org/10.5194/bg-14-5765-2017

Franklin, D. J., & Berges, J. A. (2004). Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. *Proceedings of the Royal Society of London. Biological Sciences*, *271*(1553), 2099–2107. https://doi.org/10.1098/rspb.2004.2810

Friese, U. E. (1972). *Sea Anemones*. TFH Publications.

Gabay, Y., Weis, V. M., & Davy, S. K. (2018). Symbiont identity influences patterns of symbiosis establishment, host growth, and asexual reproduction in a model Cnidarian-dinoflagellate symbiosis. *The Biological Bulletin*, *234*(1), 1–10. https://doi.org/10.1086/696365

Galasso, C., Corinaldesi, C., & Sansone, C. (2017). Carotenoids from marine organisms: biological functions and industrial applications. *Antioxidants*, *6*(4), 96. https://doi.org/10.3390/antiox6040096

Galí, M., Saló, V., Almeda, R., Calbet, A., & Simó, R. (2011). Stimulation of gross dimethylsulfide (DMS) production by solar radiation. *Geophysical Research Letters*, *38*(15). https://doi.org/10.1029/2011GL048051

Gardner, S. G., Nielsen, D. A., Laczka, O., Shimmon, R., Beltran, V. H., Ralph, P. J., & Petrou, K. (2016). Dimethylsulfoniopropionate, superoxide dismutase and glutathione as stress response indicators in three corals under short-term hyposalinity stress. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1824), 2015-2028. https://doi.org/10.1098/rspb.2015.2418

Gardner, S. G., Raina, J.-B., Nitschke, M. R., Nielsen, D. A., Stat, M., Motti, C. A., Ralph, P. J., & Petrou, K. (2017a). A multi-trait systems approach reveals a response cascade to bleaching in corals. *BMC Biology*, *15*(1), 117–127. https://doi.org/10.1186/s12915-017-0459-2

Gardner, S. G., Raina, J.-B., Ralph, P. J., & Petrou, K. (2017b). Reactive oxygen species (ROS) and dimethylated sulphur compounds in coral explants under acute thermal stress. *The Journal of Experimental Biology*, *220*(10), 1787–1791. https://doi.org/10.1242/jeb.153049

Gardner, T. A., Côté, I. M., Gill, J. A., Grant, A., & Watkinson, A. R. (2003). Long-term region-wide declines in Caribbean corals. *Science (New York, N.Y.)*, *301*(5635), 958–960. https://doi.org/10.1126/science.1086050

Garren, M., Son, K., Raina, J.-B., Rusconi, R., Menolascina, F., Shapiro, O. H., Tout, J., Bourne, D. G., Seymour, J. R., & Stocker, R. (2014). A bacterial pathogen uses dimethylsulfoniopropionate as a cue to target heat-stressed corals. *The ISME Journal*, *8*(5), 999–1007. https://doi.org/10.1038/ismej.2013.210

Gaubert, J., Payri, C. E., Vieira, C., Solanki, H., & Thomas, O. P. (2019). High metabolic variation for seaweeds in response to environmental changes: A case study of the brown algae *Lobophora* in coral reefs. *Scientific Reports*, *9*(1), 993. https://doi.org/10.1038/s41598-018-38177-z

Gladfelter, W. B. (1982). White-band disease in *Acropora palmata*: implications for the structure and growth of shallow reefs. *Bulletin of marine science*, *32*, 5.

Goreau, T. J. (1992). Bleaching and reef community change in Jamaica: 1951-1991. *American Zoologist*, *32*(6), 683–695.

Goulet, T. L., Cook, C. B., & Goulet, D. (2005). Effect of short-term exposure to elevated temperatures and light levels on photosynthesis of different host-symbiont combinations in the *Aiptasia pallida*/Symbiodinium symbiosis. *Limnology and Oceanography*, *50*(5), 1490–1498. https://doi.org/10.4319/lo.2005.50.5.1490

Graham, N. A. J., Wilson, S. K., Jennings, S., Polunin, N. V. C., Bijoux, J. P., & Robinson, J. (2006). Dynamic fragility of oceanic coral reef ecosystems. *Proceedings of the National Academy of Sciences*, *103*(22), 8425–8429. https://doi.org/10.1073/pnas.0600693103

Grajales, A. & Rodriguez, E. (2014). Morphological revision of the genus *Aiptasia* and the family Aiptasiidae (Cnidaria, Actiniaria, Matridiodea). Zootaxa. https://10.11646/zootaxa.3826.1.2.

Grawunder, D., Hambleton, E. A., Bucher, M., Wolfowicz, I., Bechtoldt, N., & Guse, A. (2015). Induction of gametogenesis in the Cnidarian endosymbiosis model *Aiptasia* sp. *Scientific Reports*, *5*, 15677–15677. https://doi.org/10.1038/srep15677

Grégoire, V., Schmacka, F., Coffroth, M. A., & Karsten, U. (2017). Photophysiological and thermal tolerance of various genotypes of the coral endosymbiont *Symbiodinium sp*. (Dinophyceae). *Journal of Applied Phycology*, *29*(4), 1893–1905. https://doi.org/10.1007/s10811-017-1127-1

Guillard, R. R. L., & Ryther, J. H. (1962). Studies of marine planktonic diatoms I. *Cyclotella nana hustedtm* and *Detonula confervacea (cleve) gran* . *Canadian Journal of Microbiology*, *8*(2), 229–239. https://doi.org/10.1139/m62-029

Hennige, S. J., Suggett, D. J., Warner, M. E., McDougall, K. E., & Smith, D. J. (2009). Photobiology of Symbiodinium revisited: Bio-physical and bio-optical signatures. *Coral Reefs*, *28*, 179–195. https://doi.org/10.1007/s00338-008-0444-x

Heron, S. F., Johnston, L., Liu, G., Geiger, E. F., Maynard, J. A., De La Cour, J. L., Johnson, S., Okano, R., Benavente, D., Burgess, T. F. R., Iguel, J., Perez, D. I., Skirving, W. J., Strong, A. E., Tirak, K., & Eakin, C. M. (2016). Validation of reef-scale thermal stress satellite products for coral bleaching monitoring. *Remote Sensing*, *8*(1), 59. https://doi.org/10.3390/rs8010059

Hoegh-Guldberg, O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Marine and Freshwater Research*, *50*, 839–866. https://doi.org/10.1071/MF99078

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K., Knowlton, N., Eakin, C. M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R. H., Dubi, A., & Hatziolos, M. E. (2007). Coral reefs under rapid climate change and ocean acidification. *Science*, *318*(5857), 1737–1742. https://doi.org/10.1126/science.1152509

Hopkins, F. E., Bell, T. G., Yang, M., Suggett, D. J., & Steinke, M. (2016). Air exposure of coral is a significant source of dimethylsulfide (DMS) to the atmosphere. *Scientific Reports*, *6*(1), 36031–36031. https://doi.org/10.1038/srep36031

Howard, E. C., Henriksen, J. R., Buchan, A., Reisch, C. R., Burgmann, H., Welsh, R., Ye, W., Gonzalez, J. M., Mace, K., Joye, S. B., Kiene, R. P., Whitman, W. B., & Moran, M. A. (2006). Bacterial taxa that limit sulfur flux from the ocean. *Science*, *314*(5799), 649–652. https://doi.org/10.1126/science.1130657

Howard, E. C., Sun, S., Biers, E. J., & Moran, M. A. (2008). Abundant and diverse bacteria involved in DMSP degradation in marine surface waters. *Environmental Microbiology*, *10*(9), 2397–2410. https://doi.org/10.1111/j.1462-2920.2008.01665.x

Howells, E. J., Ketchum, R. N., Bauman, A. G., Mustafa, Y., Watkins, K. D., & Burt, J. A. (2016). Species-specific trends in the reproductive output of corals across environmental gradients and bleaching histories. *Marine Pollution Bulletin*, *105*(2), 532–539. https://doi.org/10.1016/j.marpolbul.2015.11.034

Huffmyer, A. S., Matsuda, S. B., Eggers, A. R., Lemus, J. D., & Gates, R. D. (2020). Evaluation of laser scanning confocal microscopy as a method for characterizing reef-building coral tissue thickness and Symbiodiniaceae fluorescence. *Journal of Experimental Biology*, *223*(6), jeb220335. https://doi.org/10.1242/jeb.220335

Hughes, T. P. (1989). Community structure and diversity of coral reefs: The role of history. *Ecology*, *70*(1), 275–279. https://doi.org/10.2307/1938434

Hughes, T. P., Baird, A. H., Bellwood, D. R., Card, M., Connolly, S. R., Folke, C., Grosberg, R., Hoegh-Guldberg, O., Jackson, J. B. C., Kleypas, J., Lough, J. M., Marshall, P., Nyström, M., Palumbi, S. R., Pandolfi, J. M., Rosen, B., & Roughgarden, J. (2003). Climate change, human impacts, and the resilience of coral reefs. *Science*, *301*(5635), 929–933. https://doi.org/10.1126/science.1085046

Hughes, T. P., Bellwood, D. R., Folke, C., Steneck, R. S., & Wilson, J. (2005). New paradigms for supporting the resilience of marine ecosystems. *Trends in Ecology & Evolution*, *20*(7), 380–386. https://doi.org/10.1016/j.tree.2005.03.022

Hume, B. C. C., D'Angelo, C., Smith, E. G., Stevens, J. R., Burt, J., & Wiedenmann, J. (2015). *Symbiodinium thermophilum* sp. Nov., a thermotolerant symbiotic alga prevalent in corals of the world's hottest sea, the Persian/Arabian Gulf. *Scientific Reports*, *5*(1), 8562–8562. https://doi.org/10.1038/srep08562

IPCC (2014). The Ocean. In: Climate Change 2014: Impacts, adaptation, and vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. https:// www.ipcc.ch/site/assets/uploads/2018/02/WGIIAR5-Chap30_FINAL.pdf

Jackson, E. J., Donovan, M., Cramer, K., & Lam, V. (2014). Status and trends of Caribbean coral reefs: 1970-2012. *Global Coral Reef Monitoring Network*, 306.

Jackson, J. B. C. (1997). *Coral Reefs (1997) 16, Suppl.: S23—S32 Reefs since Columbus*.

Jackson, J. B., Kirby, M. X., Berger, W. H., Bjorndal, K. A., Botsford, L. W., Bourque, B. J., Bradbury, R. H., Cooke, R., Erlandson, J., Estes, J. A., Hughes, T. P., Kidwell, S., Lange, C. B., Lenihan, H. S., Pandolfi, J. M., Peterson, C. H., Steneck, R. S., Tegner, M. J., & Warner, R. R. (2001). Historical overfishing and the recent collapse of coastal ecosystems. *Science (New York, N.Y.)*, *293*(5530), 629–637. https://doi.org/10.1126/science.1059199

Jennings, S., & Lock, J. M. (1996). Population and ecosystem effects of reef fishing. In N. V. C. Polunin & C. M. Roberts (Eds.), *Reef Fisheries*, 193–218. Springer Netherlands. https://doi.org/10.1007/978-94-015-8779-2_8

Johnston, A. W. B., Green, R. T., & Todd, J. D. (2016). Enzymatic breakage of dimethylsulfoniopropionate—A signature molecule for life at sea. *Current Opinion in Chemical Biology*, *31*(1), 58–65. https://doi.org/10.1016/j.cbpa.2016.01.011

Johnston, A. W. B., Todd, J. D., Sun, L., Nikolaidou-Katsaridou, M. N., Curson, A. R. J., & Rogers, R. (2008). Molecular diversity of bacterial production of the climate-changing gas, dimethyl sulphide, a molecule that impinges on local and global symbioses. *Journal of Experimental Botany*, *59*(5), 1059–1067. https://doi.org/10.1093/jxb/erm264

Karim, W., Nakaema, S., & Hidaka, M. (2015). Temperature effects on the growth rates and photosynthetic activities of *Symbiodinium* cells. *Journal of Marine Science and Engineering*, *3*(2), 368–381. https://doi.org/10.3390/jmse3020368

Karsten, U., Wiencke, C., & Kirst, G. O. (1991). Growth pattern and β-dimethylsulphoniopropionate (DMSP) content of green macroalgae at different irradiances. *Marine Biology*, *108*(1), 151–155. https://doi.org/10.1007/BF01313483

Kauffmann, D. *A key to the red algae of Long Key*. Retrieved 20 July 2022, from https://www.goshen.edu/bio/marine-ecology/key-to-red-algae.htm#page95c_clavulatum

Kinzie, R. A., Takayama, M., Santos, S. R., & Coffroth, M. A. (2001). The adaptive bleaching hypothesis: Experimental tests of critical assumptions. *The Biological Bulletin*, *200*(1), 51–58. https://doi.org/10.2307/1543084

Knowlton, N. (2001). The future of coral reefs. *Proceedings of the National Academy of Sciences*, *98*(10), 5419–5425. https://doi.org/10.1073/pnas.091092998

Krueger, T., Becker, S., Pontasch, S., Dove, S., Hoegh-Guldberg, O., Leggat, W., Fisher, P. L., & Davy, S. K. (2014). Antioxidant plasticity and thermal sensitivity in four types of *Symbiodinium* sp. *Journal of Phycology*, *50*(6), 1035–1047. https://doi.org/10.1111/jpy.12232

LaJeunesse, T. C. (2002). Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. *Marine Biology*, *141*(1), 387–400. https://doi.org/10.1007/s00227-002-0829-2

LaJeunesse, T. C., Parkinson, J. E., Gabrielson, P. W., Jeong, H. J., Reimer, J. D., Voolstra, C. R., & Santos, S. R. (2018). Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Current Biology*, *28*(16), 2570-2580. https://doi.org/10.1016/j.cub.2018.07.008

Lana, A., Bell, T. G., Simó, R., Vallina, S. M., Ballabrera-Poy, J., Kettle, A. J., Dachs, J., Bopp, L., Saltzman, E. S., Stefels, J., Johnson, J. E., & Liss, P. S. (2011). An updated climatology of surface dimethlysulfide concentrations and emission fluxes in the global ocean. *Global Biogeochemical Cycles*, *25*(1). https://doi.org/10.1029/2010GB003850

Lehnert, E. M., Burriesci, M. S., & Pringle, J. R. (2012). Developing the anemone *Aiptasia* as a tractable model for cnidarian-dinoflagellate symbiosis: Generating transcriptomic resources and profiling gene expression. *BMC Genomics*, *13*(271), 1–10. https://doi.org/10.1177/1742715010368768

Lema, K. A., Clode, P. L., Kilburn, M. R., Thornton, R., Willis, B. L., & Bourne, D. G. (2016). Imaging the uptake of nitrogen-fixing bacteria into larvae of the coral *Acropora millepora*. *The ISME Journal*, *10*(7), 1804–1808. https://doi.org/10.1038/ismej.2015.229

Lesser, M. P. (2006). Oxidative stress in marine environments: Biochemistry and physiological ecology. *Annual Review of Physiology*, *68*(1), 253–278. https://doi.org/10.1146/annurev.physiol.68.040104.110001

Lesser, M. P., & Slattery, M. (2011). Phase shift to algal dominated communities at mesophotic depths associated with lionfish (*Pterois volitans*) invasion on a Bahamian coral reef. *Biological Invasions*, *13*(8), 1855–1868. https://doi.org/10.1007/s10530-011-0005-z

Lesser, M. P., Stochaj, W. R., Tapley, D. W., & Shick, J. M. (1990). Bleaching in coral reef anthozoans: Effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs*, *8*(4), 225–232. https://doi.org/10.1007/BF00265015

Lessios, H. A., Robertson, D. R., & Cubit, J. D. (1984). Spread of diadema mass mortality through the Caribbean. *Science (New York, N.Y.)*, *226*(4672), 335–337. https://doi.org/10.1126/science.226.4672.335

Levasseur, M. (2013). Impact of Arctic meltdown on the microbial cycling of sulphur. *Nature Geoscience*, *6*(9), 691–700. https://doi.org/10.1038/ngeo1910

Levasseur, M., Gosselin, M., & Michaud, S. (1994). A new source of dimethylsulfide (DMS) for the arctic atmosphere: Ice diatoms. *Marine Biology*, *121*(2), 381–387. https://doi.org/10.1007/BF00346748

Lirman, D. (2001). Competition between macroalgae and corals: Effects of herbivore exclusion and increased algal biomass on coral survivorship and growth. *Coral Reefs*, *19*(4), 392–399. https://doi.org/10.1007/s003380000125

Lirman, D., & Biber, P. (2000). *Seasonal dynamics of macroalgal communities of the Northern Florida reef tract*. *43*(4), 305–314. https://doi.org/10.1515/BOT.2000.033

Littler, D. S. & Littler, M. M. (2000). *Caribbean reef plants* (First Edition). Offshore Graphics, Inc. https://www.biblio.com/book/caribbean-reef-plants-diane-scullion-littler/d/1401083087

Loram, J. E., Trapido-Rosenthal, H. G., & Douglas, A. E. (2007). Functional significance of genetically different symbiotic algae *Symbiodinium* in a coral reef symbiosis. *Molecular Ecology*, *16*(22), 4849–4857. https://doi.org/10.1111/j.1365-294X.2007.03491.x

Lovelock, J. E., Maggs, R. J., & Rasmussen, R. A. (1972). Atmospheric dimethyl sulphide and the natural sulfur cycle. *Nature*, *237*, 452–453.

Matrai, P. A., & Keller, M. D. (1994). Total organic sulfur and dimethylsulfoniopropionate in marine phytoplankton: Intracellular variations. *Marine Biology*, *119*(1), 61–68. https://doi.org/10.1007/BF00350107

Matthews, J. L., Sproles, A. E., Oakley, C. A., Grossman, A. R., Weis, V. M., & Davy, S. K. (2016). Menthol-induced bleaching rapidly and effectively provides experimental aposymbiotic sea anemones (*Aiptasia* sp.) for symbiosis investigations. *Journal of Experimental Biology*, *219*, 306–310. https://doi.org/10.1242/jeb.128934

McClanahan, T. R., & Mangi, S. (2000). Spillover of Exploitable Fishes from a Marine Park and Its Effect on the Adjacent Fishery. *Ecological Applications*, *10*(6), 1792–1805. https://doi.org/10.2307/2641239

McCoy, C. (2019). Cayman Islands marine protected areas, their status, effects and future. PhD Thesis

McLenon, A. L., & DiTullio, G. R. (2012). Effects of increased temperature on dimethylsulfoniopropionate (DMSP) concentration and methionine synthase activity in *Symbiodinium microadriaticum*. *Biogeochemistry*, *110*(1–3), 17–29. https://doi.org/10.1007/s10533-012-9733-0

Micheli, F., Mumby, P. J., Brumbaugh, D. R., Broad, K., Dahlgren, C. P., Harborne, A. R., Holmes, K. E., Kappel, C. V., Litvin, S. Y., & Sanchirico, J. N. (2014). High vulnerability of ecosystem function and services to diversity loss in Caribbean coral reefs. *Biological Conservation*, *171*, 186–194. https://doi.org/10.1016/j.biocon.2013.12.029

Moberg, F., & Folke, C. (1999). Ecological goods and services of coral reef ecosystems. *Ecological Economics*, *29*(2), 215–233. https://doi.org/10.1016/S0921-8009(99)00009-9

Mobley, K. B., & Gleason, D. F. (2003). The effect of light and heterotrophy on carotenoid concentrations in the Caribbean anemone *Aiptasia pallida* (Verrill). *Marine Biology*, *143*(4), 629–637. https://doi.org/10.1007/s00227-003-1123-7

Muller-Parker, G., & Davy, S. K. (2001). Temperate and tropical algal-sea anemone symbioses. *Invertebrate Biology*, *120*(2), 104–123. https://doi.org/10.1111/j.1744-7410.2001.tb00115.x

Mumby, P. J., Foster, N. L., & Fahy, E. A. G. (2005). Patch dynamics of coral reef macroalgae under chronic and acute disturbance. *Coral Reefs*, *24*(4), 681–692. https://doi.org/10.1007/s00338-005-0058-5

Muscatine, L., L. (1990). The role of symbiotic algae in carbon and energy flux in reef corals. *The Role of Symbiotic Algae in Carbon and Energy Flux in Reef Corals*, *25*, 75–87.

Nesa, B., & Hidaka, M. (2009). High zooxanthella density shortens the survival time of coral cell aggregates under thermal stress. *Journal of Experimental Marine Biology and Ecology*, *368*(1), 81–87. https://doi.org/10.1016/j.jembe.2008.10.018

Nielsen, D. A., Petrou, K., & Gates, R. D. (2018). Coral bleaching from a single cell perspective. *The ISME Journal*, *12*(6), 1558–1567. https://doi.org/10.1038/s41396-018-0080-6

Nii, C. M., & Muscatine, L. (1997). Oxidative Stress in the Symbiotic Sea Anemone *Aiptasia pulchella* (Carlgren, 1943): Contribution of the Animal to Superoxide Ion Production at Elevated Temperature. *The Biological Bulletin*, *192*(3), 444–456. https://doi.org/10.2307/1542753

Obara, B., Jabeen, A., Fernandez, N., & Laissue, P. P. (2013). A novel method for quantified, superresolved, three-dimensional colocalisation of isotropic, fluorescent particles. *Histochemistry and Cell Biology*, *139*(3), 391–402. https://doi.org/10.1007/s00418-012-1068-3

Ochsenkühn, M. A., Röthig, T., D'Angelo, C., Wiedenmann, J., & Voolstra, C. R. (2017). The role of floridoside in osmoadaptation of coral-associated algal endosymbionts to high-salinity conditions. *Science Advances*, *3*(8), e1602047. https://doi.org/10.1126/sciadv.1602047

Olguín-López, N., Gutiérrez-Chávez, C., Hérnández-Elizárraga, V. H., Ibarra-Alvarado, C., & Molina, A. R. (2018). Coral reef bleaching: An ecological and biological overview. In *Corals in a Changing World, 87*(1), 11–13, InTech. https://doi.org/10.5772/intechopen.69685

Osman, E. O., Smith, D. J., Ziegler, M., Kürten, B., Conrad, C., El-Haddad, K. M., Voolstra, C. R., & Suggett, D. J. (2018). Thermal refugia against coral bleaching throughout the northern Red Sea. *Global Change Biology*, *24*(2), e474–e484. https://doi.org/10.1111/gcb.13895

Palumbi, S. R., Barshis, D. J., Traylor-Knowles, N., & Bay, R. A. (2014). Mechanisms of reef coral resistance to future climate change. *Science*, *344*(6186), 895–898. https://doi.org/10.1126/science.1251336

Peach, K. E., Koch, M. S., Blackwelder, P. L., & Manfrino, C. (2017). Calcification and photophysiology responses to elevated pCO2 in six Halimeda species from contrasting irradiance environments on Little Cayman Island reefs. *Journal of Experimental Marine Biology and Ecology*, *486*, 114–126. https://doi.org/10.1016/j.jembe.2016.09.008

Perez, S., & Weis, V. (2006). Nitric oxide and cnidarian bleaching: An eviction notice mediates breakdown of a symbiosis. *The Journal of Experimental Biology*, *209*(14), 2804–2810. https://doi.org/10.1242/jeb.02309

Pochon, X., Putnam, H. M., & Gates, R. D. (2014). Multi-gene analysis of *Symbiodinium* dinoflagellates: A perspective on rarity, symbiosis, and evolution. *PeerJ*, *2*, 1–25. https://doi.org/10.7717/peerj.394

Quinn, P. K., & Bates, T. S. (2011). The case against climate regulation via oceanic phytoplankton sulphur emissions. *Nature*, *480*(7375), 51–56. https://doi.org/10.1038/nature10580

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL *http://www.rstudio.com/*.

Raina, J.-B., Dinsdale, E. A., Willis, B. L., & Bourne, D. G. (2010). Do the organic sulfur compounds DMSP and DMS drive coral microbial associations? *Trends in Microbiology*, *18*(3), 101–108. https://doi.org/10.1016/j.tim.2009.12.002

Raina, J.-B., Tapiolas, D. M., Forêt, S., Lutz, A., Abrego, D., Ceh, J., Seneca, F. O., Clode, P. L., Bourne, D. G., Willis, B. L., & Motti, C. A. (2013). DMSP biosynthesis by an animal and its role in coral thermal stress response. *Nature*, *502*(7473), 677–680. https://doi.org/10.1038/nature12677

Reaka-Kudla, M. L., Wilson, D. E., & Wilson, E. O. (1996). *Biodiversity II: Understanding and Protecting Our Biological Resources*. Joseph Henry Press.

Ritchie, R. J. (2006). Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research*, *89*(1), 27–41. https://doi.org/10.1007/s11120-006-9065-9

Robison, J. D., & Warner, M. E. (2006). Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of *Symbiodinium* (pyrrhophyta)1. *Journal of Phycology*, *42*(3), 568–579. https://doi.org/10.1111/j.1529-8817.2006.00232.x

Rowan, R. (2004). Thermal adaptation in reef coral symbionts. *Nature*, *430*(7001), 742–742. https://doi.org/10.1038/430742a

Rusch, D. B., Halpern, A. L., Sutton, G., Heidelberg, K. B., Williamson, S., Yooseph, S., Wu, D., Eisen, J. A., Hoffman, J. M., Remington, K., Beeson, K., Tran, B., Smith, H., Baden-Tillson, H., Stewart, C., Thorpe, J., Freeman, J., Andrews-Pfannkoch, C., Venter, J. E., … Venter, J. C. (2007). The Sorcerer II global ocean sampling expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLOS Biology*, *5*(3), e77. https://doi.org/10.1371/journal.pbio.0050077

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Simó, R., Hatton, A., Malin, G., & Liss, P. (1998). Particulate dimethyl sulphoxide in seawater:production by microplankton. *Marine Ecology Progress Series*, *167*, 291–296. https://doi.org/10.3354/meps167291

Steinke, M., Brading, P., Kerrison, P., Warner, M. E., & Suggett, D. J. (2011). Concentrations of dimethylsulfoniopropionate and dimethyl sulfide are strain-specific in symbiotic dinoflagellates (*Symbiodinium* sp., Dinophyceae). *Journal of Phycology*, *47*(4), 775–783. https://doi.org/10.1111/j.1529-8817.2011.01011.x

Steinke, M., Exton, D. A., & McGenity, T. J. (2011). Marine gases: Challenges to the bio(geo)chemist. *The Biochemist*, *33*(3), 20–25. https://doi.org/10.1042/BIO03303020

Steinke, M., Stefels, J., & Stamhuis, E. (2006). Dimethyl sulfide triggers search behavior in copepods. *Limnology and Oceanography*, *51*(4), 1925–1930. https://doi.org/10.4319/lo.2006.51.4.1925

Stimson, J., Sakai, K., & Sembali, H. (2002). Interspecific comparison of the symbiotic relationship in corals with high and low rates of bleaching-induced mortality. *Coral Reefs*, *21*(4), 409–421. https://doi.org/10.1007/s00338-002-0264-3

Suggett, D. J., & Smith, D. J. (2011). Interpreting the sign of coral bleaching as friend vs. Foe. *Global Change Biology*, *17*(1), 45–55. https://doi.org/10.1111/j.1365-2486.2009.02155.x

Suggett, D. J., Warner, M. E., Smith, D. J., Davey, P., Hennige, S., & Baker, N. R. (2008). Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (Pyrrhophyta) phylotypes with different thermal tolerances. *Journal of Phycology*, *44*(4), 948–956. https://doi.org/10.1111/j.1529-8817.2008.00537.x

Sunagawa, S., Wilson, E. C., Thaler, M., Smith, M. L., Caruso, C., Pringle, J. R., Weis, V. M., Medina, M., & Schwarz, J. A. (2009). Generation and analysis of transcriptomic resources for a model system on the rise: The sea anemone *Aiptasia pallida* and its dinoflagellate endosymbiont. *BMC Genomics*, *10*, 258. https://doi.org/10.1186/1471-2164-10-258

Sunda, W., Kieber, D. J., Kiene, R. P., & Huntsman, S. (2002). An antioxidant function for DMSP and DMS in marine algae. *Nature*, *418*(6895), 317–320. https://doi.org/10.1038/nature00851

Swain, T. D., Chandler, J., Backman, V., & Marcelino, L. (2017). Consensus thermotolerance ranking for 110 *Symbiodinium* phylotypes: An exemplar utilization of a novel iterative partial-rank aggregation tool with broad application potential. *Functional Ecology*, *31*(1), 172–183. https://doi.org/10.1111/1365-2435.12694

Swan, H. B., Deschaseaux, E. S. M., Jones, G. B., & Eyre, B. D. (2017). Quantification of dimethylsulfoniopropionate (DMSP) in *Acropora* spp. Of reef-building coral using mass spectrometry with deuterated internal standard. *Analytical and Bioanalytical Chemistry*, *409*(7), 1929–1942. https://doi.org/10.1007/s00216-016-0141-5

Swan, H. B., Jones, G. B., & Deschaseaux, E. (2012). Dimethylsulfide, climate and coral reef ecosystems. *Proceedings of the 12th International Coral Reef Symposium*, *4A*(January).

Takahashi, S., Whitney, S., Itoh, S., Maruyama, T., & Badger, M. (2008). Heat stress causes inhibition of the de novo synthesis of antenna proteins and photobleaching in cultured *Symbiodinium*. *Proceedings of the National Academy of Sciences*, *105*(11), 4203–4208. https://doi.org/10.1073/pnas.0708554105

Takahashi, S., Yoshioka-Nishimura, M., Nanba, D., & Badger, M. R. (2013). Thermal acclimation of the symbiotic alga *Symbiodinium* spp. Alleviates photobleaching under heat stress. *Plant Physiology*, *161*(1), 477–485. https://doi.org/10.1104/pp.112.207480

Tanner, J. E. (1995). Competition between scleractinian corals and macroalgae: An experimental investigation of coral growth, survival and reproduction. *Journal of Experimental Marine Biology and Ecology*, *190*(2), 151–168. https://doi.org/10.1016/0022-0981(95)00027-O

Tchernov, D., Gorbunov, M. Y., de Vargas, C., Narayan Yadav, S., Milligan, A. J., Häggblom, M., & Falkowski, P. G. (2004). Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proceedings of the National Academy of Sciences*, *101*(37), 13531–13535. https://doi.org/10.1073/pnas.0402907101

Thévenaz, Philippe. (1998). *An ImageJ plugin for the recursive alignment of a stack of images.* http://bigwww.epfl.ch/thevenaz/stackreg/

Thume, K., Gebser, B., Chen, L., Meyer, N., Kieber, D. J., & Pohnert, G. (2018). The metabolite dimethylsulfoxonium propionate extends the marine organosulfur cycle. *Nature*, *563*(7731), 412–415. https://doi.org/10.1038/s41586-018-0675-0

Todd, J. D., Rogers, R., Li, Y. G., Wexler, M., Bond, P. L., Sun, L., Curson, A. R. J., Malin, G., Steinke, M., & Johnston, A. W. B. (2007). Structural and regulatory genes required to make the gas dimethyl sulfide in bacteria. *Science (New York, N.Y.)*, *315*(5812), 666–669. https://doi.org/10.1126/science.1135370

Tolleter, D., Seneca, F. O., Denofrio, J. C., Krediet, C. J., Palumbi, S. R., Pringle, J. R., & Grossman, A. R. (2013). Coral bleaching independent of photosynthetic activity. *Current Biology*, *23*(18), 1782–1786. https://doi.org/10.1016/j.cub.2013.07.041

Toole, D. A., Slezak, D., Kiene, R. P., Kieber, D. J., & Siegel, D. A. (2006). Effects of solar radiation on dimethylsulfide cycling in the western Atlantic Ocean. *Deep Sea Research Part I: Oceanographic Research*, *53*, 136–153. https://doi.org/10.1016/j.dsr.2005.09.003

Trossat, C., Nolte, K. D., & Hanson, A. D. (1996). Evidence that the pathway of dimethylsulfoniopropionate biosynthesis begins in the cytosol and ends in the chloroplast. *Plant Physiology*, *111*(4), 965–973. https://doi.org/10.1104/pp.111.4.965

Turner, J., McCoy, C., Cottam, M., Olynik, J., Timothy, A., Blumenthal, J., Bothwell, J., Burton, F., Bush, P., Chin, P., Dubock, O., Godbeer, K. D., Gibb, J., Hurlston, L.-A., Johnson, B. J., Logan, A., Parsons, G., & Ebanks-Petrie, G. (2013). Biology and ecology of the coral reefs of the Cayman Islands. https://doi.org/10.1007/978-94-007-5965-7_7

Van Alstyne, K. L., Dominique, V. J., & Muller-Parker, G. (2009). Is dimethylsulfoniopropionate (DMSP) produced by the symbionts or the host in an anemone–zooxanthella symbiosis? *Coral Reefs*, *28*(1), 167–176. https://doi.org/10.1007/s00338-008-0443-y

Van Alstyne, K. L., Schupp, P., & Slattery, M. (2006). The distribution of dimethylsulfoniopropionate in tropical Pacific coral reef invertebrates. *Coral Reefs*, *25*(3), 321–327. https://doi.org/10.1007/s00338-006-0114-9

van der Loos, L. (2021). *Identification cards: The common macroalgae of Bonaire (Rhodophyta, Chlorophyta, Phaeophyceae)*.

Van Oppen, M. J. H., Souter, P., Howells, E. J., Heyward, A., & Berkelmans, R. (2011). Novel genetic diversity through somatic Mmutations: Fuel for adaptation of reef corals? *Diversity*, *3*(3), 405–423. https://doi.org/10.3390/d3030405

Vega Thurber, R., Burkepile, D. E., Correa, A. M. S., Thurber, A. R., Shantz, A. A., Welsh, R., Pritchard, C., & Rosales, S. (2012). Macroalgae decrease growth and alter microbial community structure of the reef-building coral, *Porites astreoides*. *PloS One*, *7*(9), e44246. https://doi.org/10.1371/journal.pone.0044246

Venn, A. A., Wilson, M. A., Trapido-Rosenthal, H. G., Keely, B. J., & Douglas, A. E. (2006). The impact of coral bleaching on the pigment profile of the symbiotic alga, *Symbiodinium*. *Plant, Cell & Environment*, *29*(12), 2133–2142. https://doi.org/10.1111/j.1365-3040.2006.001587.x

Veron, J. E. N. (1995). *Corals in Space and Time: The Biogeography and Evolution of the Scleractinia* (First American Edition). Cornell University Press.

Voolstra, C. R., Buitrago-López, C., Perna, G., Cárdenas, A., Hume, B. C. C., Rädecker, N., & Barshis, D. J. (2020). Standardized short-term acute heat stress assays resolve historical differences in coral thermotolerance across microhabitat reef sites. *Global Change Biology*, *26*(8), 4328–4343. https://doi.org/10.1111/gcb.15148

Weiler, C. S., & Eppley, R. W. (1979). Temporal pattern of division in the dinoflagellate genus ceratium and its application to the determination of growth rate. *Journal of Experimental Marine Biology and Ecology*, *39*(1), 1–24. https://doi.org/10.1016/0022-0981(79)90002-9

Weis, V. M. (2008). Cellular mechanisms of Cnidarian bleaching: Stress causes the collapse of symbiosis. *The Journal of Experimental Biology*, *211*(Pt 19), 3059–3066. https://doi.org/10.1242/jeb.009597

Weis, V. M., Davy, S. K., Hoegh-Guldberg, O., Rodriguez-Lanetty, M., & Pringle, J. R. (2008). Cell biology in model systems as the key to understanding corals. *Trends in Ecology & Evolution*, *23*(7), 369–376. https://doi.org/10.1016/j.tree.2008.03.004

White, R. H. (1982). Analysis of dimethyl sulfonium compounds in marine algae. *Journal of Marine Research*, 9.

Wietheger, A., Starzak, D. E., Gould, K. S., & Davy, S. K. (2018). Differential ROS Generation in Response to Stress in *Symbiodinium* spp. *The Biological Bulletin*, *234*(1), 11–21. https://doi.org/10.1086/696977

Wilkerson, F. P., Muscatine, L., & B, P. R. S. L. (1984). Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *221*(1222), 71–86. https://doi.org/10.1098/rspb.1984.0023

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology*, *208*(15), 2819–2830. https://doi.org/10.1242/jeb.01730

Yancey, P. H., Heppenstall, M., Ly, S., Andrell, R. M., Gates, R. D., Carter, V. L., & Hagedorn, M. (2010). Betaines and dimethylsulfoniopropionate as major osmolytes in cnidaria with endosymbiotic dinoflagellates. *Physiological and Biochemical Zoology*, *83*(1), 167–173. https://doi.org/10.1086/644625

Yoch, D. C. (2002). Dimethylsulfoniopropionate: Its sources, role in the marine food web, and biological degradation to dimethylsulfide. *Applied and Environmental Microbiology*, *68*(12), 5804–5815. https://doi.org/10.1128/aem.68.12.5804-5815.2002

Yost, D., & Mitchelmore, C. (2009). Dimethylsulfoniopropionate (DMSP) lyase activity in different strains of the symbiotic alga *Symbiodinium microadriaticum*. *Marine Ecology Progress Series*, *386*, 61–70. https://doi.org/10.3354/meps08031

Zhang, T., Diaz, J. M., Brighi, C., Parsons, R. J., McNally, S., Apprill, A., & Hansel, C. M. (2016). Dark production of extracellular superoxide by the coral *Porites astreoides* and representative symbionts. *Frontiers in Marine Science*, *3*(11), 1–16. https://doi.org/10.3389/fmars.2016.00232

Ziegler, M., Seneca, F. O., Yum, L. K., Palumbi, S. R., & Voolstra, C. R. (2017). Bacterial community dynamics are linked to patterns of coral heat tolerance. *Nature Communications*, *8*(1), 14213. https://doi.org/10.1038/ncomms14213

Appendix

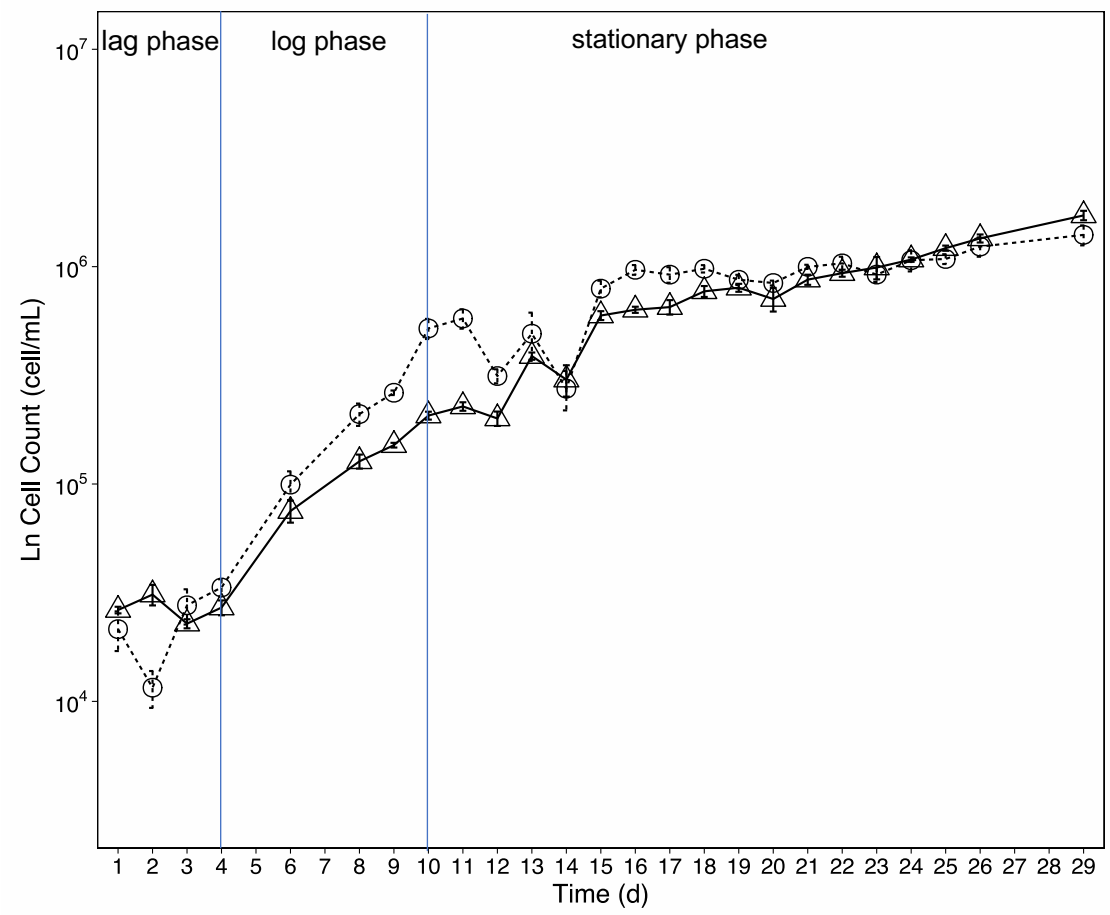


Figure 0-1. Natural logarithmic growth curve of Symbiodiniaceae *Symbiodinium linuchae* (SSA01, n=3) and *Breviolum minutum* (SSB01, n=3) at control conditions (86.29 µmol photons m^{-2} s^{-1} and 26°C). The exponential growth phase of SSA01 (triangle) and SSB01 (circle) is represented with a solid vertical line, added as text the different phases of cell growth. Some of the standard errors are sufficiently small that they are hidden by the data-points themselves.

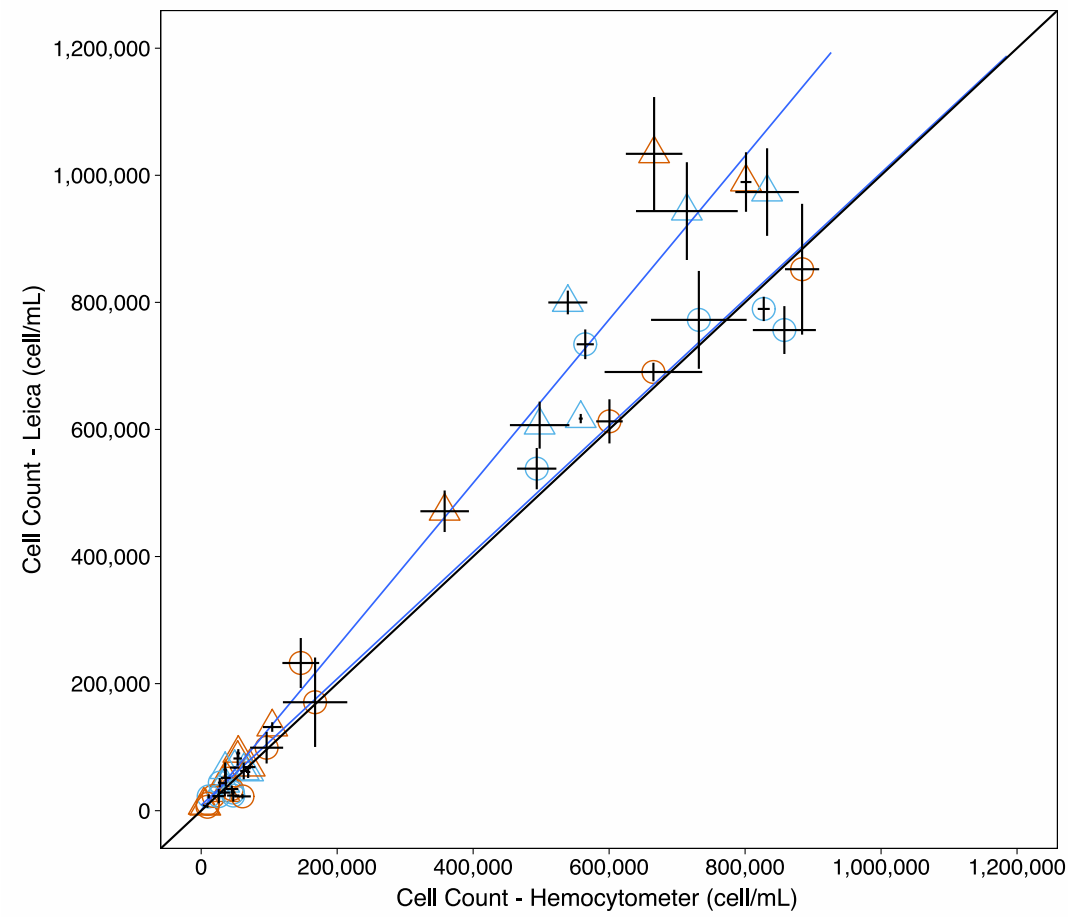


Figure 0-2. Cell count of Symbiodiniaceae using two different methods, Hemocytometer on the x-axis and Leica on the y-axis. Symbiodinium linuchae (SSA01, triangle) and Breviolum minutum (SSB01, circle) at control conditions (blue) and treatment conditions (red). Blue lines are regression lines. Some of the standard errors are sufficiently small that they are hidden by the data-points themselves.