THE PHOTOPHYSIOLOGY OF SYMBIOTIC DINOFLAGELLATES (*SYMBIODINIUM*) UNDER VARYING LIGHT AND THERMAL CONDITIONS AND THE IMPLICATIONS FOR CORAL BLEACHING

by

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ABSTRACT

Coral reefs are complex ecosystems full of extraordinary biodiversity. Reef building corals are reliant upon a symbiosis with intracellular dinoflagellates of the genus *Symbiodinium*. A considerable threat to coral reefs is the breakdown of this symbiosis, known as bleaching. Coral bleaching is characterized by the ghostly white tissue appearance resulting from loss of algal density. Over the past 20 years, there has been an increase in the frequency and magnitude of worldwide bleaching, which is strongly correlated with elevated seawater temperature, and exacerbated by increased irradiance. Bleaching has been linked to photosynthetic stress within the algae.

The capacity for photoacclimation to light at 100 or 600 μmol photons•m^{-2}•s^{-1} and the subsequent response to thermal stress was examined in four genetically distinct cultures of *Symbiodinium* (ITS2: A1, A1.1, B1, and F2). Cultures were maintained at the above light levels for at least one month prior to thermal investigation. Temperatures were ramped from the control temperature 26°C up to 32°C over 72hrs, where they remained for up to 10 days. Cultures were then monitored with chlorophyll fluorescence and protein techniques to monitor changes in photosynthesis.

While all algal types showed a reduction in chlorophyll *a* content, there was a differential response in cellular growth rate and photosystem II (PSII) activity between cultures. When maintained at 32° C for up to ten days, significant variation in the

susceptibility to thermal stress was observed in the rate of loss in PSII activity and electron transport, PSII reaction center degradation, and cellular growth rates. The order of thermal tolerance did not change between the two light levels; however high light exacerbated the effects. Phylotypes F2 and A1 showed a high degree of thermal tolerance, yet the cellular responses to light and temperature were markedly different.

The use of chlorophyll fluorescence is a common method used to evaluate photosynthesis; however, only recently has research begun to evaluate the accuracy of this technique at estimating true photosynthetic activity in *Symbiodinium*. Both chlorophyll fluorescence and oxygen evolution were monitored simultaneously in two different cultures (ITS: A1, B1) at ambient and elevated temperature. At 32°C, there was a significant decrease in both the maximum efficiency of PSII measured by chlorophyll fluorescence (F_v/F_m) and the efficiency of oxygen evolution (α) in both phylotypes: however, there was a two-fold greater decrease in α versus F_v/F_m. The relationship between oxygen evolution and electron transport was linear at low to moderate light levels, above which electron transport rates overestimates oxygen production. This study suggests that fluorescence often overestimates true values of photosynthetic activity and that care must be taken in interpreting field fluorescence data.

I. Coral Bleaching: An Introduction

Coral reefs are complex ecosystems full of extraordinary biodiversity and intricate microhabitats. While coral reefs cover only a small fraction of the ocean, they perform a number of important functions, including buffering shorelines during storms, sustaining fisheries and providing tourism based revenue for many tropical nations (Norse 1993). At the base of this ecosystem is an intriguing symbiosis of two domains, plant and animal. Reef building corals are reliant upon a symbiosis with intracellular dinoflagellates of the genus *Symbiodinium,* commonly referred to as zooxanthellae. Zooxanthellae reside within the gastrodermal tissue of the coral (Trench 1979). The symbiont translocates a significant portion of the photosynthetically derived carbon to the coral host, providing a large part of the coral's energy requirements, while the animal provides nutrients and shelter (Muscatine 1990). A considerable threat to coral reef ecosystems is the breakdown of this symbiosis, known as bleaching.

Coral bleaching is characterized by the ghostly white tissue appearance resulting from the expulsion of zooxanthellae cells and/or the loss of the photosynthetic pigments from the algae (Figure 1) (Hoegh-Guldberg and Smith 1989; Kleppel et al. 1989). Factors that can induce bleaching include, reduced salinity (Goreau 1964; Berkelmans and Oliver 1999), increased temperatures (Hoegh-Guldberg and Smith 1989; Iglesias-Prieto et al.

1992; Jones et al. 1998), high or low irradiance (Hoegh-Guldberg and Smith 1989; Iglesias-Prieto and Trench 1994), excessive ultraviolet irradiation (Lesser et al. 1990; Gleason and Wellington 1993), chemical pollution (Jones and Steven 1997; Jones and Hoegh-Guldberg 1999), and bacterial infection (Kushmaro et al. 1996). Bleaching events can lead to massive coral mortality and severely disrupt the rest of the ecosystem (Harriott 1985; Berkelmans and Oliver 1999; Marshall and Baird 2000; Loya et al. 2001). Even if colony mortality does not occur, prolonged or severe bleaching can interrupt the corals ability to produce gametes leading to unsuccessful reproduction (Baird and Marshall 2002).

While coral bleaching is caused by all of the factors listed above, over the past 20 years, there has been a rapid increase in the frequency and magnitude of worldwide coral bleaching, which has strongly correlated with elevated seawater temperature wherein temperatures rise by as little as 1-2°C (Brown 1997; Hoegh-Guldberg 1999). Bleaching is also observed when the summertime maximum temperature is not exceeded but persists for a longer duration in some years compared to others (Fitt et al. 1993). Also, coral bleaching is exacerbated by an increase in irradiance that often accompanies elevated temperatures (Bhagooli and Hidaka 2003). Thermal bleaching has been linked to the occurrence of photoinhibition and photodamage to photosystem II (PSII) within the algae, along with an increase in the production of reactive oxygen species (Brown 1997; Lesser 1997; Hoegh-Guldberg 1999; Warner et al. 1999).

Before continuing with reported photobiological changes specific in *Symbiondium* with thermal stress, it is important to review the general technique in measuring

photosynthesis. A common method for measuring photosynthesis is the use of chlorophyll fluorescence due to its quick and non-invasive nature. When light energy is absorbed by chlorophyll, there are three ways light energy is utilized: it can be used to drive photosynthesis, dissipated as heat, or re-emitted as fluorescence. A change in photosynthesis or heat dissipation is reflected as a quenching in the fluorescence trace. Light energy used to drive photosynthesis is called photosynthetic quenching, while energy dissipated away from PSII as heat is non-photochemical quenching. In the dark, PSII reaction centers are "open," meaning that the primary quinone Q_A is in the oxidized state, providing minimum fluorescence (F_o). When light excites the P_{680}, an electron is passed to Q_A which "closes" the reaction centers, once Q_A has accepted an electron it cannot accept another until the electron has passed to the secondary quinone, Q_B. The closure of reaction centers creates an increase in fluorescence and provides a maximal fluorescence value (F_m). The difference in fluorescence values between open and closed reaction centers is used for the calculation of maximum quantum yield ($F_v/F_m = (F_m - F_o)/F_m$), which is a measure of the efficiency of photon capture by PSII (Gentry et al. 1989).

Briefly, the remaining flow of electrons through PSII is as follows: once Q_B has bound within the D1 core protein, it accepts the electron from Q_A re-opening the reaction center. Q_B then disassociates from the protein and passes the electron on where it eventually enters photosystem I. The excited electron from P_{680} is replaced by the splitting of water and generates molecular oxygen (Figure 2) (Krause and Weis 1991; Maxwell and Johnson 2000). In continual light, the above process continues repeatedly so

that reaction centers are never fully open, raising the minimal fluorescence level, which is used to calculate the effective quantum yield ($\Delta F/F_m'$). The effective quantum yield is related to the proportion of light absorbed and the efficiency of PSII in the light acclimated state. In some cases, this value is proportional to the rate of linear electron transport and thus total photosynthesis (Gentry et al. 1989).

A decrease in the quantum yield of photosystem II in *Symbiodinium* during thermal stress has been demonstrated repeatedly in culture and *in hospite* under both laboratory and field conditions (Hoegh-Guldberg and Smith 1989; Iglesias-Prieto et al. 1992; Fitt and Warner 1995; Warner et al. 1996; Jones et al. 2000). Light-saturated maximal photosynthesis (net P_{max}) is significantly reduced at 30°C and ceases completely at 36°C in zooxanthellae cultured from the jellyfish *Cassiopeia xamachana*. This results in a significant loss in the net ratio of photosynthesis to respiration such that photosynthetic production cannot overcome the respiratory requirements of the cell (Iglesias-Prieto et al. 1992). Experimental exposure to elevated temperature has revealed that a decrease in the photosynthetic yield and electron flow between photosystem I (PSI) and PSII, and this tends to precede any significant symbiont expulsion and visible bleaching (Hoegh-Guldberg and Smith 1989; Warner et al. 1996).

Three non-mutually exclusive characteristic photosynthetic responses of zooxanthellae during thermal stress have been suggested: 1) increase in non-photochemical quenching not accompanied by a significant decrease in photochemical quenching, 2) a decrease in quantum yield of PSII, and 3) a decrease in photosynthetic oxygen evolution (Jones et al. 1998). Photoinhibition caused by the breakdown of

photosystem II reaction centers, specifically the breakdown of the reaction center protein D1, has also been suggested as a cause of thermal bleaching (Warner et al. 1999). Additionally, an increase in the generation of reaction oxygen species (ROS), such as superoxide radicals and hydrogen peroxide, has been recorded during thermal stress (Lesser 1996; Brown et al. 2002a; Franklin et al. 2004). Reactive oxygen species can cause physical membrane damage which may be leading to the loss of PSII reaction centers noted in other studies (Smith et al. 2005). It has been shown that thermally sensitive and tolerant *Symbiodinium* have different compositions of membrane lipids, with the thermally sensitive alga showing an irreversible loss in membrane integrity that corresponded with an increase in ROS production (Tchernov et al. 2004).

Photoinhibition has been categorized as being chronic or dynamic, with the former characterized as damage at the protein level that requires *de novo* protein repair which takes hours to days to recover (Osmond 1994). The PSII reaction center protein, D1 is the primary target of chronic photoinhibition damage (Ohad et al. 1994). Damage to the D1 protein during thermal bleaching has been observed in zooxanthellae both from naturally bleaching field collected corals and from cultures exposed to thermal stress (Warner et al. 1999). This protein is considered to be a key player in the breakdown of photosynthesis during bleaching, though direct measurements of D1 protein levels have been investigated in few studies (Warner et al. 1999; Lesser and Farrell 2004).

In contrast to chronic photoinhibition, dynamic photoinhibition lasts for mere minutes to hours (Osmond 1994). It is often related to non-photochemical quenching, where the excess light excitation energy is diverted away from PSII. Non-photochemical

quenching has three components defined by their reaction kinetics: energy-dependent quenching (qE), state-transition quenching (qT), and photoinhibitory quenching (qI). qE is the quickest component, relaxing in seconds to minutes, while qI is the longest taking several hours (Müller et al. 2001). The most common component of non-photochemical quenching measured and discussed is qE, causing a decrease in the pH of the lumen which is believed to trigger xanthophyll conversion, wherein the pigment diadinoxanthin is converted to diatoxanthin. Diatoxanthin is then able to absorb excess photon energy that would otherwise be damaging to active chlorophyll molecules (Brown et al. 1999; Winters et al. 2003), and correlates quite strongly to the daily drop and subsequent recovery in the effective quantum yield of chlorophyll fluorescence . The absolute quantum yield of PSII (F_v/F_m) in zooxanthellae *in hospite* is inversely related to irradiance levels; in simpler terms, the activity of PSII decreases as light increases (Brown et al. 1999; Gorbunov et al. 2001; Warner et al. 2002).

There is no set temperature at which bleaching will occur in all locations or within every coral species; in fact the occurrence and severity of bleaching varies significantly between and within coral species (Edmunds 1994; Fitt and Warner 1995; Berkelmans and Oliver 1999; Loya et al. 2001). Bleaching has been noted to occur at 28°C in *Plesiastrea versipora,* while no significant bleaching was recorded until 34°C in *Platygyra ryukuensis,* demonstrating the wide range of thermal tolerances in different species of coral (Jones et al. 2000; Bhagooli and Hidaka 2004) and their symbionts. Photosynthetic changes due to thermal stress vary across Caribbean reef-building coral species (Fitt and Warner 1995; Warner et al. 1996). For example, the zooxanthellae residing in

Montastraea annularius seem to be thermally damaged at or near the reaction center of photosystem II (PSII), while zooxanthellae of *Siderastrea radians* are able to dissipate the excess excitation energy preventing photodamage under the same experimental conditions (Warner et al. 1996). There are several hypotheses to explain the observed differences in bleaching onset and severity, including, genetic differences in the hosts (Edmunds 1994), genetic differences within *Symbiodinium* (Rowan et al. 1997), different coral morphology and host tissue thickness which may provide differential protection for the zooxanthellae (Loya et al. 2001), and previous exposure to thermal and irradiance stress (Brown et al. 2002c).

Recently, the hypothesis that a diverse assemblage of zooxanthellae inhabit some corals, and that this diversity may explain observed bleaching patterns has received a great deal of attention. Historically, zooxanthellae were classified based on morphological differences, however, recent attention has turned to molecular methods to elucidate the taxonomic diversity of these algae. There were four original species of *Symbiodinium* described using morphological methods: *S. microdiraticum*, *S. kawagutii*, *S. pilosum, S.goreauii* (Freudenthal 1962; Trench and Blank 1987). Rowan and Powers (1991) were the first to create a molecular phylogeny of zooxanthellae based on restriction fragment length polymorphisms (RFLP) and sequence analysis of the small subunit ribosomal RNA (ssRNA) nuclear gene. Their limited data set, taken largely from cultured zooxanthellae, split the algae into three predominant clades, A, B, and C. The use of the rapidly evolving ITS region of the ribosomal gene has brought a more refined separation of species within clades that are not resolved using either small or large

subunit rDNA sequence (LaJeunesse 2001; LaJeunesse 2002; LaJeunesse et al. 2003). To date, the genus *Symbidodinium* has been split into eight clades (A – H), only 5 of which have been discovered in corals: A, B, C, D and F (Baker 2003; LaJeunesse 2004; Coffroth and Santos 2005).

Current algal typing methods have allowed a better understanding of the potential ecological distribution of zooxanthellae. For example, shallow environments have higher symbiont diversity than deeper environments (LaJeunesse 2002). The distribution of coral species across a depth gradient is based upon the symbionts ability for photoacclimation in eastern Pacific corals (Iglesias-Prieto et al. 2003). Likewise, Rowan et al. (1997) found zonation of symbionts within two species of the predominant Caribbean coral genus *Montastraea* were based on light levels. They summarized that clades A and B are found in shallow, high light environments, while clade C is located in deeper low light environments. They also suggested that during bleaching events the clade C alga was the most thermally sensitive and was expelled in higher proportions. It is now recognized that this interpretation is an overgeneralization, as physiological differences are now known at the intracladal level (e.g. one B type alga may be more thermally tolerant than another B type). It has also been shown in transplant and toppling experiments that corals can exchange their zooxanthellae for types that are more favorable to the new environment leading to the so called "adaptive bleaching hypothesis" (Rowan et al. 1997; Baker 2001; Toller et al. 2001). The adaptive bleaching hypothesis suggests that bleaching events allow the coral to exchange current symbionts for less sensitive ones (Baker 2001). In a reciprocal transplant depth experiment, Baker

(2001) showed that zooxanthellae within clade D appears in corals recovering from experimental bleaching. However, experimental data suggests only temporary algal "reshuffling", that is an already present cryptic algal population temporarily becomes dominant and after a few weeks the original dominant compliment of zooxanthellae returns (Lewis and Coffroth 2004; Thornhill et al. 2005).

The understanding of changes in photophysiology of individual phylotypes of zooxanthellae is important to understanding and predicting global bleaching events. Research of photobiology of individual types of *Symbiodnium* has become a subject of interest in recent years. Iglesias-Prieto and Trench (1994) demonstrated that three cultured species of *Symbiodinium* have very different patterns of photoacclimation unique to each particular species. Savage et al. (2002) showed that variations in the photosynthetic response to irradiance of *Symbiodinium* were not consistent with cladal designation. Their study only evaluated the resolution of the large subunit RNA nuclear gene, not the more variable ITS region, which may tease out fine scale differences within clades. Brown et al. (2002c) saw no difference in cladal genotype of the zooxanthellae residing within the coral *Goniastrea aspera,* yet they demonstrated different bleaching susceptibilities on the west versus east facing sides of coral colonies. This work showed a clear phenotypic difference in thermal stress response among the same type of zooxanthellae acclimated to different irradiance levels. To date most of the work performed has focused on one or two photosynthetic parameters utilizing short and acute stress periods and have investigated either thermal or irradiance stress but rarely both.

This study endeavors to describe the photosynthetic changes associated with photoacclimation in different phylotypes of *Symbiodinium* and compare the photosynthetic responses of these acclimated algae to thermal stress over a timeframe comparable to observed global bleaching conditions. In addition, this research attempts to further elucidate the relationship between chlorophyll fluorescence, the most common technique to measure photosynthesis in coral reefs, and oxygen evolution, traditional photosynthesis measurements, within *Symbiodinium*. Also to evaluate if this relationship changes during increased thermal conditions, such as those that may be experienced during coral bleaching events.

Figures

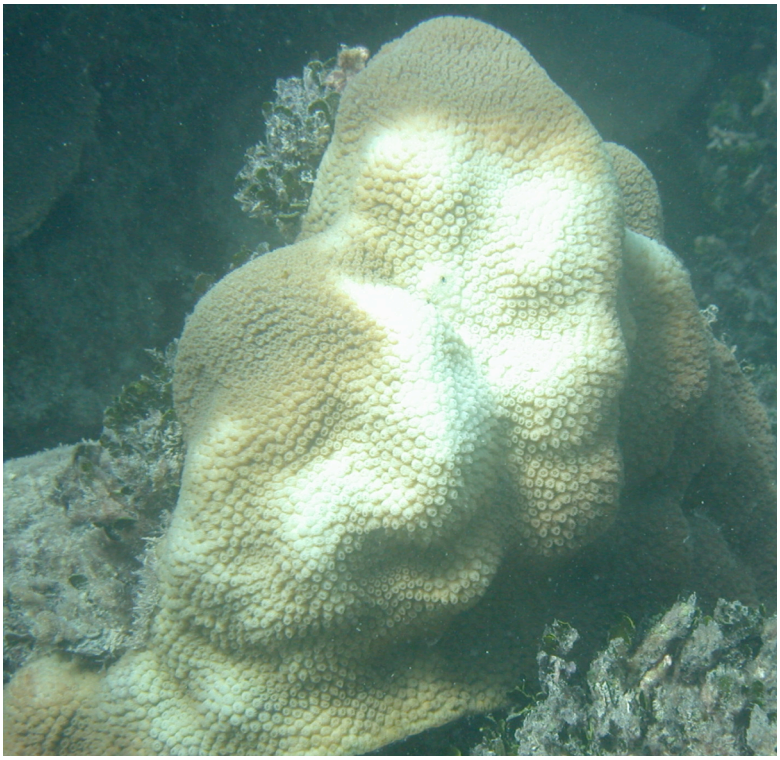

Figure 1: Partially bleached colony of *Montastraea faveolata* in Key Largo Florida. Photograph courtesy Dr. Mark E. Warner.

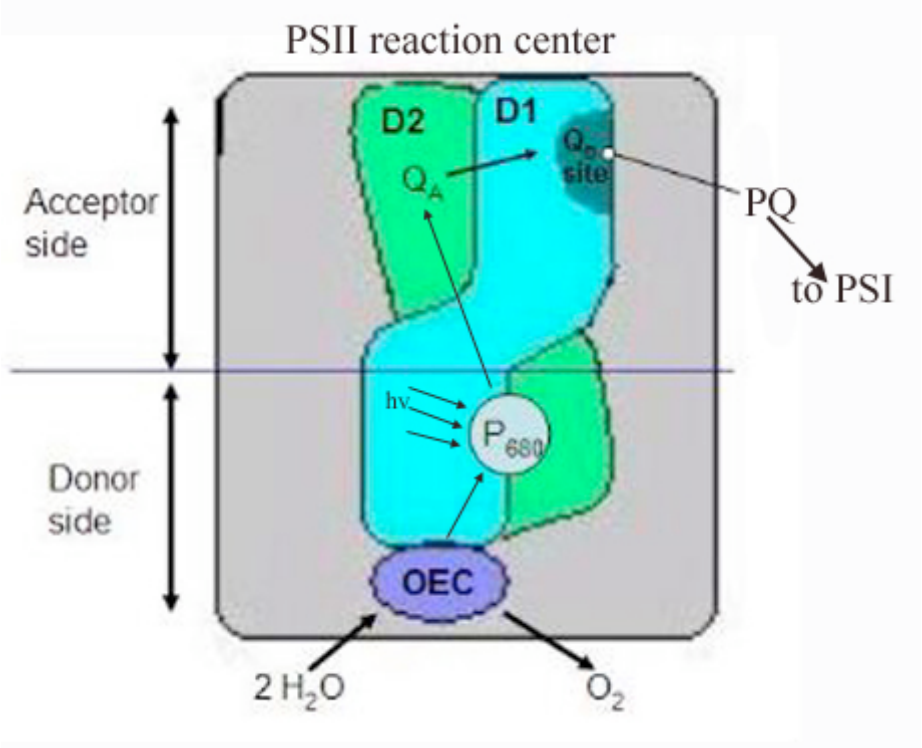

Figure 2: Electron transport through photosystem II (PSII) reaction centers. Light (hv) excites an electron in P_{680} reaction center, which reduces the primary acceptor Q_A. The secondary acceptor Q_B binds to the D1 protein and accepts the electron from Q_A. Q_B then disassociates from the protein and electrons continue into the plastoquinone (PQ) pool and the electrons continue onto photosystem I (PSI). The electron in P_{680} is replaced by the splitting of water in the oxygen evolving compound (OEC) eventually causing the generation of molecular oxygen.

II: Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of *Symbiodinium* (Pyrrhophyta)

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Abstract

The capacity for photoacclimation to light at 100 or 600 µmol photons•m^{-2}•s^{-1} and the subsequent response to thermal stress was examined in four genetically distinct cultures of symbiotic dinoflagellates in the genus *Symbiodinium* with the ITS2 designations A1, A1.1, B1, and F2. While all algal types showed typical signs of photoacclimation to high light via a reduction in chlorophyll *a*, there was a differential response in cellular growth, photosystem II (PSII) activity, and the chlorophyll *a* specific absorption coefficient between cultures. When maintained at 32° C for up to ten days, significant variation in the susceptibility to thermal stress was observed in the rate of loss in PSII activity and electron transport, PSII reaction center degradation, and cellular growth. The order of thermal tolerance did not change between the two light levels. However, as expected, loss in photosynthetic function was exacerbated in the thermally sensitive phylotypes (B1 and

A1.1) when acclimated to the higher light intensity. There was no consistent relationship between thermal tolerance and changes in light energy dissipation via non photochemical pathways. Phylotypes F2 and A1 showed a high degree of thermal tolerance, yet the cellular responses to light and temperature were markedly different between these algae. The F2 isolate showed the greatest capacity for photoacclimation and growth at high light and temperature, while the A1 isolate appeared to adjust to thermal stress by a slight decline in PSII activity and a significant decline in growth, possibly at the expense of increased photosystem and cellular repair.

Introduction

Coral reefs represent an important ecosystem with respect to both biodiversity and human dependence. Reef building corals are reliant upon a symbiosis with intracellular dinoflagellates in the genus *Symbiodinium,* commonly referred to as zooxanthellae. These algae translocate a significant portion of their photosynthetically derived carbon to the coral host, providing a large part of the coral's daily energy requirements (Muscatine 1990). A considerable threat to coral reefs is the breakdown of this symbiosis due to coral bleaching. Coral bleaching is characterized by a loss of either zooxanthellae from the coral and/or loss of photosynthetic pigments in the algae (Fitt et al. 2001). Bleaching events can lead to massive coral mortality and further reef degradation (Harriott 1985; Berkelmans and Oliver 1999; Marshall and Baird 2000; Loya et al. 2001), and severe

bleaching can suppress gametogenesis and therefore lead to lower fecundity (Baird and Marshall 2002).

There has been a rapid increase in the frequency and magnitude of global bleaching events (Glynn 1993; Brown 1997; Hoegh-Guldberg 1999) that strongly correlate with elevated seawater temperature, wherein temperatures rise by as little as 1-2° C above the annual maximum or when maximum temperatures persist for extended times (Fitt et al. 1993; Brown 1997; Hoegh-Guldberg 1999). Many of these events have occurred during doldrums, thereby leading to excessive irradiance which exacerbates the impact of thermal stress. Several studies have shown a disruption of photosynthesis in *Symbiodinium* spp. during exposure to temperatures above 32° C (Coles and Jokiel 1977; Iglesias-Prieto et al. 1992). Elevated temperature can lead to a decrease in the dark acclimated quantum yield of PSII (F_v/F_m) in *Symbiodinium* in culture and *in hospite* under both laboratory and field conditions (Hoegh-Guldberg and Smith 1989; Iglesias-Prieto et al. 1992; Fitt and Warner 1995; Warner et al. 1996; Jones et al. 2000; Bhagooli and Hidaka 2003; Hill et al. 2004), and significant photoinactivation is noted prior to symbiont expulsion and visible bleaching (Hoegh-Guldberg and Smith 1989; Warner et al. 1996).

While in many cases thermal bleaching is linked to photoinhibition (Brown 1997; Lesser 1997; Hoegh-Guldberg 1999; Warner et al. 1999), the specific cellular pathways responsible for the decline in photosynthesis is an active area of study. In *Symbiodinium*, excessive temperature leads to a significant loss in the primary PSII reaction center protein, D1, which may result from a lesion at some point in the reaction center repair

cycle (Warner et al 1999). Others have postulated that the dark reactions of photosynthesis are initially affected, leading to further sink limitation of electron flow that results in damage to PSII (Jones et al. 1998; Yakovleva and Hidaka 2004). Of particular interest is the impact of reactive oxygen species (ROS), including superoxide radicals and hydrogen peroxide, both of which have been implicated in the loss of photosynthesis in zooxanthellae exposed to elevated temperature, excessive light, and ultraviolet radiation (Lesser 1996; Lesser 1997). Importantly, oxygen radicals have been implicated in directly cleaving D1 peptide bonds (Lupínková and Komenda 2004) and blocking D1 translation in *Synecocystis* sp. (Nishiyama et al. 2004). Likewise, ROS have been implicated in initiating cascades of intracellular degradation in *Symbiodinium* that closely resemble programmed cell death discovered in other microalgae (Bidle and Falkowski 2004; Franklin et al. 2004). Recently, Tchernov et al (2004) have noted significant differences in the fatty acid profiles in thylakoid membrane lipids from thermally sensitive and tolerant *Symbiodinium*, and suggested that elevated levels of specific polyunsaturated fatty acids could enhance membrane stability and lower the potential for damage by ROS during thermal stress.

The genus *Symbiodinium* represents a highly diverse group of dinoflagellates that are divided into eight large clades (denoted clades A–H) (Rowan and Powers 1991; Coffroth and Santos 2005), and it is clear that thermal sensitivity is not relegated to any one clade of zooxanthellae (Tchernov et al 2004). Natural zooxanthellae distributions are strongly influenced by both light across depth gradients found on a reef as well as over larger geographic scales (LaJeunesse 2002; Ulstrup and van Oppen 2003; Iglesias-Prieto

et al. 2004). Further, light can directly influence the micro-scale distribution of zooxanthellae in corals that simultaneously harbor genetically different *Symbiodinium* populations, as well as lead to differential algal loss across a coral colony during natural thermal bleaching (Rowan et al. 1997).

Reef building corals inhabit a wide range of irradiance levels spanning roughly two orders of magnitude across their respective depth gradients (Wyman et al. 1987). Unlike phytoplankton, incident irradiance on benthic organisms such as corals is relatively stable, thus making photoacclimation to long term (e.g. several days to months) shifts in light exposure more relevant to *Symbiodinium* versus more temporally variable shifts in light intensity that many free living microalgae must face (Macintyre et al. 2000). The photochemical efficiency of zooxanthellae typically increases with depth (Dubinsky et al. 1984; Warner et al. 1999), thus photochemical capacity in shallow corals is further reduced during bleaching (Bhagooli and Hidaka 2003). In some cases of large-scale bleaching, shallow corals appear to be affected greater than deeper corals (Marshall and Baird 2000). The effect of thermal stress on *Symbiodinium* acclimated to various irradiance levels is poorly understood, yet may be very important given the substantial differences noted in the capacity for photoacclimation between different species in this genus (Iglesias-Prieto and Trench 1994).

In other algal systems, acclimation to temperature at the level of the photosynthetic apparatus is quite similar to photoacclimation. In particular, growth at low temperature can lead to a reduction in light absorption and a concomitant increase in photosynthetic capacity and a decline in cellular chlorophyll and light harvesting protein

abundance (Maxwell et al. 1994). Much less is known concerning the interaction of light and elevated temperature and possible adjustments to light harvesting and photosynthetic capacity. The objectives of this study were to investigate the changes in photochemical potential associated with photoacclimation to high light in four different phylotypes of *Symbiodinium* and then compare the impact of elevated temperature on the photosynthetic capacity, potential for photoinactivation, and long term resilience of these algae during thermal perturbation.

Materials and Methods

Four cultures of *Symbiodinium* from three different genetic clades, A, B, and F, were used in this study. These isolates were previously characterized by analysis of the ribosomal ITS2 sequence as A1, A1.1, B1 and F2 (LaJeunesse 2001). The A1 culture was previously described as *Symbiodinium microadriaticum,* originally isolated from the jellyfish *Cassiopeia xamachana* (Freudenthal 1962). The other cultures were originally isolated from the sea anemones *Condylactis gigantea* (A1.1), *Aiptasia pallida* (B1), and the scleractinian coral *Meandrina meandrites* (F2). These isolates were part of the original *Symbiodinium* culture collection of Dr. Robert Trench and have been held in culture for approximately 17-20 years.

Cultures were maintained in artificial seawater media (ASP-8A) (Blank 1987) at 26° C on a 14:10 light:dark cycle in temperature controlled incubators with high output fluorescent bulbs set to 80 μmol photons•m^{-2}•s^{-1} measured with a 4π light sensor (Li-Cor

193SA). Cultures were shifted to incubators set to either 100 or 600 μmoles photons• m^{-2}•s^{-1} PAR (for simplicity, these levels are referred to hereafter as low and high light respectively). Replicate flasks (n=4 for all treatments $isolate^{-1}$) were acclimated to the two light settings for at least one month prior to experimentation and maintained in log-phase batch growth by replenishing media every ten days to minimize the effects of self-shading and prevent nutrient limitation. Cell densities were reduced to approximately 5 x 10^4 cells•ml^{-1} with each addition of media. Following light acclimation, thermal exposure was gradually applied by raising the temperature in 2° C increments from 26 to 32° C over 72 h, where the temperature was raised once every 24 h at 09:00. Once 32° C was reached, samples for fluorescence analysis (described below) were taken every 24 hours for the first 72 hours, after which, samples were removed every 48 hours for a total exposure time of 240 hours. All samples were collected at 09:00 to eliminate any possible variation due to natural diel periodicity in the measured parameters. Cell number was measured microscopically with a hemacytometer (8 counts $sample^{-1}$), and cell specific growth rate (μ, day^{-1}) was calculated during the first five days of growth at 32° C.

Chlorophyll fluorescence analysis.

At each sampling time, PSII activity was monitored by pulse amplitude modulation fluorometry with a laboratory fluorometer fitted with a liquid suspension cuvette with gentle stirring (PAM 101, Walz, Germany). All fluorescence measurements were performed at room temperature (24-26° C). The continual 1.6 kHz measuring light

of the fluorometer had a peak wavelength of 470nm (intensity < 1 μmoles photons•m^{-2}•s^{-1}). Chlorophyll fluorescence ≥ 645 nm was detected via a photodiode, and the zero off-set of the fluorometer was adjusted with a filtered (0.2 μm) media blank each day prior to sampling. Samples were dark acclimated for 12 minutes prior to measuring the maximum quantum yield of PSII fluorescence ($F_v/F_m = F_m-F_o/F_m$) with a brief saturation light pulse (400 ms, 3000 μmol photons•m^{-2}•s^{-1}) provided by a blue LED array (470 nm peak, HPL-L470, Walz, Germany). Immediately after recording F_v/F_m, fluorescence induction during steady state fluorescence was recorded under the respective growth light intensities (either 100 or 600 μmol photons•m^{-2}•s^{-1}). Light for fluorescence induction was provided by the same LED array used for saturation pulses, and the effective quantum yield of PSII ($\Delta F/F_m$') as well as photochemical and nonphotochemical quenching of the fluorescence signal were recorded. Photochemical quenching (qP) was corrected for the F_o' signal with a short (1 s) dark period and pulse of far red light, and calculated as $qP = (F_m'-F_s)/(F_m'-F_o')$, where F_m' is the maximal fluorescence yield in the light acclimated state, Fs is the steady state fluorescence, and F_o' is the minimal fluorescence signal in the light acclimated state. Nonphotochemical quenching was calculated as $NPQ = (F_m - F_m')/F_m'$ where F_m is the dark acclimated maximal fluorescence (van Kooten and Snell 1990; Kromkamp and Forester 2003)

Fluorescence relaxation kinetics were used to assess the rate of electron transport between the primary and secondary quinone acceptors of PSII (Q_A to Q_B). Fluorescence decay was recorded following a single turnover saturation flash provided by a xenon strobe (XST-103, Walz, Germany, 8 μs half rise time) using a sampling rate of 20 μs.

Each sample was dark acclimated for 12 minutes and the fluorescence decay was recorded for six single turnover flashes with a dark acclimation period of 30 s between each flash. The mean re-oxidation curve of each sample was analyzed by a non-linear second order exponential decay (Origin, Microcal) to obtain the electron transfer rate (τ) from Q_A to Q_B as the initial time constant of the fitted curve (Schofield et al. 1998).

Pigment and protein analysis.

Samples were removed for both pigment and protein analysis just prior to thermal ramping (26° C) and after 120 and 240 h of exposure to 32° C. Due to the rapid loss in PSII function and cell growth in the A1.1 isolate at 600 µmol photons•m^{-2}•s^{-1} and 32° C (see results) this alga was not sampled at 240 h. The average (400-700 nm) chlorophyll *a* specific absorption coefficient (ā*), was calculated by the quantitative filter pad method with adjustments for the spectrophotometer used (Cleveland and Weidemann 1993). Samples (5-15mL) were filtered onto GF/F glass fiber filters and scanned between 380 and 750 nm in a spectrophotometer fitted with an integrating sphere (UV-2401PC, Shimadzu, USA). Duplicate filters were used for each sample. Absorption spectra were corrected for light scattering by subtracting the absorption at 750 nm prior to ā* calculation. The filters were then ground in 100% acetone and extracted pigments recorded by spectrophotometry, and chlorophyll *a* content was calculated using the equations of Jeffrey and Humphrey (1975). Algal proteins were resolved by 1-D gel electrophoresis (15 % acrylamide) with equivalent protein loading between samples (15

μg•lane^{-1}), followed by Western blotting in order to quantify D1 protein content, using previously published methods (Warner et al. 1999) with the exception that algal cells were immediately lysed in the detergent-SDS buffer mixture prior to electrophoresis.

Statistics

Statistical comparisons were made by analysis of variance (ANOVA), with Tukey post-hoc analysis when necessary, using SPSS version 11.0 (SPSS Inc., USA). Data were examined to ensure normality and when necessary log transformations were applied. Non transformed data are shown in all figures.

Results

Cell growth and chlorophyll a

There was no significant effect of increasing light from 100 to 600 μmol photons•m^{-2}•s^{-1} on growth rates of A1 and A1.1 algae at 26° C. Only the growth rate of the F2 isolate increased significantly in high light, while that of the B1 isolate decreased (Table 1). There was no change in growth rate in any alga acclimated to low light during the first five days at 32° C. However, there was a significant decline in growth rate in isolates A1, B1, and A1.1 at high light and 32° C (Table 1). As expected, chl *a* cell^{-1} decreased by 50-60% in all algae when grown at high light at 26° C ($p<0.01$, Table 2). At

32° C, chlorophyll *a* decreased significantly in A1 and F2 algae grown in low light by 42 and 22% respectively, while thermal exposure under high light resulted in a significant decline in chlorophyll *a* within types A1.1 and B1 by 43 and 71% respectively ($p<0.01$, Table 2).

Chlorophyll fluorescence

At the start of the experiment, the maximum quantum efficiency of PSII (F_v/F_m) was significantly lower in all high light acclimated cells compared to cells acclimated to low light. The extent of this decrease was different between isolates, and types F2 and A1 demonstrated a smaller decrease in F_v/F_m compared to types B1 and A1.1 ($p <0.01$, compare closed symbols at -48 h in Figure 1 A–D). At 32° C, F_v/F_m did not change in the F2 alga at either light level and decreased by 10% in the A1 alga at high light ($p = 0.703$, <0.01 respectively, Figure 1A, B). F_v/F_m decreased in B1 and A1.1 algae by 29 and 44% respectively at 32° C and 100 µmol photons•m^{-2}•s^{-1}. Loss of PSII activity at 32° C in these algae was amplified further to 41 and 53% of the control values respectively at high light ($p<0.01$, Figure 1C, D). In particular, F_v/F_m in the A1.1 alga declined at 32° C and high light in half the time compared to the low light treatment. (Figure 1D). The experiment with the high light acclimated A1.1 alga was terminated at 120 h since continued measurements indicated that the fluorescence signal was close to the limit of accurate detection with the photodiode sensor of the PAM fluorometer.

When measured at the respective growth light intensity, the effective quantum yield, $\Delta F/F_m'$, was significantly lower in high light acclimated samples relative to low light acclimated algae prior to thermal ramping (Figure 2, compare closed symbols at -48 h). Similar to F_v/F_m, elevated temperature had no significant impact on $\Delta F/F_m'$ in the F2 alga at either light level (Figure 2A). Low light acclimated A1, B1, and A1.1 algae all demonstrated a significant decrease in $\Delta F/F_m'$ relative to control treatments when grown at 32° C ($p<0.01$, Figure 2B-D). However, the rate of decline in effective quantum yield was different between each isolate and not always as drastic as the loss in dark acclimated F_v/F_m. In particular, $\Delta F/F_m'$ in the A1 alga began to decrease by 24 h at 30° C, while culture B1 and A1.1 showed significant declines relative to control values at 24 and 240 h respectively (Figure 2 B, C and D, square symbols). In contrast to low light treatments, there was no significant decline in $\Delta F/F_m'$ in any culture acclimated to high light at 32° C.

When measured at the respective growth light intensity, all high light acclimated cultures at 26° C displayed significantly greater nonphotochemical quenching (NPQ) relative to low light cultures, with the A1 and B1 isolates having the highest levels of NPQ at 600 μmol photons•m^{-2}•s^{-1} (Figure 3 A–D, closed symbols at -48 h). At low light, the B1 alga displayed a significant rise in NPQ when held at 32° C ($p<0.01$), while the F2 alga had a slight increase in NPQ under the same treatment conditions. Elevated temperature had no effect on NPQ in algae grown under high light.

When grown at high light at 26° C, photochemical quenching (qP) in A1 and A1.1 algae was significantly lower ($p<0.01$) as compared to qP values recorded at low

light (Figure 4 B and D, closed symbols), while there was no difference in qP between high and low light acclimated cultures in F2 or B1 algae (Figure 4A and C). Elevated temperature had no effect on qP in the F2 or A1.1 algae at either light intensity (Figure 3A, D, open vs. closed symbols). However, qP declined significantly in the low light acclimated A1 alga after 48 h at 32° C, and continued to decline by 36% relative to the control temperature ($p<0.01$), yet there was no change in qP in this alga at 32° C and 600 μmol photons•m^{-2}•s^{-1} (Figure 4B). Conversely, there was no change in qP in the low light acclimated B1 alga under elevated temperature, while it declined by 12.7% in the high light and 32° C ($p<0.01$, Figure 4C).

At high light and 26° C, the fast component of the reoxidation curve (representing one electron transfer from Q_A to Q_B or Q_B^-) increased by 3, 17, 30 and 12 % in F2, A1, B1, and A1.1 algae respectively (Figure 5, closed symbols). There was no significant change to the reoxidation time in the A1 alga regardless of light intensity, nor was there a change in the F2 culture when exposed to 32° C at low light (Figure 5). Electron transport time rose significantly in the B1 and A1.1 algae at low light and elevated temperature. This loss in electron transport at 32° C was further exacerbated in the A1.1 alga when acclimated to high light, resulting in a 53% increase in τ in half the time as that seen at low light (Figure 5).

Cellular absorption

Prior to thermal ramping, acclimation to high light resulted in a significant increase in the spectrally averaged chlorophyll *a* specific absorption coefficient (ā*) in the F2, A1 and B1 algae ($p<0.02$), while there was no change in the A1.1 alga (Table 3). By the end of the thermal treatment, there was a significant increase in ā* in the F2 alga at both light levels. For all other algae, ā* increased significantly following exposure to 32° C only at high light (Table 4). The absorption coefficient at the wavelength specific chl *a* peak of 676 nm in the F2 isolate showed a similar degree of change as the total ā* ($p> 0.05$, data not shown). However there was less change in $a^*_{(676)}$ relative to wavelengths 400-500 nm in the other algae, possibly indicating a greater loss in accessory and non photosynthetic pigments relative to chl *a* during the thermal treatment at high light.

D1 protein content

There was a significant reduction in the amount of D1 protein at 26° C in all high light acclimated cultures ($p<0.01$) (Figure 6). Thermal exposure had no significant effect of on the amount of D1 protein in the F2 alga at either light intensity or in the A1 alga when grown under low light (Figure 7). However at high light and 32° C, D1 declined significantly ($p<0.01$) in the A1 alga by 120 h , and then remained constant through the end of the experiment (240 h). There was a significant loss of D1 protein in the B1 alga at

32° C at both light levels ($p<0.01$), however, this loss was considerably faster in high light. Likewise, D1 loss during thermal exposure was exacerbated in high light in the A1.1 culture as well, resulting in a 74% decline in D1 by 120 h at 32° C (Figure 7).

Discussion

Previous work with *Symbiodinium* in culture and *in hospite* has shown interspecific differences in the capacity to photoacclimate to low light, and that zooxanthellae can differentially adjust both the size and/or number of photosynthetic units (PSU's) (Chang et al. 1983; Iglesias-Prieto and Trench 1994). As the average E_k (minimum irradiance to reach maximal photosynthesis) for three species of zooxanthellae acclimated to 250 μmol photons•m^{-2}•s^{-1} was 133.9 ± 17.5 μmol photons•m^{-2}•s^{-1} (Iglesias-Prieto and Trench 1994), the experimental design in this present study was testing the potential for further acclimation to supersaturating irradiance. The decline in cellular chl *a* when grown at 600 μmol photons•m^{-2}•s^{-1} is a typical photoacclimation response for many microalgae (Falkowski and Raven 1997). However, the clear differences in growth between the isolates indicated that not all algae were performing in a similar fashion under elevated light. Analysis of the efficiency of light harvesting capacity by changes in ā* between the two light intensities tended to show an increase (cultures F1, A1, and B1) or no change (A1.1). While all cultures contained less D1 protein when acclimated to high light, there was a clear difference in the degree to which this occurred. The lowered D1 content in high light is a function of a decrease in the number of PSU's due to

photoacclimation (Iglesias-Prieto and Trench 1994; Fisher et al. 1998). In this regard, other methods (e.g. oxygen flash yield analysis) would prove useful in confirming the total number of PSU's (Iglesias-Prieto and Trench 1994). The small decline in D1 content and minimal change in ā* when grown at high light suggests that the A1.1 isolate had a limited capacity to acclimate to this light level.

At 26° C, maximal photochemical efficiency of PSII (F_v/F_m) in all zooxanthellae cultures at high light was significantly lower as compared to F_v/F_m at low light. This is in contrast to other types of algae in which F_v/F_m does not change when acclimated to a broad range of light intensities (Harris et al. 2005), but is in general agreement for natural *Symbiodinium* spp. populations found *in hospite*, where F_v/F_m in shallow water corals is lower than in deeper corals (Warner et al. 2002). This decline in maximal PSII efficiency under high light may be considered a photoacclimatory response, however some photoinactivation was most likely present in some cultures (discussed further below). Further, it is important to note that F_v/F_m at 26° C under high light was significantly lower in the algae that showed the greatest thermal sensitivity (isolates B1 and A1.1).

The exacerbated loss in F_v/F_m at 32° C in some high light acclimated algae, and the fact that D1 protein closely matched the inactivation of PSII in two isolates (B1 and A1.1, Fig 8) corroborates the hypothesis that thermal stress enhances photoinactivation via a loss in the capacity for D1 repair and physical loss of PSII reaction centers in zooxanthellae within bleached corals (Warner et al. 1999; Takahashi et al. 2004). Similar results were also noted in the Caribbean coral *Montastraea faveolata,* where the loss in D1 was correlated with an increase in coral DNA damage and apoptotic activity when

exposed to elevated temperature and PAR + ultraviolet radiation (Lesser and Farrell 2004). Though not directly measured here, enhanced cell death and necrosis in thermally exposed zooxanthellae similar to that recently noted in intact symbioses (Dunn et al. 2004; Franklin et al. 2004; Strychar et al. 2004) was most likely occurring within some algae in this study, as growth decreased rapidly (isolate A1.1) or ceased completely (isolate B1) at 32° C and high light.

Interestingly, the A1 alga, *Symbiodinium microadriaticum*, displayed a much slower decline in F_v/F_m when grown at elevated temperature. This alga was subsequently grown for up to twenty days at 32° C, which resulted in no further significant loss in PSII function than that noted for the initial 10 days of thermal exposure (data not shown). That cellular growth decreased substantially in this alga despite the minimal loss in F_v/F_m indicates that photosynthesis and growth had become uncoupled. Thus, elevated cellular demand for maintaining active protein repair, as well as respiratory losses (Goulet et al. 2005) would contribute to this decline in growth. This is in contrast to the B1 alga which had approximately the same decline in growth yet a much lower PSII efficiency when acclimated to high light alone, indicating that the B1 alga was experiencing a larger degree of PSII photoinactivation at the higher light level before the temperature was raised.

The lower $\Delta F/F_m$' noted in all high light acclimated cultures prior to thermal ramping is similar to former comparisons between corals sampled at different depths, wherein a decline in effective quantum yield represents an increase in energy dissipation via nonphotochemical pathways while net productivity remains high (Lesser and

Gorbunov 2001). Some studies have documented a significant rise in NPQ shortly after exposing corals to elevated temperature (Warner et al. 1996; Hill et al. 2004), and this may have provided a transitory level of photoprotection from excess excitation pressure on PSII during thermal stress in the B1 alga at low light. However, this current work clearly demonstrates that elevated NPQ is not a ubiquitous response in zooxanthellae during thermal stress. While there does seem to be a good relationship between thermal tolerance and high effective quantum yield in the F2 alga, the B1 alga had a higher $\Delta F/F_m'$ than that of the A1 alga after 240 h of exposure to 32° C, yet it is clear that the B1 alga was suffering from chronic photoinactivation at this time. Importantly, shifts in $\Delta F/F_m'$, especially when recorded at light levels approaching or above E_k, cannot be used as a reliable indicator of photochemical electron transport linked solely to carbon fixation (Flameling and Kromkamp 1998; Gilbert et al. 2000). These results call into question the reliability of using maximal relative electron transport rates derived solely by chlorophyll fluorescence as a metric for total photosynthetic activity or stability of the dark reactions of photosynthesis in thermally stressed *Symbiodinium* spp. (Ralph et al. 2001; Yakovleva and Hidaka 2004).

Thermally induced losses in photochemical quenching (qP) have been noted in zooxanthellae in culture and *in hospite* (Warner et al. 1996; Jones et al. 1998; Warner et al. 1999), however, only the A1 and B1 algae displayed any loss in qP when held at 32° C at low and high light respectively. Photochemical quenching has been used as an approximate index for the fraction of open PSII reaction centers, however, this is correct only if there is minimal connectivity (energy transfer) between centers, while prevailing

evidence suggests that this is not the case (Bernhardt and Trissl 1999, Blankenship 2002). Further, qP does not necessarily equate to the efficiency of reaction centers, and several studies have shown that qP may not approximate overall photochemical activity during physical stress (Juneau et al. 2005). Similarly, Eggert et al (2003) found a good correlation between the loss in effective quantum yield and qP after exposing the chlorophyte, *Valonia utricularis*, to low temperature, but no loss in qP was noted when this alga was exposed to elevated temperature between 30 and 35° C despite a loss in PSII function.

Light and temperature both impacted Q_A reoxidation in the PSII reaction center, resulting in a slower rate of electron transport from Q_A to Q_B (Figure 5). The significant rise in τ in the B1 alga in high light at 26° C provides further evidence that this alga was already experiencing photoinhibition before thermal ramping, and correlates with the lower amount of D1 protein and significant loss in growth as compared to the other algae tested. The slower decline in F_v/F_m in the B1 alga compared to the A1.1 alga during thermal stress at high light may have been due to further nonphotochemical energy dissipation by damaged reaction centers (Chow et al. 2002), as D1 loss in the B1 culture was slower relative to the loss in D1 in the A1.1 alga. The loss in electron flow out of PSII was exacerbated by thermal stress most prominently in the B1 alga at the low light level and in the A1.1 alga at both light intensities. These findings are in contrast to those of Tchernov et al (2004) who noted a significant decrease in τ in cultured *Symbiodinium* spp. following exposure to 32° C and 100 μmol photons$\bullet m^{-2}\bullet s^{-1}$ for 168 h. Given the different algae and instrumentation used between the two studies, these differences are

not easy to reconcile, however, our results are in agreement with other studies using similar methods with plant thylakoids and microalgae that have shown an enhanced loss in electron flow in reaction centers altered by heat (Pospísil and Tyystjärvi 1999) or D1 protein mutations (Govindjee et al. 1992).

Few studies have investigated changes in ā* under elevated temperature, however, our results are similar to those seen for microalgae exposed to low temperatures where a decline in chl *a* and accessory pigments leads to a reduction in pigment packaging and an increase in ā* (Sosik and Mitchell 1994; Stramski et al. 2002). In addition to the loss in chl *a* cell^{-1}, disruption in thylakoid stacking (Tchernov et al. 2004) and further morphological changes were most likely occurring in the chloroplasts of the B1 and A1.1 algae during thermal stress. Thus, while the rise in ā* in high light acclimated B1 and A1.1 algae during thermal treatment represents a physical increase in absorption efficiency, photosynthetic activity was severely disrupted in these algae. If similar alterations in light absorption occur during natural bleaching they may have substantial impacts for *Symbiodinium in hospite*, as significant scattering by the coral skeleton can greatly enhance the total light reaching zooxanthellae (Kühl et al. 1995, Enriquez et al. 2005), which would lead to further photoinactivation. Further work is needed in this area of coral photobiology, as it is not known if similar physiological differences as noted here would be maintained in the host. Likewise, this work cannot discount the potential influence the host coral may have in modulating the photosynthetic response of these algae (Bhagooli and Hidaka 2003) either by behavioral changes such as polyp contraction

(Brown et al. 2002b) or by different pigments (e.g. pocilloporins or green fluorescent proteins) which may dampen or enhance the effects of available light.

This work places the concepts of coral bleaching and thermal stress in *Symbiodinium* into a broader context that accounts for differences in photoacclimation between different zooxanthellae and shows how these processes are interrelated. The ability of a particular *Symbiodinium* "type" to acclimate to elevated light intensities appears to parallel the ability to maintain active PSII function during thermal stress. The results presented here are in agreement with the general idea that exposure to high irradiance can significantly lower the thermal threshold for photoinactivation in some *Symbiodinium*. However, the rate of loss in PSII activity and the level of PSII damage behind this loss, are not necessarily similar between different zooxanthellae, and this could have a significant impact on the overall rate and extent of bleaching. The capacity for a coral to withstand future bleaching events has been a central focus of the "adaptive bleaching hypothesis," which suggests that corals that harbor multiple phylotypes of zooxanthellae may acclimatize to future warming events by harboring "new" more thermally tolerant algae following bleaching (Buddemeier and Fautin 1993; Baker 2003). It is still not known if a particular algal type found within a coral after bleaching is "new" or simply part of the remnant algal population. There is mounting evidence to suggest that the latter scenario may be applicable in some cases, considering that resident algal populations can change on a seasonal scale (Chen et al. 2005; Thornhill et al. 2005). If algal growth is affected during natural bleaching similar to that noted here, such results

could directly influence the outcome of competition between distinct *Symbiodinium* populations and further impact successful recovery form coral bleaching.

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Tables and Figures

Table 1: Cell growth rate (μ, day^{-1}) for four *Symbiodinium* isolates (designated by ITS2) maintained at two light levels (100 or 600 μmol photons•m^{-2}•s^{-1}) and temperatures (26 or 32° C). Superscripts [a] and [b] represent significant differences ($p < 0.05$) for within culture comparisons between light at 26° C and temperature at the same light level respectively. Rates are calculated from the first five days of exposure (time 0–120 h) once 32° C was reached (n = 4 ± SD).

Symbiont	Light (μmol photons•m^{-2}•s^{-1}) and temperature (° C)			
	100 – 26	600 – 26	100 – 32	600 – 32
F2	0.19 ± 0.04	0.40 ± 0.07[a]	0.19 ± 0.06	0.44 ± 0.08
A1	0.17 ± 0.03	0.19 ± 0.02	0.16 ± 0.02	0.07 ± 0.02[b]
B1	0.20 ± 0.04	0.09 ± 0.06[a]	0.21 ± 0.03	-0.09 ± 0.04[b]
A1.1	0.14 ± 0.05	0.13 ± 0.02	0.18 ± 0.05	0.02 ± 0.003[b]

Table 2: Chlorophyll *a* content (pg cell^{-1}) in four *Symbiodinium* isolates (designated by ITS2) maintained at two light levels (100 or 600 µmol photons•m^{-2}•s^{-1}) and temperatures (26 or 32° C). Superscripts [a] and [b] represent significant differences ($p < 0.05$) for within culture comparisons between light at 26° C and temperature at the same light level respectively. Samples are from 240 h exposure, except for A1.1, 600 µmol photons•m^{-2}•s^{-1} , which was removed at 120 h (n = 4 ± SD).

Symbiont	Light (µmol photons•m^{-2}•s^{-1}) and temperature (° C)			
	100 – 26	600 – 26	100 – 32	600 – 32
F2	1.99 ± 0.14	1.01 ± 0.24[a]	1.56 ± 0.14[b]	0.77 ± 0.20
A1	1.86 ± 0.25	0.84 ± 0.18[a]	1.07 ± 0.23[b]	0.72 ± 0.06
B1	0.58 ± 0.09	0.19 ± 0.02[a]	0.50 ± 0.12	0.05 ± 0.01[b]
A1.1	0.91 ± 0.12	0.39 ± 0.09[a]	0.68 ± 0.09	0.22 ± 0.03[b]

Table 3: Mean chlorophyll *a*-specific absorption coefficient ā* (m^{-2}•mg chl a^{-1}) in four *Symbiodinium* isolates (designated by ITS2) maintained at two light levels (100 or 600 µmol photons•m^{-2}•s^{-1}) at 26° C prior to thermal ramping. Superscript [a] represents significant difference ($p < 0.05$) between light treatments within each isolate. ($n = 4 \pm$ SD).

Symbiont	Light (µmol photons•m^{-2}•s^{-1})	
	100	600
F2	0.0129 ± 0.0014	0.0177 ± 0.0023[a]
A1	0.0123 ± 0.0009	0.0184 ± 0.0025[a]
B1	0.0172 ± 0.0015	0.0253 ± 0.0017[a]
A1.1	0.0112 ± 0.0015	0.0089 ± 0.0016

Table 4: Mean chlorophyll *a*-specific absorption coefficient ā* (m^{-2}•mg chl a^{-1}) in four *Symbiodinium* isolates (designated by ITS2) maintained at two light levels (100 or 600 µmol photons•m^{-2}•s^{-1}) at 26 and 32° C. Superscript [a] represents significant differences ($p < 0.05$) for within culture comparisons between light. Samples are from 240 h exposure, except for isolate A1.1, 600 µmol photons•m^{-2}•s^{-1}, which was removed at 120 h (n = 4 ± SD).

Symbiont	Light (µmol photons•m^{-2}•s^{-1}) and temperature (° C)			
	100 – 26	100 – 32	600 – 26	600 – 32
F2	0.0123 ± 0.0015	0.0155 ± 0.0011[a]	0.0138 ± 0.0011	0.0205 ± 0.0016[a]
A1	0.0117 ± 0.0003	0.0111 ± 0.0020	0.0152 ± 0.0016	0.0189 ± 0.0005[a]
B1	0.0168 ± 0.0016	0.0187 ± 0.0005	0.0238 ± 0.0012	0.0569 ± 0.0112[a]
A1.1	0.0211 ± 0.0035	0.0209 ± 0.0021	0.0106 ± 0.0022	0.0178 ± 0.03[a]

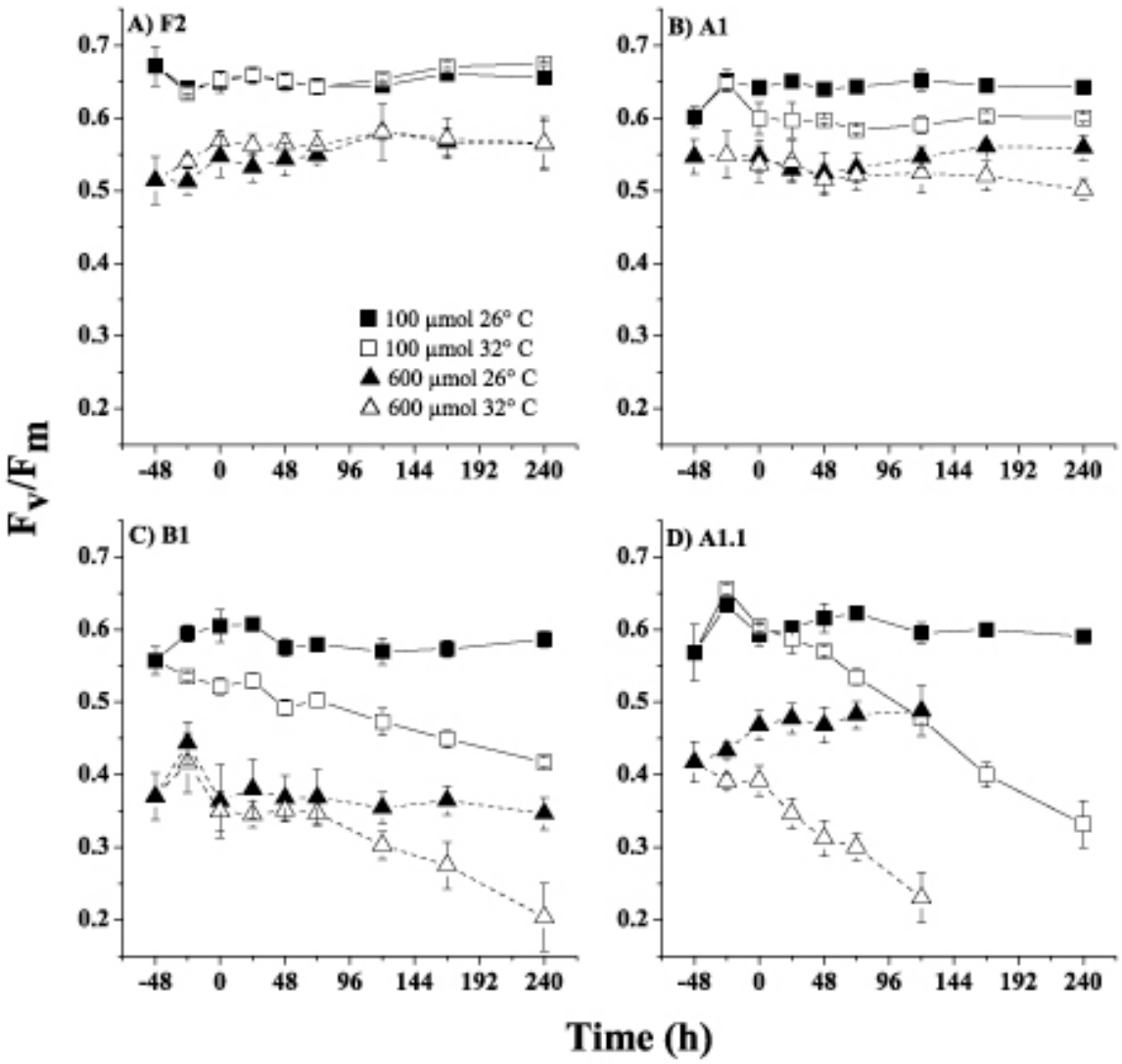

Figure 1: Dark-acclimated PSII quantum yield (F_v/F_m) in four phylotypes of *Symbiodinium* (designated by ITS2 assignment as F2, A1, B1, and A1.1 for panes A–D respectively) grown at 26° C or gradually exposed to elevated temperature and held at 32° C at either 100 or 600 µmol photons•m^{-2}•s^{-1}. Data from -48 to 0 h represent the temperature ramping period from 26–30° C, after which, the temperature was raised to 32° C at time 0 and held at 32° C for the rest of the experiment (n = 4 ± SD).

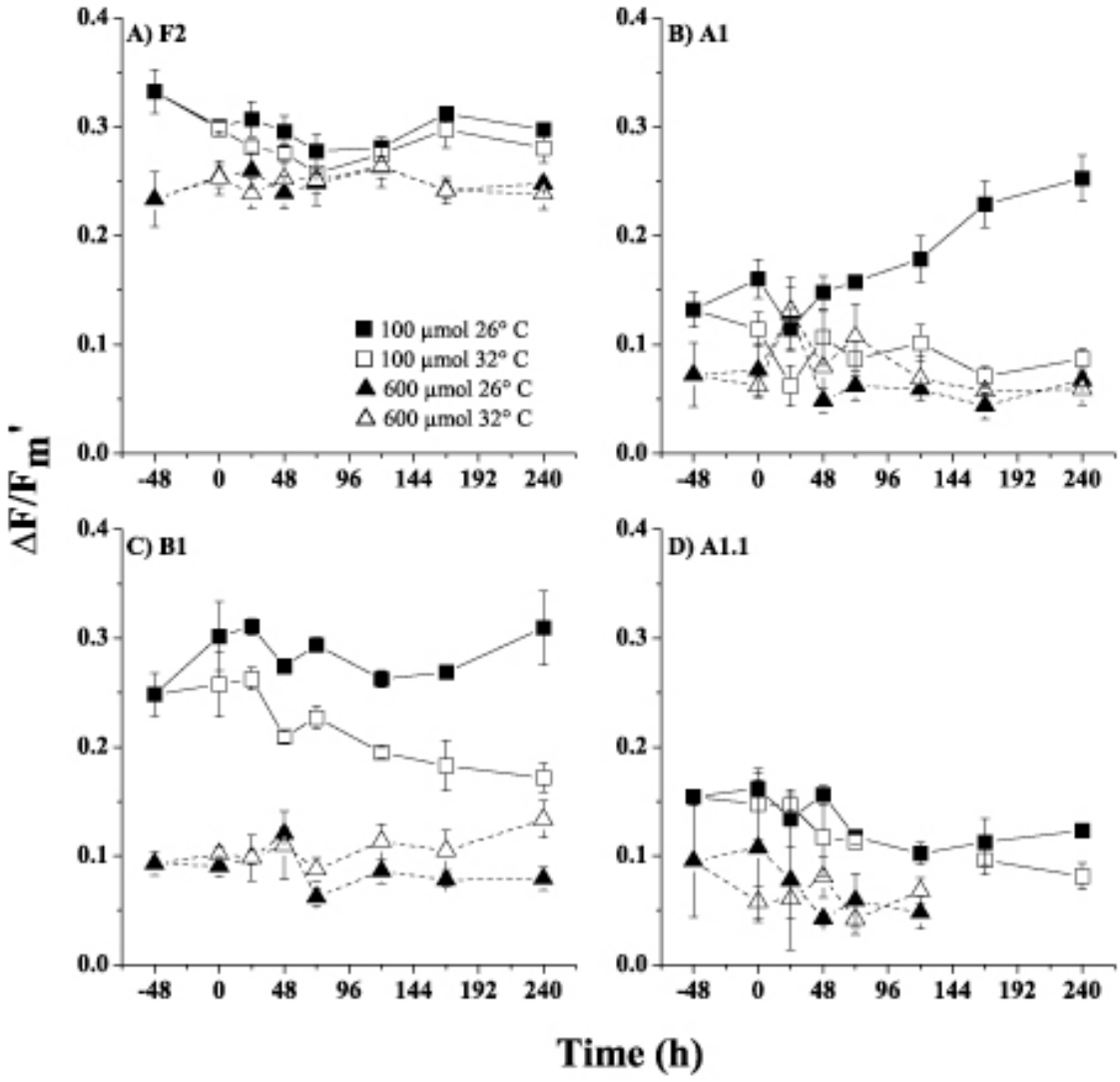

Figure 2: Effective quantum yield ($\Delta F/F_m'$) in four phylotypes of *Symbiodinium* grown at two light levels at control (26° C) or elevated temperature. Panes A–D and legend are the same as in Figure 1 (n = 4 ± SD).

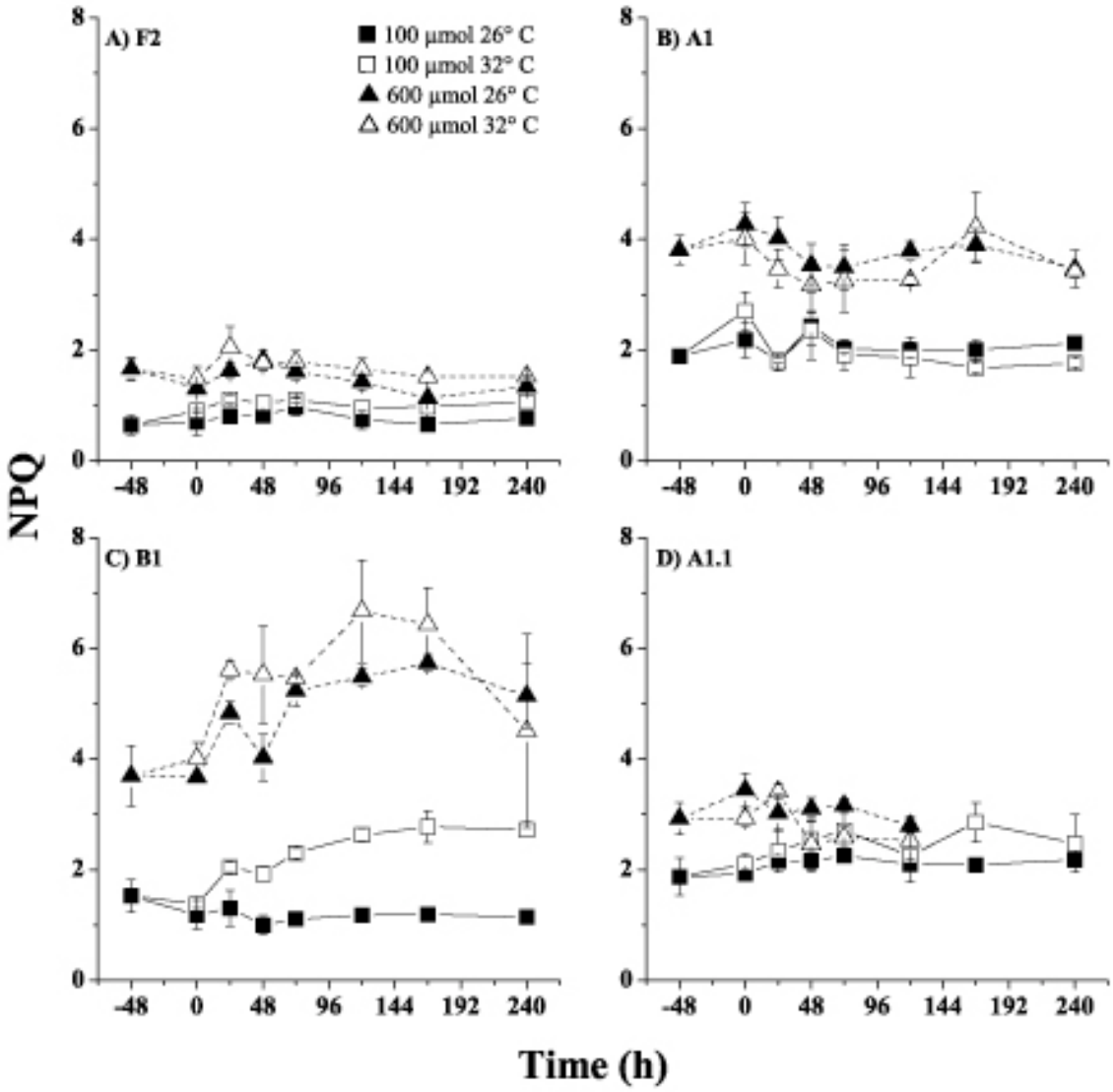

Figure 3: Non-photochemical quenching (NPQ) of chlorophyll fluorescence in four phylotypes of *Symbiodinium* at two light levels at control (26° C) or elevated temperature. Panes A–D and legend are the same as in Figure 1 (n = 4 ± SD).

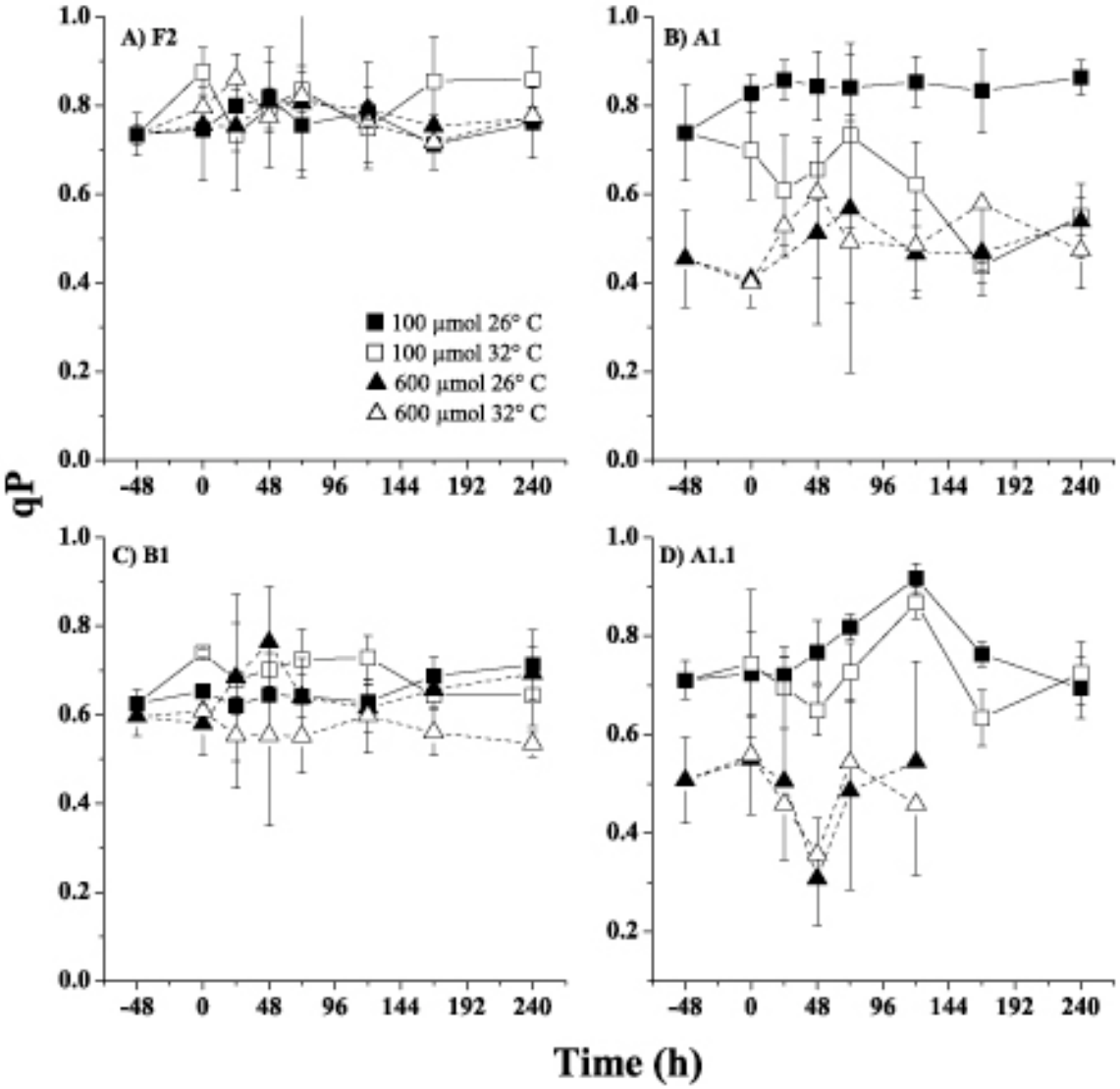

Figure 4: Photochemical quenching (qP) of chlorophyll fluorescence in four phylotypes of *Symbiodinium* grown at two light levels at control (26° C) or elevated temperature. Panes A–D and legend are the same as in Figure 1 (n = 4 ± SD).

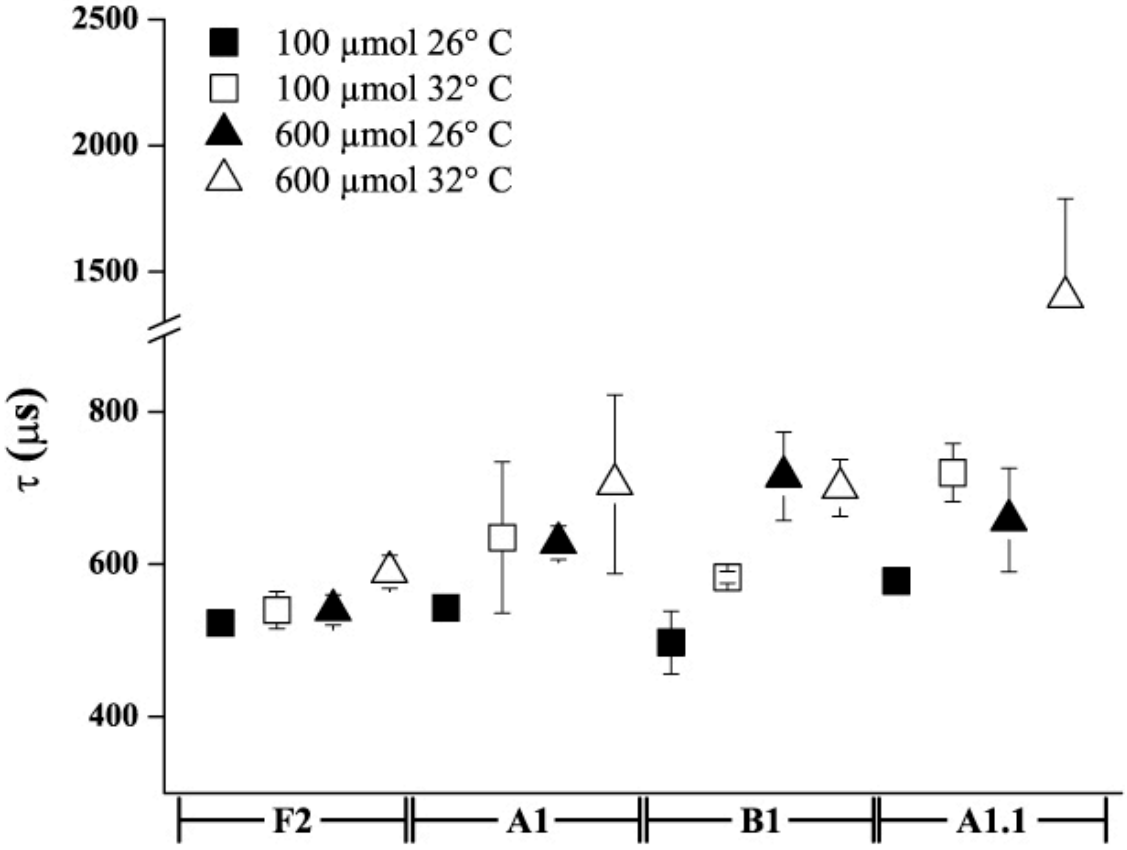

Figure 5: Electron transfer rate (τ) between the primary and secondary quinones ($Q_A \rightarrow Q_B$) in four phylotypes of *Symbiodinium* at 26 or 32° C at two light intensities. Data are from 240 h, except for the A1.1 alga at 600 µmol photons•m^{-2}•s^{-1} which was sampled at 120 h (n = 4 ± SD).

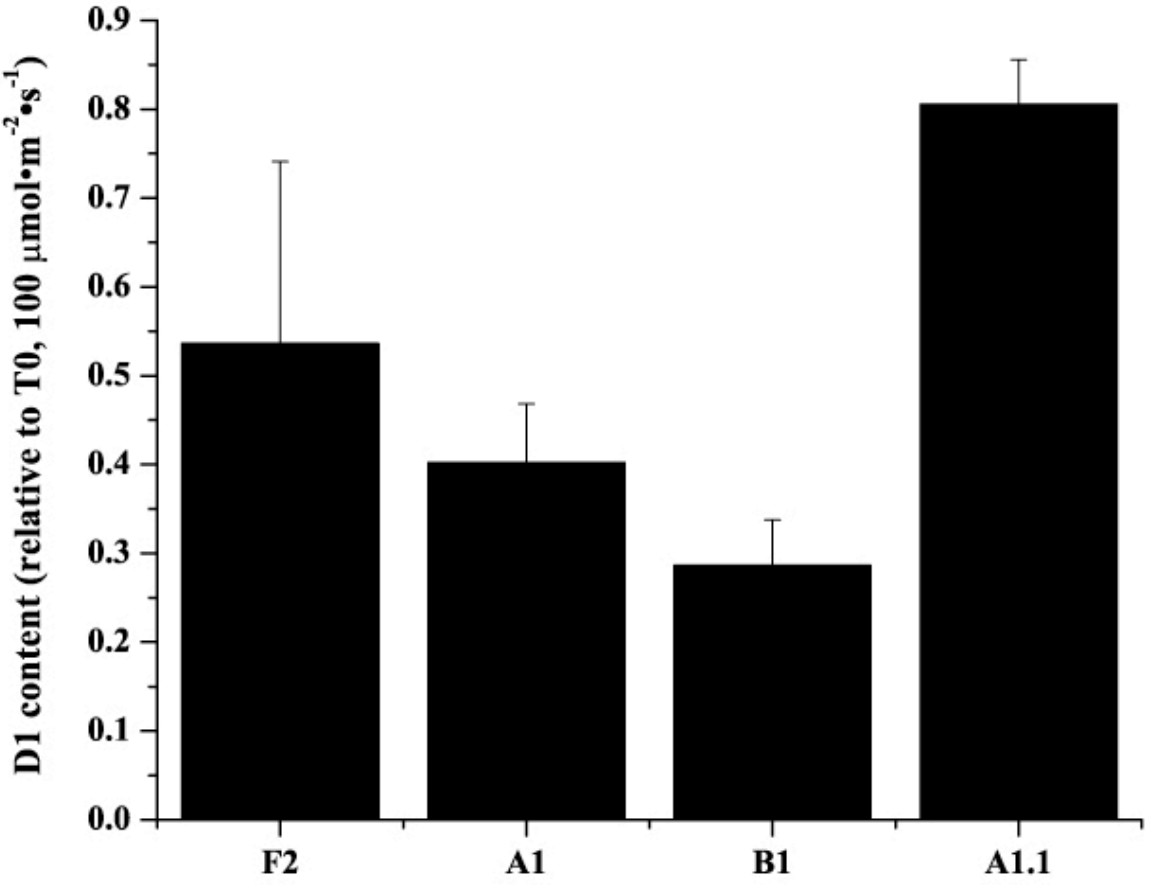

Figure 6: D1 protein content in four phylotypes of *Symbiodinium* grown at 600 µmol photons•m^{-2}•s^{-1} and 26° C. Data are presented as a percentage of D1 relative to 100 µmol photons•m^{-2}•s^{-1} at the start of the experiment (n = 4 ± SD).

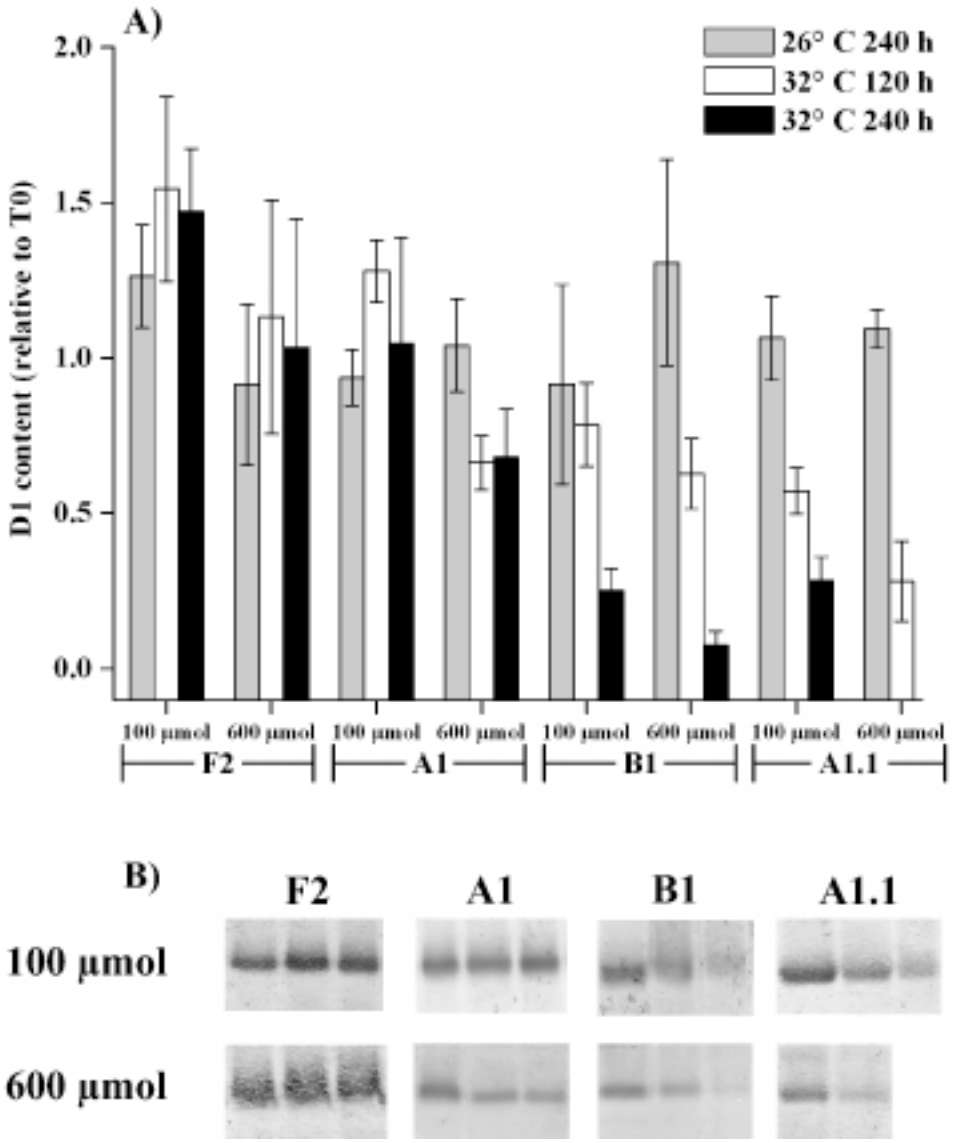

Figure 7: Change in D1 protein in four phylotypes of *Symbiodinium* at two light levels at 26 or 32° C (A) and representative western blots for each isolate and treatment (B). Data are presented as a percent of D1 content relative to time 0 and are from 240 h, except for the A1.1 alga at 600 µmol photons•m^{-2}•s^{-1} which was sampled at 120 h (n = 4 ± SD). Blot series for each isolate and light level shown in (B) are (from left to right) control, 32° C 120h, and 32° C 240 h.

III: Evaluating the reliability of chlorophyll fluorescence to infer changes in photosynthesis in *Symbiodinium* under increased temperatures

Abstract

Non-invasive chlorophyll fluorescence techniques have become a common method to evaluate photosynthesis within the algal symbionts of reef-building corals (*Symbiodinium* spp.). However, only recently has research begun to evaluate the accuracy of these methods for inferring total photosynthetic activity in comparison to more traditional methods like oxygen evolution. Both chlorophyll fluorescence parameters and oxygen evolution were monitored simultaneously in two different isolates of *Symbiodinium* (phylotypes A1 and B1) with different thermal tolerance levels maintained at either 26 or 32 °C for 10 days. There was a significant decrease in both maximum quantum yield of PSII (F_v/F_m) and the efficiency of photosynthesis (α) in both phylotypes A1 and B1; however, there was a two-fold greater decrease in α versus F_v/F_m. There was a significant decrease in maximal photosynthesis (P_{max}) in phylotype B1 while there was no change in A1. Both phylotypes demonstrated a significant decrease in maximal electron transport (ETR_{max}); however, the change in ETR_{max} underestimated that of P_{max} in phylotype B1 while it overestimated P_{max} in A1. The relationship between oxygen evolution and

electron transport is linear at light levels <200 μmol m^{-2} s^{-1} under control temperatures and <60 μmol m^{-2} s^{-1} with increased temperature above which ETR overestimates oxygen evolution. Conversely, the effective quantum yield of PSII overestimates the operation quantum yield of oxygen production at light levels less than <300 μmol m^{-2} s^{-1} in both thermal and control samples. The results of this study suggest that a disconnect between chlorophyll fluorescence and oxygen evolution exists in *Symbiodnium* and suggests possible causative mechanisms.

Introduction

Coral reefs are an important ecosystem based upon a symbiotic relationship between the coral host and symbiotic dinoflagellates from within the genus *Symbiodinium*, commonly known as zooxanthellae. Over the past 20+ years there has been an increase in coral bleaching on coral reefs worldwide (Hoegh-Guldberg 1999) which has been strongly correlated with an increase in seawater temperature and exacerbated by increased irradiance (Fitt et al. 1993; Bhagooli and Hidaka 2003). Coral bleaching is characterized by the loss of either entire zooxanthellae cells and/or the loss of photosynthetic pigments within the algae leaving the tissue a distinctive white color (Harriott 1985; Berkelmans and Oliver 1999; Loya et al. 2001). Thermal bleaching has been connected with photosynthetic breakdown within the algal cells, specifically occurring at photosystem II (PSII) along with the production of reactive oxygen species (Lesser 1997; Hoegh-Guldberg and Jones 1999; Warner et al. 1999). A decrease in both

the maximal and effective quantum yield of photosystem II during thermal stress has been demonstrated both in culture and *in hospite* (Hoegh-Guldberg and Smith 1989; Iglesias-Prieto et al. 1992; Fitt and Warner 1995; Warner et al. 1996; Robison and Warner 2006).

Currently, a common method of measuring and monitoring photosynthesis within corals is the use of non-invasive chlorophyll fluorescence techniques, such as pulse amplitude modulation (PAM) fluorometry or fast repetition rate (FRR) fluorometry (Fitt and Warner 1995; Hoegh-Guldberg and Jones 1999; Brown et al. 2000; Jones et al. 2000; Gorbunov et al. 2001; Tchernov et al. 2004). The effective quantum yield measured with fluorescence techniques reports the charge separation in PSII reaction centers (Gentry et al. 1989). As both charge separation and oxygen evolution are coupled processes of PSII activity, there should be little variability between the quantum yields of charge separation and the quantum yield of oxygen production. However, research into this relationship has been contradictory, with both linear and non-linear relationships reported between measured quantum yields using active chlorophyll fluorescence and traditional methods of measuring photosynthesis via O_2 respirometry and ^{14}C uptake (Kroon et al. 1993; Flameling and Kromkamp 1998; Franklin and Badger 2001; Figueroa et al. 2003).

The effective quantum yield ($\Delta F/F_m'$) or the PSII efficiency in the light acclimated state as measured by chlorophyll fluorescence techniques can be used to calculate the photosynthetic electron transport rate (ETR) with knowledge of the absorptance of the sample and the incident irradiance (Gentry et al. 1989). Due to the coupling of charge separation and oxygen production mentioned above, electron transport

rates are often used as an approximation of total photosynthetic rates, particularly that of oxygen evolution rates. The relationship between ETR and oxygen evolution must be understood for ETR values to be effectively interpreted as an estimate of photosynthetic capacity. This relationship has been found to be linear, non-linear, and curvilinear and is highly variable based on species and environmental conditions (Geel et al. 1997; Beer et al. 2000; Figueroa et al. 2003; Kromkamp and Forester 2003; Suggett et al. 2003). It has typically been shown to be linear at low to moderate light levels that are below E_k, the light intensity needed to saturate photosynthesis, and the upper irradiance limit for linearity varies on a species by species basis (Geel et al. 1997; Beer et al. 2000).

Despite the prevalent use of chlorophyll fluorescence techniques to infer photosynthetic activity beyond PSII, the relationship between chlorophyll fluorescence parameters and oxygen evolution has only been recently examined in *Symbiodinium.* The relationship between quantum yields of PSII and oxygen evolution has been measured in the coral *Turbinaria mesenterina*, in which a non-linear relationship was shown (Hoogenboom et al. 2006). The relationship between relative ETR and oxygen evolution in these symbionts was directly linear at low light, though at high light the relationship was non-linear, with ETR changing up to 50% with no effect on oxygen evolution and thus overestimating oxygen evolution (Hoogenboom et al. 2006). Conversely, this relationship was linear at low light, while ETR underestimated oxygen evolution under increased irradiance in the symbionts of the coral *Pocillopora damicornis* (Ulstrup et al. 2006). These conflicting results indicate that further research is needed in this important area of photobiology.

This research attempts to further elucidate the relationship between chlorophyll fluorescence and oxygen evolution within *Symbiodinium* and investigate if this relationship changes during increased thermal conditions, such as those that may be experienced during coral bleaching events. These patterns were examined within two previously investigated phylotypes of *Symbiodinium* where one is known to be thermally tolerant and one thermally sensitive (Robison and Warner 2006).

Materials and Methods

Two phylotpyes of cultured *Symbiodinium,* previously classified by the analysis of the ribosomal ITS2 sequence to be types A1 and B1 (LaJeunesse 2001), were utilized in this study. Isolate A1 was formally described as *Symbiodinium microadriaticum,* originally isolated from the jellyfish *Cassiopeia xamachana* (Freudenthal 1962) while the B1 isolate was isolated from the sea anemone *Aiptasia tagetes*, and has not been given a formal species name.

Cultures were maintained in log-phase batch growth in artificial sweater media (ASP-8A) (Blank 1987) at 26 °C on a 14:10 light:dark cycle in temperature controlled incubators with high output fluorescent bulbs providing 100 μmoles photons•m^{-2}•s^{-1} PAR. A portion of media was replenished every ten days to minimize the effects of self-shading and maintain exponential growth rates. Cultures were held in this condition for at least 4 weeks prior to experimentation. Thermal exposure was gradually applied by raising the temperature daily at 09:00 in 2 °C increments from 26 to 32 °C over 72 hr.

Samples for oxygen evolution and fluorescence analysis (described below) were taken prior to the thermal increase, hereafter referred to as 0, and at 120 hrs and 240 hrs at 32°C. All samples were collected at 09:00 to eliminate any possible variation due to natural diel periodicity in the measured parameters.

Oxygen evolution and chlorophyll fluorescence technique

Samples were removed and concentrated via gentle centrifugation (500g, 3min) and re-suspended to yield a final concentration of 1.5-2 million cells ml^{-1} in fresh media. Sodium bicarbonate was added to the sample (final concentration of 1 mM) to eliminate the possibility of carbon limitation within the chamber. Oxygen evolution was measured in an aqueous-phase Clark type electrode chamber (Oxylab, DW-2, Hansatech, England) maintained at the growth temperature by a thermostatically controlled water jacket. Photosynthesis was measured as O_2 production during 10 light steps lasting for 5 minutes each, ranging from 0 – 600 μmol photons•m^{-2}•s^{-1}. Respiration was measured for 5 minutes in the dark at the start of the respirometer run. Actinic light was provided by an automated red LED array with a peak wavelength of 650±25nm (LH11 Mk2, Hansatech, England). While respirometry was recorded, chlorophyll fluorescence was monitored by pulse amplitude modulation fluorometry (PAM 101, Walz, Germany) by using two customized Perspex rods designed to sit flush against the outer wall of the electrode chamber. One rod supplied the continual 1.6 kHz measuring light of the fluorometer (peak wavelength 470nm, < 1 μmol photons•m^{-2}•s^{-1}). Chlorophyll fluorescence ≥ 645 nm

was detected via a photodiode connected to the other Perspex rod, which was placed 90° relative to the red LED to eliminate interference due to spectral overlap of the red LED's and fluorescence detector. The maximum quantum yield ($F_v/F_m = F_m\text{-}F_o/F_m$) was recoded at 4.5 min into the dark respiration curve. For each additional light step the effective quantum yield ($\Delta F/F_m' = (F_m'–F)/F_m'$) was recorded 4.5 min into each light step with a brief saturation light pulse (400 ms, 3000 μmol photons•m^{-2}•s^{-1}) provided by a blue LED array (470 nm peak, HPL-L470, Walz, Germany). The excitation pressure on PSII was calculated as $Qm = 1 – [\Delta F/F_m'/ (F_v/F_m$ prior to thermal ramping)] (Iglesias-Prieto et al. 2003).

Pigment analysis

Cellular absorption of the cell suspension was measured immediately following respirometry and chlorophyll fluorescence readings. Absorbance was measured from 400 – 750 nm (1 cm quartz cuvette) in a spectrophotometer fitted with an integrating sphere (UV-2401PC, Shimadzu, USA). The resulting raw absorbance values were corrected for turbidity by subtracting the average absorbance 700 – 750nm followed by the calculation of average absorbance from 625 – 675nm (corresponding to the wavelength range of the measuring light). The amount of incident light absorbed by the algae (absorptance (abst)) was then calculated by:

$$1 – 10^{\text{abs 625-675nm}}$$

The average (400-700 nm) chlorophyll *a* specific absorption coefficient (ā*), was calculated by the quantitative filter pad method using previously published equations (Cleveland and Weidemann 1993). The samples from the electrode chamber were filtered onto GF/F glass fiber filters and scanned between 380 and 750 nm in the same spectrophotometer as above. Absorption spectra were corrected for particle scattering by subtracting the absorption at 750 nm prior to ā* calculation. The filters were then ground in 100% acetone and extracted pigments recorded by spectrophotometry, and chlorophyll *a* content was calculated using the equations of Jeffrey and Humphrey (1975).

Data Analysis

Gross photosynthesis was calculated and P:E curves are presented as μmol O_2 $cell^{-1}$ min^{-1}. The curves were plotted and non linear fits were performed using the equations of Jassby and Platt (1976) to determine P_{max} and α (KaleidaGraph ver. 3.6, Synergy Software, USA) as well as $E_k = P_{max}/\alpha$. The operational quantum yield for O_2 evolution (Φ_{O2}) Flameling and Kromkamp (1998) was calculated as: $\Phi_{O2} = P^B/(115 \times \bar{a}^* \times E)$, where P^B is net photosynthesis mg^{-1} chl *a* at irradiance E. The electron transport rate of PSII was calculated as:

$$ETR = \Delta F/F_m' \times E \times abst \times 0.5$$

where E is the measured irradiance and 0.5 is the fraction of photons assumed to be directed to PSII. ETR curves were plotted using the modified Jassby and Platt (1976) equation to fit ETR curves as described by Figueroa et al. (2003) to determine ETR_{max}

and α^{ETR} (KaleidaGraph ver. 3.6, Synergy Software, USA) from which $E_k^{ETR} = ETR_{max}/\alpha^{ETR}$.

Data were examined to ensure normality and log-transformed when needed. Non-transformed data are shown in all figures. One-way analysis of variance (ANOVA) were preformed using SPSS version 11.0 (SPSS Inc., USA) for statistical comparisons.

Results

As expected, B1 was the most susceptible of the two phylotypes to thermally induced photoinhibition, with a significant decrease in both maximum quantum yield of PSII (F_v/F_m, 36% decline) and the efficiency of photosynthesis (α, 66% decline) measured at the end of thermal exposure. In addition to the loss in the efficiency of photosynthesis in phylotype B1, there was a 60% decrease in P_{max} ($p < 0.01$) and the irradiance needed to saturate photosynthesis (E_k) increased by almost 50% at 32°C ($p = 0.03$), yet there was no significant change in respiration (Table 1, Figure 1A). In contrast, phylotype A1 also showed a significant decrease in F_v/F_m (22%) and α (44%); however, there was no thermal effect on P_{max}, E_k, or respiration rates (Figure 1B, Table 1).

Unlike in the P:E curves, both phylotypes showed a significant decrease in the electron transport rate parameters. Both phylotypes demonstrated approximately a 50% decrease in the efficiency of electron transport (α^{ETR}, $p < 0.01$) as well as a roughly 30% increase in E_k^{ETR} ($p < 0.01$,Figure 2, Table 1) at 32°C and 240h of exposure. The

irradiance level needed to saturate photosynthesis (E_k) was lower than that needed to saturate electron transport (E_k^{ETR}) regardless of phylotype or temperature (Table 1).

When comparing curve parameters, the percent change in both ETR_{max} and P_{max} during thermal stress was different between the phylotypes. In phylotype B1, P_{max} decreased by 60% while ETR_{max} decreased by 34%, thus ETR_{max} underestimated the thermal effect on masimal photosynthesis by 25%. In contrast, phylotype A1 demonstrated a 25% decrease in P_{max} and a 36% decrease in ETR_{max}, meaning the change in ETR_{max} overestimated the thermal effect on maximal photosynthesis by 10%.

The relationship between oxygen evolution and electron transport rate was linear at low light in both phylotypes (Figure 3). Under control conditions (26°C) linearity was lost around 200 μmol m^{-2} s^{-1} in both phylotypes. However, with increased temperature this loss occurred at 60 μmol m^{-2} s^{-1} in both algae (Figure 3), yet the driving force behind this decline in ETR was different between the two isolates. Phylotype B1 was driven primarily by a loss in oxygen evolution under thermal stress (Figure 3A). Conversely, there was no significant loss in oxygen evolution at increased temperature in phylotype A1 while there was a loss in maximum electron transport rate (Figure 3B).

Comparison of the effective quantum yield of chlorophyll fluorescence ($\Delta F/F_m'$) and operational quantum yield of oxygen evolution (Φ_{O2}) revealed that fluorescence quantum yield consistently overestimated oxygen quantum yields at irradiance levels less than 300μmol m^{-2} s^{-1} (Figure 4). While the trend remained the same, the disparity between the methods was reduced for both algae when compared at 32°C. In phylotype A1, there was a noticeable decrease in effective quantum yield with increased

temperature yet there was no change in Φ_{O2} suggesting a disconnect between the efficiency of photosynthetic oxygen evolution and photosynthetic efficiency derived by active chlorophyll fluorescence measurements. While this trend was also present in phylotype B1, there was a simultaneous decrease in operational quantum yields of oxygen, albeit the degree of decrease was much less than the loss noted in effective quantum yield.

In some cases, we found that F_v/F_m in culture A1 was reduced greater than expected based on a previous experiment run in the same manner with similar initial algal concentrations. In these cases, we utilized the F_v/F_m from the previous thermal experiment for the calculation of excitation pressure (Robison and Warner 2006). In phylotype A1, there was no significant effect on excitation pressure during thermal exposure. However, with increased temperature the excitation pressure over PSII increased significantly at irradiances lower than 300 μmol m^{-2} s^{-1} in phylotype B1, which include the growth irradiance (Figure 5).

Discussion

As anticipated, the photosynthesis to irradiance parameters reported here for *Symbiodinium microadriaticum* are in agreement with the values reported by Iglesias-Prieto and Trench (1994) and fall within the range of light levels used to measure photosynthesis in the previous work. The difference in susceptibility to thermal induced photoinhibition was as anticipated from previous work, with phylotype B1 being more

susceptible than phylotype A1 at 32°C (Robison and Warner 2006). The occurrence of photoinhibition in phylotype B1 is further confirmed in this present study as indicated by the significant loss in P_{max}, α, and F_v/F_m during exposure to increased temperature. The trends presented here are similar to those found in the symbionts isolated from two populations of the sea anemone *Aiptasia pallida* harboring either clade A or B *Symbiodinium* (Goulet et al. 2005). At 32°C, clade B demonstrated a dramatic decrease in P_{max}, though there was no decrease in α and a significant increase in respiration rate was noted in the previous study. In contrast, the clade A algae used in their work demonstrated no significant change in P_{max}, α, or respiration rates; however, in the current study phylotype A1 showed a decrease in α. A major difference between the two studies that may explain the difference between the results of these two studies is the length of thermal exposure. The previous study utilized a short-term acute exposure of 1h prior to measurement, while the current study utilized a long-term approach of 240 h prior to respiration measurements.

The percent decrease in F_v/F_m during thermal stress was greater in phylotype A1 in the current study versus our previous work using the same alga and experimental design for thermal exposure (Robison and Warner 2006). However, there were methodological differences between these studies which may have contributed to this difference. Dark acclimation times were shorter (5 min vs 12 min) in the current study, with a much greater cell density in the measuring apparatus. A shortened dark acclimation time may not have allowed all PSII reaction centers to re-open and thus artificially raised the minimum fluorescence value and decreased F_v/F_m. Another possible

explanation for lower dark-acclimated fluorescence yields is chlororespiration which has been proposed to occur in *Symbiodinium* in the reef building corals, *Montipora digitata* and *Stylophora pistillata* (Jones and Hoegh-Guldberg 2001). The chlororespiratory pathway transports electrons from NADPH to molecular oxygen via the photosynthetic electron chain in the dark (Bennoun 2002). One step in chlororespiration reduces the plastiquinone pool which affects the redox state of Q, the primary reducer of PSII, and thus can affect F_v/F_m. The occurrence of chlororespiration in *Symbiodinium* has been observed to be more prevalent in clade A algae (Warner, personal communication) which may explain why a similar artificial decrease in F_v/F_m was not noted in phylotype B1.

Maximal photosynthesis, P_{max}, is related to both the number of photosynthetic units and their maximum turnover rate, and is assumed to be limited by the supply and fixation of CO_2, thus changes may be indicative of either a change in the number of functional units and/or the turnover rate (Falkowski and Raven 1997). It has been shown that P_{max} can be insensitive to the loss of 50 – 65% of functional PSII reaction centers by increasing the turnover rate through the remaining functional reaction centers (Leverenz et al. 1990; Behrenfeld et al. 1998). Such a phenomenon likely occurred in phylotype A1, where increased temperature had no significant effect on P_{max}, yet there was a significant (40%) decrease in α. As elevated temperature was noted to have no significant effect on the quinone turnover rate within PSII (initial time for an electron to pass from Q_A to Q_B) in this alga (Robison and Warner 2006) this suggests that the increase in turnover rate is most likely not occurring within the photosystem II reaction centers and therefore the

increase may occur down-stream of PSII, possibly by enhanced cyclic electron transport at photosystem I (Munekage et al. 2004) and carbon fixation pathways.

The initial slope of the photosynthesis to irradiance curve (α) is limited by photon absorption and it is often proportional to the maximum quantum yield of photosynthesis (Falkowski and Raven 1997). However, in the current study there was an observed two-fold greater decrease in α as compared to the decrease of F_v/F_m in both phylotypes. This suggests that the efficiency of photosynthesis measured by photosynthesis to irradiance (P:E) curves is more sensitive to the changes occurring under increased temperatures. This is in contrast to the association between F_v/F_m and photosynthetic efficiency (α) found in two populations of the kelp *Laminaria saccharina* where both parameters decreased equally over the range of temperatures investigated (Bruhn and Gerard 1996).

The results presented here indicate that in both phylotypes of *Symbiodinium,* ETR overestimates photosynthetic activity at high to moderate light levels. Despite the different saturation intensities (E_k) noted between the phylotypes, the loss of linearity between oxygen and fluorescence measurements occurs at the same light intensities for both phylotypes. This overestimation at high to moderate lights contradicts published results of an underestimation in high light with *in hospite Symbiodinium* from the coral *P. damicornis* (Ulstrup et al. 2006), however, it matches reports for *Symbiodinium* from the coral *T. mesenterina (Hoogenboom et al. 2006)*. Many other studies using other species of micro and macro algae have noted higher ETR relative to O_2 produced as found in this work (Geel et al. 1997; Flameling and Kromkamp 1998; Beer et al. 2000; Franklin and Badger 2001; Longstaff et al. 2002). There are several cellular processes which can

create this disconnect between oxygen evolution and PSII electron transport including rubisco oxygenase activity, the Mehler reaction, and nitrogen assimilation.

Rubisco oxygenase activity can consume oxygen via photorespiration without affecting electron transport or the quantum yield of charge separation of PSII. In this case, Rubisco can bind alternatively with carbon dioxide or oxygen based on the concentration of the substrate (Falkowski and Raven 1997). When oxygen concentrations are high within the cell and/or the supply of inorganic carbon is reduced, Rubisco can potentially function as an oxygenase instead of a carboxylase. While there is limited evidence for detection for the byproducts of photorespiration, such as glycolate and phosphoglycolate in *Symbiodinium* (Streamer et al. 1993), it is also important to note that these algae utilize a Form II Rubisco which shows a poor discrimination between CO_2 and O_2 (Whitney et al. 1995). Yet other evidence suggests that it is unlikely that rubisco oxygenase activity is affecting the relationship between oxygen evolution and electron transport in *Symbiodinium*, as some algae in this genus do contain an efficient carbon concentrating mechanism (e.g. carbonic anhydrase) that has been shown to maintain high carbon dioxide concentrations within the chloroplast, thus minimizing the chance for Rubisco to act as an oxygenase (Leggat et al. 2002). Clearly, future work is needed in order to confirm if thermal perturbation may alternatively impact the capacity for carbon delivery.

In the Mehler reaction, photosystem I donates electrons to oxygen instead of $NADP^+$ and the reduction of dioxygen at PSI produces free superoxide radicals. These radicals are converted rapidly in a series of reactions via superoxide dusmutase and

ascorbate peroxidase to result in a net oxygen yield of zero (Asada 2000). Despite the net production of zero oxygen, the Mehler reaction has the potential to increase ETR as electrons are still proceeding completely through the photosystems. It has been shown that Mehler reaction electron transport may contribute from 10 – 50% of total electron transport rates, and can be especially high in some cyanobacteria (Kana 1993). Thus, the Mehler reaction is a possible mechanism causing the observed overestimation of oxygen production by chlorophyll fluorescence techniques. During increased temperature, there may be an increase in electron flow through this cycle which may explain why linearity disintegrates at a lower light level versus control temperatures. An increase in this pathway may lead to an over-production of ROS that can not be scavenged quick enough to prevent photo-oxidative damage that has been observed in reef building corals (Lesser 1996; Lesser 1997; Downs et al. 2002).

The nitrogen assimilation pathway directs electrons from photosystem I towards the conversion of nitrate into ammonia via the enzyme nitrate reductase. It has been shown that greater ETR versus oxygen evolution correlates with macroalgae with higher nitrogen assimilation values (Figueroa et al. 2003). A higher nitrogen assimilation rate determines a higher sink of electrons that could drive ETR higher without affecting oxygen evolution. Rodríguez-Román and Iglesias-Prieto (2005) showed that two cultures of *Symbiodinium* demonstrated a loss in electron transport consistent with sink limitations under nitrate starvation, suggesting that nitrogen does play an important role as a electron acceptor in *Symbiodinium*. It has been shown in diatoms that an increase in nitrogen

assimilation can prevent photoinhibition during cold stress by utilizing excess electrons that may otherwise cause photodamage (Lomas and Gilbert 1999).

While ETR overestimates oxygen production at moderate to high light (>200 μmol m^{-2} s^{-1}), the effective quantum yield of photosystem II ($\Delta F/F_m'$) overestimates the operational quantum yield of oxygen production (Φ_{O2}) under low to moderate light levels (<300 μmol m^{-2} s^{-1}). This is in contrast to the results of Hoogenboom et al. (2006) where no relationship was noted between quantum yields. However, the results of this study are not directly comparable to those presented here, as Hoogenboom et al (2006) compared the total daily changes in oxygen production and fluorescence yield. The disparity at low light may be due to the multiple turnover methodology of the PAM. The multiple turnover flash can lead to an overestimation of the reduction state of PSII and thus of the effective quantum yield, which is not noticed with single turnover methods (Suggett et al. 2003). In the current study, thermal exposure decreased the disparity between the quantum yields, meaning that under increased temperatures $\Delta F/F_m'$ is a better approximation of Φ_{O2}. However, $\Delta F/F_m'$ was shown to be more susceptible to thermal stress. There was no decrease in Φ_{O2} despite the loss in $\Delta F/F_m'$ in phylotype A1 and a greater decrease in $\Delta F/F_m'$ was noted in B1 versus Φ_{O2}, though the quantum yield did decrease.

Regardless of the mechanism causing the overestimation of photosynthesis by electron transport, the disparity between the two methods suggests that care must be taken when interpreting fluorescence data. The trends observed in the current study were varied by thermal exposure. This indicates that indices such as ETR_{max} cannot be used to

infer maximal photosynthetic activity in these algae and may not be a reliable index for measuring the response to elevated temperature in coral algal symbionts.

Tables and Figures

Table 1: Photosynthetic parameters for two *Symbiodinium* isolates (designated by ITS2). [a] represent ($p < 0.05$) for within culture comparisons. Units are as follows: chl *a* - pg chl *a* $cell^{-1}$; P_{max}, respiration - µmol O_2 $cell^{-1}$ m^{-2} s^{-1}; E_k, E_k^{ETR} - µmol m^{-2} s^{-1}; ETRmax - µmol electrons m^{-2} s^{-1}. Samples are from 240 h exposure (n = 3 ± SD).

	Phylotype B1		Phylotype A1	
	26°C	32°C	26°C	32°C
F_v/F_m	0.619 ± 0.005	0.399 ± 0.009[a]	0.569 ± 0.006	0.447 ± 0.016[a]
Chl *a*	0.365 ± 0.028	0.205 ± 0.033[a]	1.18 ± 0.231	0.608 ± 0.348
P_{max} (10^{-9})	9.3 ± 1.2	3.9 ± 0.20[a]	8.6 ± 1.7	6.5 ± 0.72
α (10^{-10})	0.96 ± 0.10	0.28 ± 0.02[a]	0.92 ± 0.08	0.66 ± 0.11[a]
E_k	103 ± 9	141 ± 19[a]	93 ± 12	99 ± 12
respiration (10^{-9})	2.1 ± 0.25	1.4 ± 0.36	2.8 ± 0.50	3.8 ± 0.32
ETR_{max}	5.0 ± 0.67	3.3 ± 0.47[a]	11 ± 0.37	7.2 ± 1.0[a]
α^{ETR} (10^{-2})	2.5 ± 0.39	1.1 ± 0.17[a]	0.06 ± 0.01	0.03 ± 0.004[a]
E_k^{ETR}	201 ± 6	317 ± 23[a]	185 ± 26	263 ± 1[a]

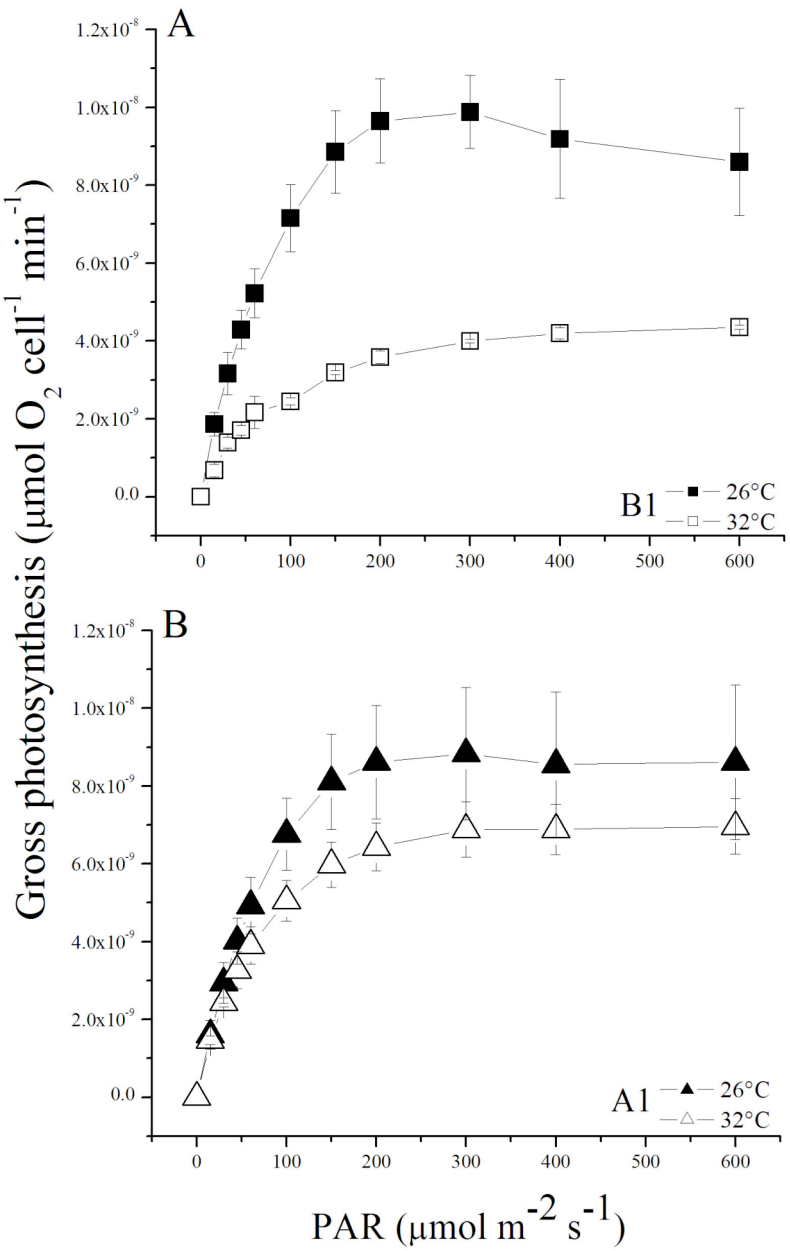

Figure 1: Gross photosynthesis to irradiance (P:E) curves for two phylotypes of *Symbiodinium.* **A**) Phylotype B1, closed symbols represent 26ºC (■) and open symbols represent 32ºC (□) **B**) Phylotype A1, closed symbols represent 26ºC (▲) and open symbols represent 32ºC (△) Samples from T=240hrs. Error bars represent 1 standard deviation, n = 3.

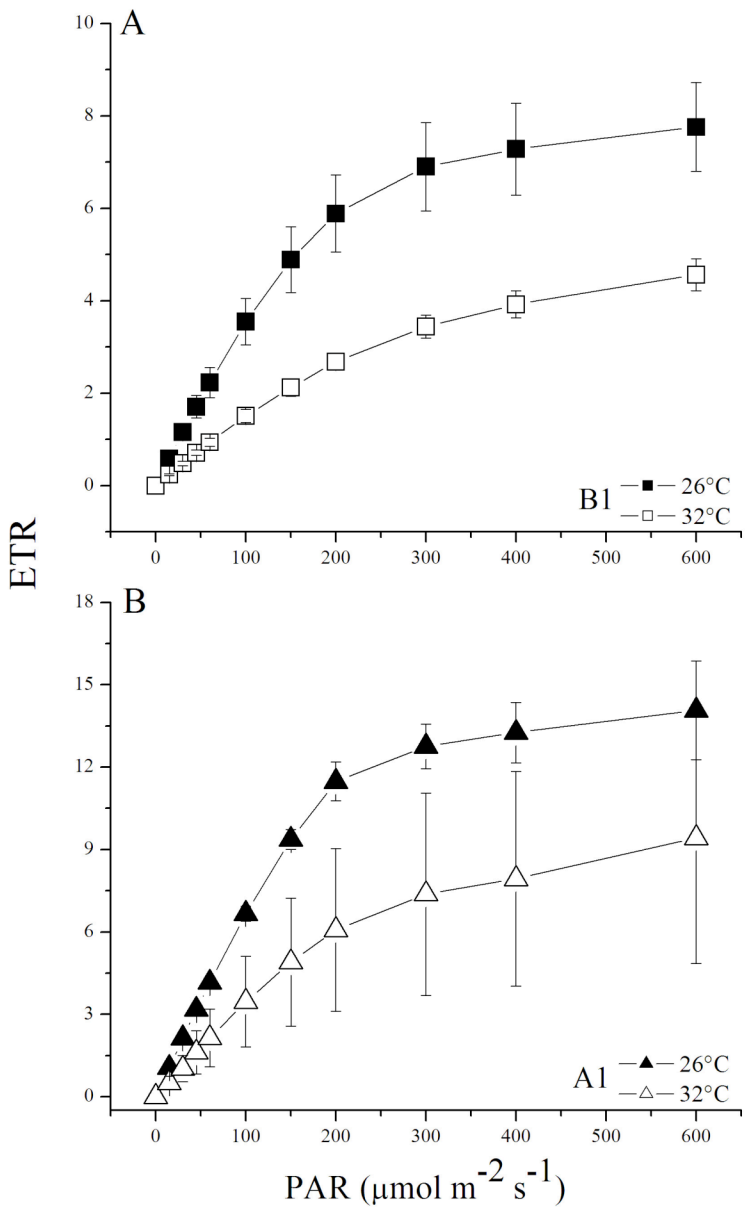

Figure 2: Electron transport rate (ETR) curves for two phylotypes of *Symbiodinium.* **A**) Phylotype B1, closed symbols represent 26ºC (■) and open symbols represent 32ºC (□) **B**) Phylotype A1, closed symbols represent 26ºC (▲) and open symbols represent 32ºC (△) Samples from T=240hrs. Error bars represent 1 standard deviation, n = 3.

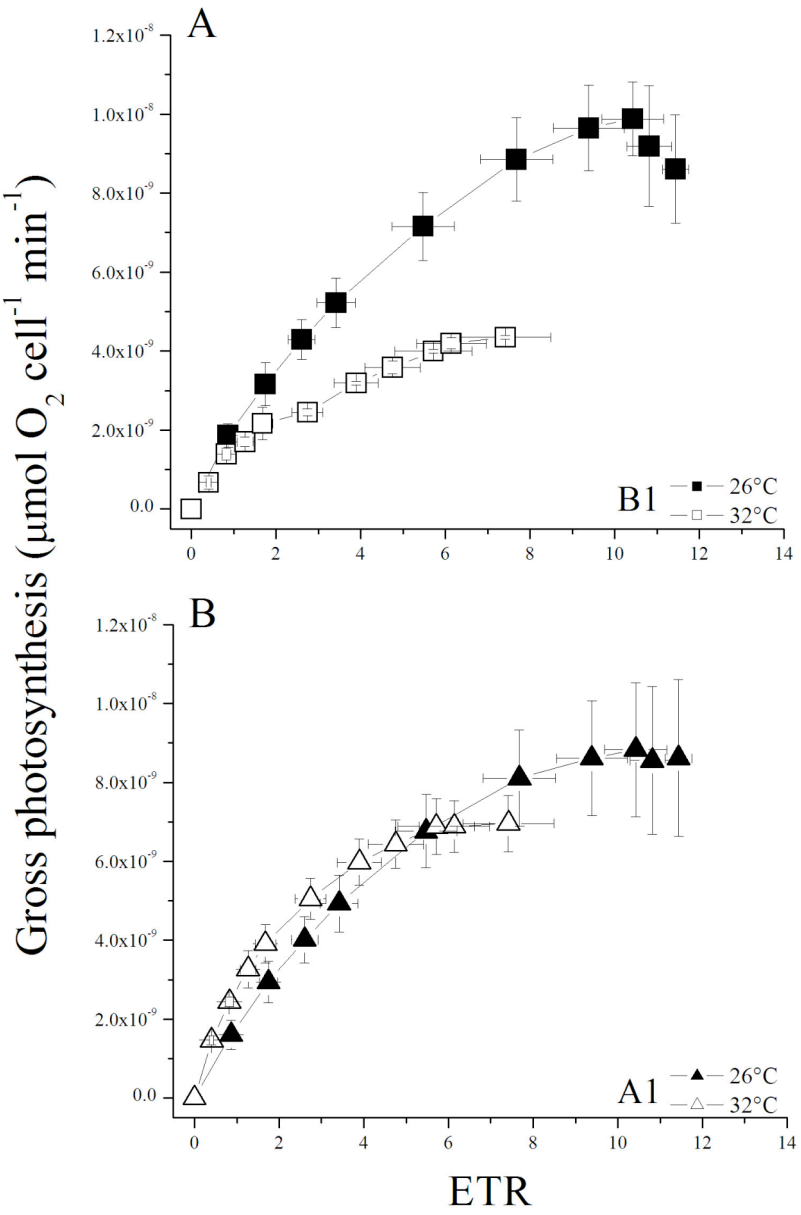

Figure 3: Gross oxygen evolution versus electron transport rate (ETR) in two phylotypes of *Symbiodinium.* **A**) Phylotype B1, closed symbols represent 26ºC (■) and open symbols represent 32ºC (□) **B**) Phylotype A1, closed symbols represent 26ºC (▲) and open symbols represent 32ºC (△) Samples from T=240hrs. Error bars represent 1 standard deviation, n = 3.

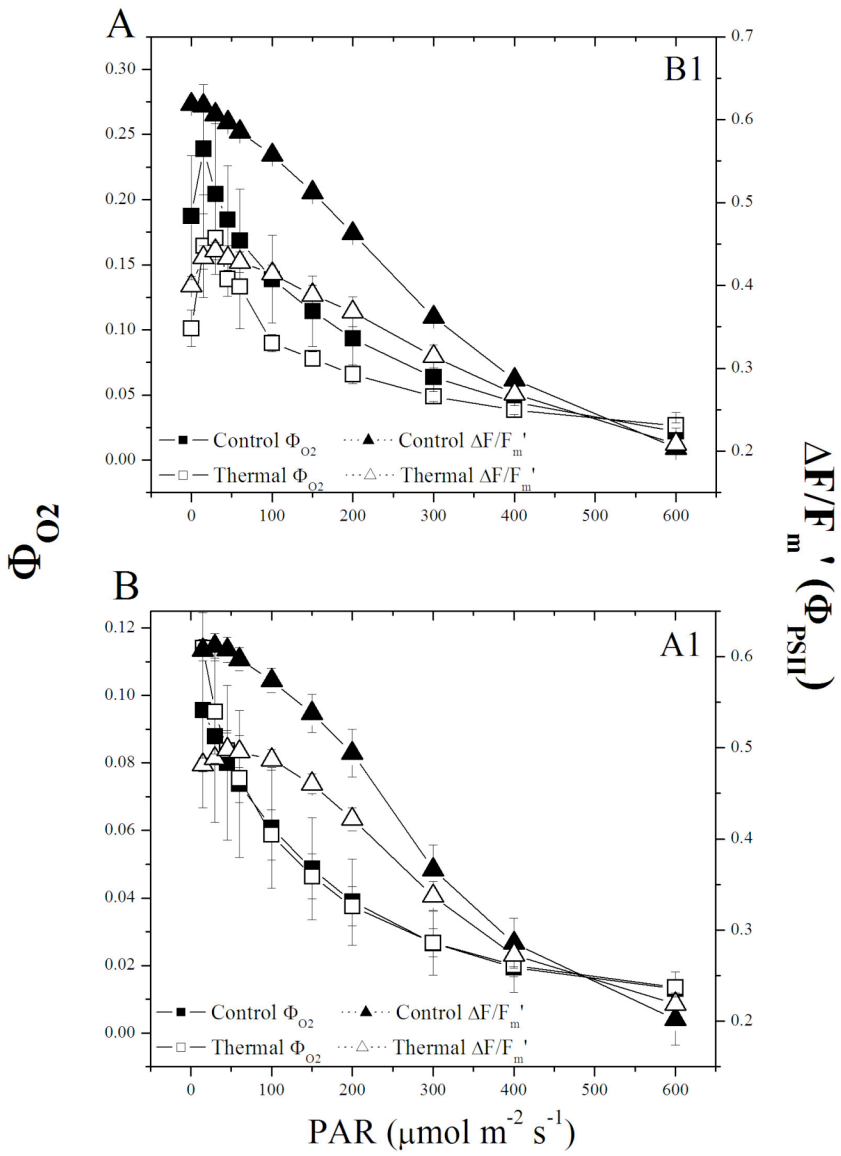

Figure 4: Operational quantum yields of oxygen evolution (Φ_{O2}) and chlorophyll fluorescence ($\Phi_{PSII}/\Delta F/F_m'$, effective quantum yield) in two phylotypes of *Symbiodinium.* Closed symbols represent 26ºC (■, ▲ - Φ_{O2} and Φ_{PSII} respectively). Open symbols represent 32ºC (□,△ - Φ_{O2} and Φ_{PSII} respectively). **A**) B1 **B**) A1. Samples from T=240hrs. Error bars represent 1 standard deviation, n = 3.

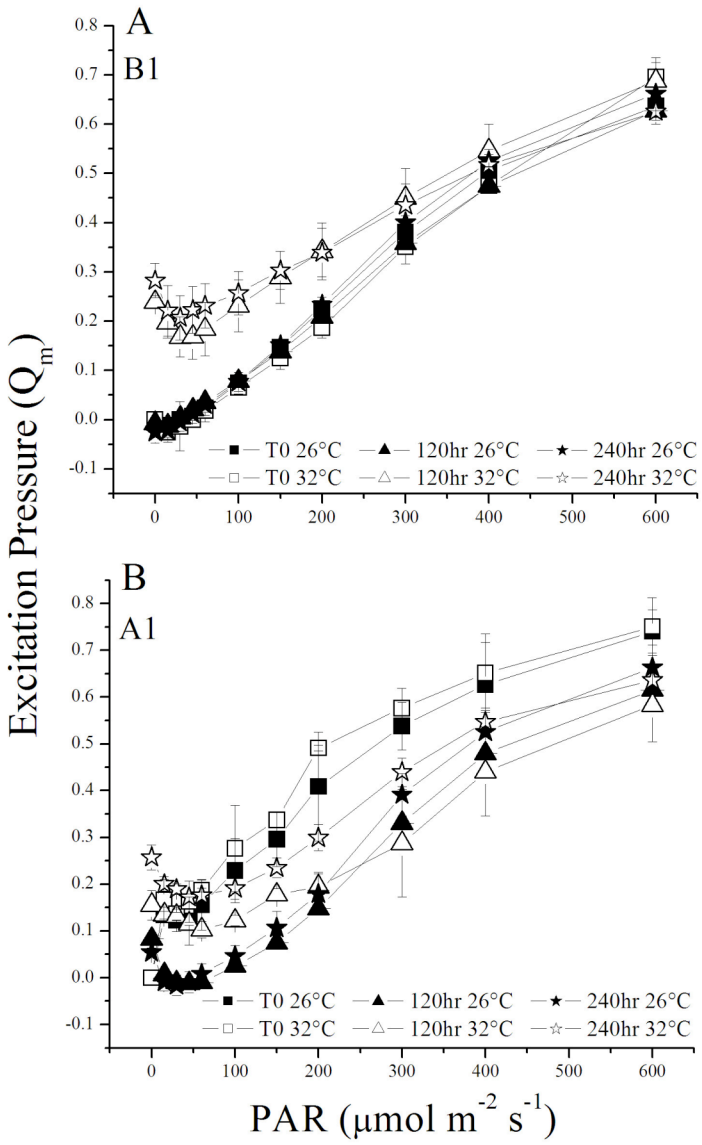

Figure 5: Excitation pressure over PSII in two phylotypes of *Symbiodinium*. **A**) B1 **B**) A1. Closed symbols represent 26ºC (■, ▲,★ - T0, 120 and 240hr respectively). Open symbols represent 32ºC (□,△,☆ - T0, 120 and 240hr respectively). Data from time 0 represents the point prior to thermal ramping. Error bars represent 1 standard deviation, n = 3.

IV: Summary and Further Research

Coral bleaching is a complex problem resulting in several physiological responses within the symbiotic algae, zooxanthellae as well as the coral host. The research presented here has focused on the thermally induced changes in the photobiology of zooxanthellae, specifically at the level of photosystem II (PSII hereafter) in different phylotypes of cultured zooxanthellae grown under different light intensities. Additionally the reliability of using chlorophyll fluorescence, one of the main techniques used in monitoring reef corals today, to infer photosynthetic efficiency was investigated.

Differential thermal tolerance was noted across the four phylotypes utilized, and acclimation to higher light exacerbated the effect of increased temperature indicating that previous light history should be taken into account when interpreting field data based on bleaching experiments with corals. Interestingly, the phylotypes with the greater capacity to photoacclimate to higher light were also thermally tolerant. It appears that there is more than one cellular pathway by which PSII may be protected from thermal breakdown as the thermally tolerant phylotypes had markedly different responses to thermal exposure. Phylotype F2 showed the greatest capacity for photoacclimation and growth at high light while phylotype A1 appeared to adjust to thermal stress by a slight decline in PSII activity coupled with a significant decline in growth rate, possibly at the expense of

elevated photosystem and cellular repair. The observed differences in growth rate between the two thermally tolerant phylotypes have intriguing implications toward predicting the outcome of bleaching events and the potential for successful recovery from such events. An alga capable of sustaining higher growth rate has the potential to out compete other phylotypes within the tissue of a host coral capable of harboring more than one symbiont at a time. This provides evidence supporting the idea that changes in algal types observed in the field during both bleaching and seasonal conditions are due to a "reshuffling" of dominant algal populations within the coral due to a change in environmental conditions (Lewis and Coffroth 2004; Thornhill et al. 2005).

In contrast to the thermally tolerant algae studied, thermally sensitive isolates A1.1 and B1 had a complete photosynthetic breakdown under thermal stress, as indicated by the sharp decline in the PSII quantum efficiency (F_v/F_m), loss of the primary reaction center D1 protein, and a significant decline in growth rate at high light. However, B1 demonstrated a loss in the stability of D1 protein and a significant decline in growth rate at high light prior to any thermal ramping as compared to low light growth, suggesting that this alga is unable to photoacclimate to high light levels. The observation that the ability of the investigated alga to photoacclimate to higher temperatures mirrors its respective thermal tolerance is an important finding. This research suggests that irradiance levels play a key role in defining the state of the photosystems prior to thermal exposure.

The observed loss in the D1 protein confirms that a change in photosynthetic units is occurring during thermal stress and photoacclimation. As D1 is the core protein of

photosystem II, the change in D1 content is evidence of changes in photosystem II but gives no information on how or if photosystem I is changing. This may be of particular importance in phylotype A1 which shows a decrease in D1 at high light and thermal stress yet does not show a large decrease in photosynthetic (by PSII function) activity suggesting that photosystem I activity may be increasing to compensate for the loss in PSII reaction centers. Further research evaluating the changes in photosystem I content and activity relative to PSII during thermal stress should be performed.

The results of investigations into the use of chlorophyll fluorescence as a proxy of oxygen evolution revealed that fluorescence parameters often overestimate photosynthesis regardless of phylotype studied. At irradiance levels above 300 μmol m^{-2} s^{-1}, the effective quantum yield was a reliable approximation of the quantum yield of oxygen evolution. Alternatively, the use of electron transport rates (ETR) was shown to be an accurate predictor of oxygen evolution rates only at light levels less than 200 μmol m^{-2} s^{-1}, after which ETR consistently overestimated oxygen evolution. However, when exposed to increased temperature, ETR was shown to be accurate at light levels less than 60 μmol m^{-2} s^{-1} only. As these low light levels are rarely found on a coral reef flat, this suggests that during bleaching conditions ETR is not a good approximation for oxygen production and that a decrease in ETR does not necessarily reflect a decrease in photosynthesis.

The comparison of fluorescence versus oxygen production was only performed with PAM flourmetry in the current study. Due to the multiple turnover nature of the PAM, an overestimation of electron transport through PSII has been reported compared

to single turnover fluorescence (Suggett et al. 2003). The use of a single turnover device, such as fast repetition rate fluorometry (FRR), to re-examine the relationship between flourescence and oxygen production may reveal a closer coupling. However, as the PAM is still the most utilized fluorometer in the field, the results of this study have widespread applications for field data.

While the current study provided valuable information on the reliability of current techniques and specific photobiological changes during thermal stress in cultured algae, the effect of the host coral can not be ignored. Further research to better understand the thermal stress response of the intact symbioses must be established, as the response of the intact symbiosis is central to understanding the physiological changes for the entire holobiont during bleaching and is necessary for better prediction of the severity and ultimate outcome of future coral bleaching events. The interaction between host and symbiont is critical as the symbiont may have a certain environmental range, the host a different one, and the holobiont a different tolerance than either alone.

In addition to more in depth studies of photosystem activity within the different phylotypes of *Symbiodinium*, the photosynthetic recovery after thermal stress is an important factor to explore. Would either thermally sensitive phylotype studied be able to recover to pre-bleaching levels? How corals recover from bleaching is an important question as the frequency and severity of bleaching events increase worldwide. An understanding of the changes in both the photosynthesis of the symbiont as well as the overall effect on the coral holobiont is critical for predictions as to how increased bleaching will affect worldwide coral distribution in the future.

Another large segment needing investigation is the ability of *Symbiodinium* to acclimate to higher temperature, which may lead to a long-term evolutionary adaptation, to the effects of thermal stress. Will certain phylotypes be unable to keep up with current increases in seawater temperature and face extinction? Or does repeated thermal exposure lead to a resistance? Will host corals be able to acquire new symbionts from the environment as predicted by the adaptive bleaching hypothesis (Baker 2001) or will the resilience of cryptic populations that could become dominant within the coral prove to be more important? Understanding how or if a particular symbiont can acclimatize to thermal stress is an important piece of the puzzle for worldwide coral reef survival.

Overall this research has answered many questions about how the photobiology of several types of *Symbiodinium* is affected by both acclimation to different light levels and the subsequent effect of thermal stress. However, there are still several questions remaining to be answered before accurate predictions of the outcome of coral bleaching events can be made.

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