# PHOTOSYNTHETIC AND RESPIRATORY RESPONSES TO THERMAL STRESS IN THE CORAL SYMBIONT SYMBIODINIUM

by

CLINTON ALEXANDER OAKLEY

(Under the Direction of Gregory W. Schmidt)

## ABSTRACT

The symbiotic algae of corals, dinoflagellates of the genus *Symbiodinium*, exist in a harsh environment characterized by high temperatures, hyperoxia, ultraviolet radiation and irradiance. Despite these stressors, *Symbiodinium* is able to fix inorganic carbon with an apparently inferior Form II Rubisco and metabolically drive one of the earth's most diverse and productive ecosystems. Coral reefs are threatened by coral bleaching, a physiological response characterized by the death or expulsion of the algal symbionts, which has been increasing in frequency in recent decades. As the coral mutualism is based upon the exchange of carbon, the effects of thermal stress on the carbon concentrating mechanism and photosynthetic parameters of cultured *Symbiodinium* were assayed. A novel apparatus was devised to simultaneously measure dissolved inorganic carbon and  $O_2$  flux while simultaneously measuring photosynthetic electron transport via PAM fluorometry in small algal culture samples. There has been considerable debate whether the initial point of thermal damage during high temperature coral bleaching is in the photosynthetic apparatus or in the "dark reactions" of the Calvin-Benham-Bassham

cycle. Surprisingly, the inorganic carbon requirement for maximal photosynthetic and electron transport rates was found to be reduced in Symbiodinium cultures at high temperatures, with no evidence to support damage to Rubisco or carbon fixation. High inorganic carbon levels were found to be photoprotective, implying that inorganic carbon supply by the host is central for stabilizing the symbiosis. The hyperoxic conditions of coral tissues in the light combined with high temperatures and ultraviolet radiation are conducive to the formation of damaging reactive oxygen species (ROS). Excessive generation and release of ROS by symbionts in warm conditions triggers the coral bleaching response. Here, a mitochondrial terminal alternative oxidase, widespread among plants, was detected by inhibitor assay and transcriptome analysis and described for the first time in Symbiodinium cultures. Acclimation to both high and low temperatures induced greater terminal alternative oxidase capacity, which likely has a role in mitigating mitochondrial ROS generation. High affinity for inorganic carbon and enhanced mitochondrial alternative oxidase activity of Symbiodinium are demonstrated mechanisms by which these algae are capable of maintaining physiological function under thermal stress.

INDEX WORDS: Coral bleaching, coral reefs, inorganic carbon, alternative oxidase, photosynthesis, *Symbiodinium* 

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# DEDICATION

To my wife Kate, for her unwavering support and love. Thank you.

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## CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

## Introduction

The symbiotic algae of corals, genus Symbiodinium, exist in a harsh environment characterized by high temperatures, ultraviolet radiation and irradiance. Despite these stressors, Symbiodinium is able to fix inorganic carbon with an apparently inferior Rubisco and drive one of the earth's most productive ecosystems. Symbiodinium possesses many unusual photosynthetic features, including the light-harvesting pigment peridinin and a Form II Rubisco that is unique among eukaryotes. This Rubisco is characterized by a lower  $CO_2$  specificity than that utilized by other algae and land plants, especially at high temperatures. Temperature stress is of particular importance due to the implications of anthropogenic climate change over the next century and the increasing frequency of large-scale coral bleaching events. Coral reefs contain a genetically diverse assemblage of *Symbiodinium* types, but the relationship between genetic and functional diversity is less clear. The presence of a single diverse algal genus in symbioses, often obligate mutualisms, with such a highly diverse assemblage of organisms suggests that they possess trait(s) amenable to both the establishment and maintenance of mutualism. Here, the carbon fixation of *Symbiodinium* and its implications for symbiosis stability, especially in regards to climate change, are discussed.

### The coral/algal mutualism

Coral reefs cover only 0.2 percent of the Earth's surface but are the most diverse ecosystems on the planet (Veron et al. 2009), including the tropical rain forests. Coral reefs are highly productive, both ecologically (Odum and Odum 1955) and economically, providing over 10% of the world's fisheries (Moberg and Folke 1999). This diversity and productivity is built upon the photosynthetic production of the symbiotic dinoflagellate Symbiodinium. Each square centimeter of coral tissue contains millions of these algal symbionts, also known as zooxanthellae, with coral cells hosting groups of one to four dinoflagellate cells within specialized vacuoles known as symbiosomes. The mutualism between cnidarians and dinoflagellates is characterized by the translocation of fixed carbon from the symbiont to the animal host and the provision by the host of nutrients, habitable space, protection from microfauna and a constant light field to the algae. This process can provide more than 95% of the metabolic requirements of dominant reefbuilding corals (Muscatine et al. 2008), with the rest derived from heterotrophic feeding. Within cnidarians symbionts are located intracellularly, while Symbiodinium hosted by molluscs, such as the giant tridacnid clams, are located in intercellular sinuses. In addition to hermatypic (reef-building) corals, *Symbiodinium* is also found in symbiosis with other cnidarian groups (many other Anthozoa, Hydrozoa) as well as molluscs and foraminiferans.

#### Carbon fixation in Symbiodinium

Energy and carbon flow in the biosphere are driven by the autotrophic fixation of carbon dioxide by ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco), nearly all

of which is a result of photosynthesis. Its ecological function, ubiquity among primary producers and kinetic properties make Rubisco the most abundant protein on the planet. Rubisco is a large enzyme with a low activity, typically less than 10 turnovers per second, requiring large concentrations of the enzyme for sufficient carbon fixation. Rubisco is an ancient enzyme that has evolved, along with a suite of auxiliary systems and enzymes (Badger and Bek 2008), to optimally fit atmospheric and ocean carbon dioxide/DIC (dissolved inorganic carbon) concentrations.

Peridinin-containing dinoflagellates are unique among eukaryotes in their possession of a Form II Rubisco (Whitney et al. 1995). Form I Rubisco is utilized by the land plants and all other algae, including other dinoflagellate groups. Otherwise only found in proteobacteria, many of which also possess the Form I variant, the Form II enzyme is a homodimer of two large subunits, without the additional small subunits that characterize the Form I Rubisco complex of eight large subunits and eight small subunits (Tabita et al. 2008). All Rubisco forms suffer from competitive interactions between the favored substrate, CO<sub>2</sub>, and O<sub>2</sub> due to the featureless nature of the uncharged, linear molecules (Tcherkez et al. 2006). The oxygenase reaction to produce 2-phosphoglycolate is metabolically wasteful, requiring further processing to recoup the phosphorous while the remaining glycolate is often excreted from the organism rather than used as a reductant (Falkowski and Raven 2007). Form II Rubisco is functionally characterized as having a much lower CO<sub>2</sub> specificity ( $S_{C/O}$ ) than Form I Rubisco.  $S_{C/O}$  values for terrestrial  $C_3$  plants range from 82-90 (Tcherkez et al. 2006), while those from Form IIutilizing bacteria are a much lower 6-12 (Kane et al. 1994), (Robinson et al. 2003);  $S_{C/O}$ values of some non-green algae can be as high as 167 (Whitney et al. 2001). The oxidase

activity is higher than the carboxylase activity in a solution at air equilibrium (Falkowski and Raven 2007). The specificity of Rubisco in peridinin-containing dinoflagellates is difficult to determine due to the instability of the enzyme *in vitro* (Whitney et al. 1995), necessitating the use of approximate figures from bacteria.

The low  $S_{C/O}$  of Form II Rubisco makes its presence in the tissues of coral symbionts even more curious. The dissolved inorganic carbon (DIC) and oxygen fluxes in the host tissue can vary widely. When sufficiently illuminated, the  $O_2$  levels within coral tissues can reach 250% of saturation, collapsing to 2% saturation within minutes of cessation of illumination (Kuhl et al. 1995). It is unlikely that respiratory DIC alone is capable of saturating Form II Rubisco, as Symbiodinium has been shown to utilize a carbon concentration mechanism consisting of both internal and external carbonic anhydrases, allowing for the utilization of  $CO_2$  and bicarbonate (Leggat et al. 1999). These exist in addition to carbonic anhydrases produced by the animal host. Carbonic anhydrases function to convert DIC between the carbon dioxide and bicarbonate forms, but importantly it is not coupled to ATP oxidation, allowing for conversion only in the direction dictated by the pH gradient. Photoautotrophs frequently utilize carbon concentration mechanisms (CCMs), a term for a varied collection of methods for concentrating  $CO_2$  around the Rubisco enzyme to maximize the carboxylase reaction. The C4 and Crassulacean acid metabolism (CAM) pathways of terrestrial plants are highly developed CCM systems. It was long assumed that the C4 pathway could not exist in unicellular algae due to the requirement in land plants for large-scale multicellular anatomy and spatial separation between cells, but the use of a C4 pathway has been recently demonstrated in the marine diatom *Thalassiosira weissflogii* (Reinfelder et al.

2004). The CAM mechanism, widespread in terrestrial plant groups adapted to arid, high temperature environments, is present in some aquatic true plants but has not been found in algae.

Form II Rubisco in dinoflagellates is thought to be the result of horizontal gene transfer to the nucleus (Janouškovec et al. 2010). The phylogeny of Rubisco subforms IA-ID maps with high confidence to the major algal lineages (Tabita et al. 2008). Intriguingly, Form II Rubisco has been found to be utilized by the chemoautotrophic bacterial symbionts of the vestimentiferan tubeworm *Riftia pachyptila*, a dominant species and ecosystem engineer in deep sea hydrothermal vent communities (Robinson et al. 2003). These ecosystems exist in areas of extremely low  $O_2$  concentrations where the low  $S_{C/O}$  values of Form II Rubisco are presumably of trivial importance and the lower metabolic cost of the smaller Rubisco enzyme may be advantageous over a Form I Rubisco.

Tcherkez *et al.* argue that all naturally occurring Rubiscos are tuned to maximize fitness between two competing interests, specificity and reaction rate (Tcherkez et al. 2006). This balance is mediated by the difficulty in distinguishing between the two competing substrates,  $CO_2$  and  $O_2$ . The carboxylation reaction has several ephemeral intermediates, and any increase in  $CO_2$  specificity would require tighter binding of the  $CO_2$  transition state, reducing the reaction rate. They posit that Rubiscos from various organisms are optimized to their respective thermal and substrate environments. The logical extension of this hypothesis is that the Form II Rubisco of invertebrate symbionts—or its suite of accessory mechanisms, such as CCMs—is somehow functionally adaptive.

#### Genetic diversity of Symbiodinium

Zooxanthellae were originally classified as members of the genus Gymnodinium before being determined as a single novel genus and species Symbiodinium microadriaticum Freudenthal (Freudenthal 1962). Symbiodinium nomenclature accommodates considerable uncertainty in taxonomic levels. The basic unit of diversity is the "type", which make up multiple clades lettered A-I (Pochon and Gates 2010), with clade A considered ancestral. The phylogeny of *Symbiodinium* is rapidly becoming defined. Unfortunately, no Symbiodinium genome yet exists. Symbiodinium diversity has been largely based on the use of the nuclear ribosomal intertranscribed spacer (ITS) regions ITS1 and ITS2 (LaJeunesse 2001), though the chloroplast large ribosomal subunit (23S) rDNA sequences have also been used (Santos et al. 2002). The ITS regions allow for differentiation between algal "types" via denaturing gradient gel electrophoresis (DGGE), band excision and sequencing (LaJeunesse 2001), though qPCR is gaining utility as well (Mieog et al. 2007). Each clade consists of many types, particularly clade C, which is known to contain dozens of types based on ITS2 sequences (Lajeunesse 2004). Not all Symbiodinium types are found in symbiosis; clades A-D are of primary importance in corals while other clades are much less common in symbiosis or are free living and presumably of more marginal ecological significance.

The evolutionary history of coral taxa is complex and it is hypothesized that reticulate evolution has played a major role in coral evolution (Veron 1995). These hypotheses emphasize the effective splitting and joining of populations by changes in ocean currents, which would drastically change the path of gametes and larvae. Most coral species are found in obligate mutualisms with only a single type of *Symbiodinium*. The strength of these mutualisms is emphasized by their persistence even among broadcast spawning coral taxa, whose gametes fuse in the water column and must acquire symbionts post-settlement, as opposed to the "brooding" corals which release packets of larvae already infected with *Symbiodinium* from the parent colony. A recent paper analyzing population structure of a common Pacific scleractinian coral, *Seriatopora hystrix*, determined that shallow-water populations tens of kilometers apart were homogenous but hosted distinct symbionts, and were genetically distinct themselves, from populations at a depth of 30m in the same locations, providing evidence of depth gradation as a driver for ecological speciation (Bongaerts et al. 2010).

The requirement for post-settlement acquisition of larvae in broadcast spawning corals raises the question of the source of symbionts and the reservoir of *Symbiodinium ex hospite*. To date the few studies that have searched for environmental *Symbiodinium* have mostly found success in the algal mats adjacent to live corals. Scleractinian coral larvae without symbionts have been shown to accept symbionts nonselectively, but the typical symbiont type for the coral species rapidly becomes dominant. Intracolony symbiont population dynamics and the relative contributions of host selection, competition between symbiont populations, and physiological response to environmental pressures in determining *Symbiodinium* populations are poorly understood, though advances in qPCR methods and symbiont typing are advancing the field. Direct inhibitory effects between algae, such as allelopathy, have not been documented.

There are large differences in distribution and abundance at the cladal level between Indo-Pacific coral symbiont populations and Caribbean populations (LaJeunesse et al. 2003). Clade C symbionts are dominant in the Pacific, existing in symbiosis with the ecologically dominant and diverse acroporids, mainly genus *Acropora* (LaJeunesse et al. 2003). The formerly dominant *Acropora* species of the Caribbean universally host A3 types, related to those found in tridacnid clams in the Pacific. The Caribbean *Montastraea* species complex, now elevated to dominant status in many areas after the *Acropora* collapse, shows remarkable flexibility in its symbiont partners. *Montastraea* associates most commonly with clade B symbionts (which are relatively uncommon in the Pacific) but can also be found with symbionts from clades A, C and D, often with multiple clades inhabiting the same colony (Kemp et al. 2008). These flexible associations present great opportunities for experimental investigations into the functional importance of *Symbiodinium* diversity. It is hypothesized that corals often possess multiple algal types in symbiosis, and that the dominant strains may "shuffle" due to severe stress events (Mieog et al. 2007).

#### Functional diversity of Symbiodinium

The genetic diversity and host specificity of *Symbiodinium* would lead one to surmise that the genus would contain considerable functional diversity, particularly to temperature and light regimes. Host specificity, however, confounds many studies of *Symbiodinium in hospite* as the effects of host versus those of the symbiont are difficult to tease apart. Several avenues allow for the detection of functional diversity, including geographic/depth distributions (Iglesias-Prieto et al. 2004), intracolony distributions (Kemp et al. 2008) and studies *ex hospite* (McCabe Reynolds et al. 2008). To date, studies have investigated differential functional diversity with regards to thermal protection of the light reactions, depth/light regime zonation, mucous layer bacterial

diversity, disease resistance, photosynthate quality and reactive oxygen species production.

The photosynthate translocated from symbiont to host is poorly understood but is known to consist primarily of glycerol (Gattuso and Jaubert 1990). Whether or not the translocated compounds vary in type or ratio of components by algal type is unknown. Notably *Symbiodinium* in culture do not release translocated photosynthate unless host homogenate is added, suggesting a requirement for a putative host release factor, the chemical identity of which has not yet been deduced. It is likely that symbionts require a combination of chemical cues rather than a universal host release factor to initiate photosynthate release.

## **Coral bleaching**

Coral bleaching is a generalized stress response characterized by the rapid loss of coloration of corals due to loss or expulsion of zooxanthellae or their photosynthetic pigments. Bleaching has been documented to be caused by sedimentation, disease and, most commonly, extreme temperature stress. Bleaching due to high temperature stress can occur simultaneously across many coral species spanning from the reef to regional scale, and these mass bleaching events are becoming more common due to increased sea surface temperatures (SSTs) as a result of anthropogenic climate change. Bleaching events can be triggered by sea surface temperatures only 1-2°C above the typical summer maximum (Hoegh-Guldberg 1999). These events typically occur in the late summers due to high irradiance, low wind speed and minimal wave action, all contributing to elevated SSTs.

Coral bleaching has been linked to high SST events and there have been numerous proposed mechanisms of high-temperature bleaching, including photoinhibition, breakdown of the light harvesting mechanisms, breakdown of the thylakoid membranes, inhibited repair of the photosynthetic apparatus, increased reactive oxygen species production, ultraviolet damage, and impairment of the carbon fixation reactions. These mechanisms are all interrelated with multiple feedbacks upon one another, making the detection of the initial point of thermal impairment difficult to determine, if a single point exists.

There has been considerable attention to whether the initial point of thermal breakdown lies within the light harvesting complexes or in the dark reactions, particularly due to the extreme thermal sensitivity of Form II Rubisco in extracts (Whitney et al. 1995; Jones et al. 1998). Lilley et al. have pioneered a new method of Rubisco extraction and activity determination using a chemiluminescence protocol (Lilley et al. 2010) and propose that either the Rubisco protein or a Rubisco repair mechanism is the site of initial thermal damage. Once the Calvin cycle is inhibited, whether by Rubisco degradation, carbon limitation or a combination of the two, feedback effects may begin to rapidly produce conditions that are damaging to the light reactions. The lack of an electron sink for the electron transport chain would require the initiation of alternate means of dissipating excess energy, such as nonphotochemical quenching (NPQ), photorespiration, or cyclic electron transport to prevent enhanced production of reactive oxygen species and direct thermal damage to the photosynthetic apparatus. One model-based approach (Wooldridge 2009) has proposed that feedback between the light reactions and Calvin cycle would escalate, triggering coral bleaching, until either thermal conditions improve

or the symbiont population is reduced to a level that can be sustained by ambient  $CO_2$  concentrations, alleviating competition among symbionts for carbon.

#### **Oxidative stress**

Reactive oxygen species (ROS) generation is influenced by the state of both the light harvesting and carbon fixation reactions and is known to be one of the primary determinants in coral bleaching. The warm hyperoxic microenvironment inside coral tissues is ideal for ROS generation by photosynthesis, as well as through normal host cellular function. In this scenario, host bleaching is a direct response to ROS toxicity generated by the symbionts (Lesser 1996, 1997; Richier et al. 2005). Light exposure, particularly during the summer months, increase ROS production by approximately two orders of magnitude (Saragosti et al. 2010). The addition of antioxidant compounds such as acorbate and catalase to corals in closed chambers can maintain their photosynthetic performance even when exposed to temperatures higher than the bleaching threshold (Lesser 1997). Photosystem II (PSII) is proposed to be the primary site of ROS generation (Downs et al. 2002), potentially resulting in positive feedback in which ROS production damages the D1 protein of PSII and associated reaction center components, inhibiting the transfer of excitation energy to PSI and producing more ROS.

The great risk to the coral mutualism of ROS production and the low specificity of the Form II Rubisco suggest a mechanism by which Form II Rubisco may confer an adaptive benefit to a high-light, high-temperature environment. A relatively high oxygenase activity would result in a higher photorespiration rate, producing 2phosophoglycolate and potentially reducing intracellular oxygen concentrations (and therefore potential ROS production) through a combination of direct fixation and enhanced respiration due to the metabolism of glycolate by glycolate dehydrogenase. Glycolate is oxidized to glycerate, recovering the phosphorous, some NADH, and CO<sub>2</sub> through the intermediate glyoxylate (Beardall and Quigg 2003). This glycerate can then reenter the Calvin cycle. Intriguingly, dinoflagellates, together with some chlorophytes, cyanobacteria and other disparate groups, and unlike land plants, do not possess glycolate oxidase (Beardall and Quigg 2003). Glycolate oxidase produces hydrogen peroxide, resulting in enhanced ROS production via photorespiration. Furthermore, unlike many groups, dinoflagellates, diatoms and tribophytes possess malate synthase, allowing for the complete oxidation of glyoxylate through the tricarboxylic acid cycle and the production of NADH, ATP and CO<sub>2</sub> (Beardall and Quigg 2003).

Also unlike most photosynthetic eukaryotes, evidence of a terminal alternative oxidase (AOX) in dinoflagellates is slight (Eriksen and Lewitus 1999, Vanlerberghe et al. 2006, Butterfield et al. 2012), which has been proposed to mediate oxidative stress in higher plants. AOX oxidizes the mitochondrial ubiquinone pool between Cytochrome II and Cytochrome III in the mitochondrial electron transport pathway. This action does not pump protons across the interior mitochondrial membrane and therefore does not drive ATP synthase. This enzyme is universal in plants, widespread in algae and central to the metabolism of some apicomplexan parasites, such as *Trypanosoma brucei*, the causative agent of African sleeping sickness. The oxidative stress experienced by *Symbiodinium* would imply that an active AOX would serve a valuable function and have important implications as a major metabolic pathway in the primary producer of coral reefs.

## Conclusion

Hermatypic corals exist in a highly energetic and stressful environment, with high irradiance and temperature both increasingly contributing to coral bleaching and mortality. The combination of Form II Rubisco, cyclic electron transport and malate synthase suggests a photosynthetic apparatus tuned to reduce excitation pressure on the photosynthetic electron transport chain and reactive oxygen species generation. Studies are needed to better characterize these photosynthetic features of *Symbiodinium* to better model carbon dynamics within the host tissue and responses to elevated temperatures. As seen in regional bleaching events over the past twenty years, projected anthropogenic climate change is likely to challenge corals further, necessitating greater physiological understanding of coral sensitivity to elevated temperatures.

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# CHAPTER 2

# A MODULAR SYSTEM FOR THE MEASUREMENT OF $CO_2$ AND $O_2$ GAS FLUX AND PHOTOSYNTHETIC ELECTRON TRANSPORT IN MICROALGAE<sup>1</sup>

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## Abstract

Conventional means of assessing net photosynthetic rates of microalgae have largely relied upon the use of oxygen electrodes or carbon isotope radiolabeling. These methods are simple but inadequately resolve simultaneous fluxes of the gaseous substrates and products of photosynthesis,  $CO_2$  and  $O_2$ . Fluorometric methods allow for assessment of the photosynthetic efficiency of excitation energy capture by the light harvesting complexes and electron transport chain but do not directly measure photosynthetic rates as they do not resolve the fate of excitation energy downstream of electron transport chain. Here we describe and validate the accuracy of a compact system for the simultaneous, real-time and comprehensive measurement of photosynthetic  $CO_2$ and  $O_2$  flux as well as photosynthetic electron transport in microalgae, from which net and gross rates of carbon fixation and oxygen evolution can be derived. Essential components include an infrared gas analyzer, gas-phase galvanic oxygen electrode and pulse-amplitude modulated fluorometer.

## Introduction

Accurate assessment of the photosynthetic performance of aquatic organisms requires comprehensive monitoring of multiple components of the photosynthetic process, including oxygen evolution, carbon fixation and activity of the photosynthetic electron transport chain. For net primary production estimations, traditional studies have employed either oxygen electrodes (or, more recently, probes utilizing oxygen-sensitive fluorescent dyes) or carbon isotope labeling (Steemann-Nielsen 1952) for photosynthesis and respiration measurements. Although these methods are of great utility, non-invasive gas exchange technology has been inadequately developed for algae and other aquatic photosynthetic organisms. We have successfully modified commercially available photosynthetic instrumentation systems and describe an apparatus array that simultaneously records  $CO_2$  and  $O_2$  flux in a small chamber in near real-time and, additionally, is coupled to a pulse-amplitude modulated fluorometer.

Oxygen evolution reflects production of the byproduct of water oxidation during photosynthesis but may not relate directly to the amount of carbon fixed at the end of the complex chain of reactions in the Calvin-Benson-Bassham cycle. Carbon labeling, whether via the stable isotope <sup>13</sup>C or the radioisotope <sup>14</sup>C, directly quantifies the amount of carbon momentarily fixed by the organism and allows for the eventual determination of carbon fate but requires tedious sample processing and costly instrumentation for sample processing and analyses. Isotope pulse/chase labeling techniques, moreover, only determine the amount of isotope remaining in the organism regardless of respiratory release, refixation or excretion of photosynthates. Isotope labeling also requires periodic and destructive sampling of the organism and, in the case of radioactive isotopes, have

safety, hazardous waste and environmental restrictions, which limit their use. More recently, membrane-inlet mass spectrometry (MIMS) instrumentation and methods have improved and are becoming more common in microalgal research (Beckmann et al. 2009). MIMS is highly sensitive to simultaneous fluxes of dissolved gases (e.g. O<sub>2</sub>, CO<sub>2</sub>, H<sub>2</sub>) in liquid samples but requires an expensive mass spectrometer.

Gas exchange systems potentially ameliorate many issues of assessment of photosynthetic efficiency. Infrared gas analysis (IRGA) has found widespread use in the photosynthetic assessment of terrestrial plants but rarely in algae as they measure  $CO_2$ concentrations in the gas phase. IRGA systems which determine photosynthetic or respiratory gas flux may be run in open, closed or compensating configurations (Koller and Samish 1964). Open systems determine gas flux by pumping gas, often ambient air, into the chamber and measuring gas concentrations in both the influent and effluent gas stream. This configuration necessitates either two gas analyzers per sample for simultaneous measurement or alternating the gas stream through the instrument, which is both technically more complex and introduces time lag and sensitivity loss due to switching and purging the gas line and instrument. Compensating systems operate similarly to open systems but determine flux by calculating, releasing and recording the amount of a standard gas required to maintain a constant concentration of a given gas. These are useful in that they allow for physiological experiments in tightly controlled conditions but require either complex gas mixing systems or gas canisters mixed to exact specifications.

In closed systems the gas portion of the system (the head space) is constantly recirculated throughout the system in a loop, passing through the gas analyzer(s) in the

process. Consequently, gas concentrations change from their initial levels, forgoing experiments under steady-state gas environments but allowing direct, real-time measurement of changes in CO<sub>2</sub> concentrations due to photosynthesis and respiration (Van et al. 1976). A complex closed-loop system has been described (Birmingham and Colman 1979) as well as systems which combine compensating and closed-loop approaches at different timescales (Červený et al. 2009). Inexpensive open IRGA systems for algal studies are commercially available (Qubit S151, Qubit Systems). The sensitivity of the latter for aquatic use is limited by the slow exchange of dissolved CO<sub>2</sub> with the liquid medium and slow conversion between the different forms of dissolved inorganic carbon: CO<sub>2</sub>, HCO<sub>3</sub> and CO<sub>3</sub><sup>2-</sup>.

Chlorophyll fluorometry is an invaluable tool in the study of plant and algal physiology, operating by measuring the fluorescence of chlorophyll in the presence of excitation light (Maxwell and Johnson 2000). Excitation energy absorbed by chlorophyll may be quenched by plastoquinone at the reaction center (photochemical quenching), quenched by non-photochemical quenching pathways such as the xanthophyll cycle, or released as photons by fluorescence. By comparison of chlorophyll fluorescence under a low-irradiance measuring light and intense actinic light the proportion of absorbed light that passes into the photosynthetic electron transport chain (the quantum yield), as well as many other parameters, may be calculated. Combined with a known amount of absorbed quanta, the rate of linear photosynthetic electron transport may also be estimated. This rate, however, must not be interpreted as rate of photosynthesis, as energy harvested by the antenna complexes has many potential fates other than carbon fixation. Fluorometry measurements are rapid and nondestructive but are insufficient to fully characterize photosynthetic performance.

We describe here an apparatus utilizing a closed-loop design which overcomes many of the obstacles previously outlined by combining a small headspace, rapid pumping and enzymatic enhancement of dissolved inorganic carbon exchange. This apparatus is capable of rapidly and nondestructively measuring the CO<sub>2</sub>, O<sub>2</sub> and fluorometric parameters of algal photosynthesis of a very small quantity (1 mL) of freshwater and marine microalgae.

## **Materials and Procedures**

#### Algal culture

Cultures of the peridinin-containing dinoflagellate *Symbiodinium* were grown axenically in ASP-8A artificial seawater medium (Zahl and McLaughlin 1957) buffered to pH 8.5 with Tris. Dinoflagellates were maintained in 250mL flasks at 26°C and 30 µmol quanta m<sup>-2</sup> s<sup>-1</sup> with a 12h:12h light/dark photoperiod with new medium added twice weekly, as well as the day before experimentation, to maintain a constant cell growth phase. Experiments were conducted at 26°C and 100 µmol quanta m<sup>-2</sup> s<sup>-1</sup>. For measurements utilizing *Chlamydomonas reinhardtii*, cultures were grown in minimal pH 7.0 Tris-buffered TAP medium (Gorman and Levine 1965) in agitated 250 mL flasks at 30 µmol quanta m<sup>-2</sup> s<sup>-1</sup> continuous light. This minimal medium lacks acetate to avoid confounding additional respiration from non-photosynthetic acetate uptake and oxidation. Algal cultures were transferred and diluted into new medium weekly as well as the day before experimentation to ensure that cells were in the logarithmic growth phase and not
nutrient-limited. All experiments were conducted only during the acclimated photoperiod to minimize photoperiodicity effects.

# Apparatus

 $CO_2$  and  $O_2$  flux are determined by measuring the concentrations of  $CO_2$  and  $O_2$ in air bubbled through a vial of microalgal culture in a closed loop (Fig. 2.1). Instrumentation includes an infrared gas analyzer (IRGA, Qubit S151, Qubit Systems) and galvanic gas-phase oxygen electrode (Qubit S102, Qubit Systems). This IRGA unit has a [ $CO_2$ ] precision of  $\pm 2$  ppm and the galvanic  $O_2$  electrode a precision of  $\pm 50$  ppm. A multichannel, variable speed peristaltic pump (Buchler Polystaltic Pump, Buchler Instruments) was used to circulate the gas at a rate that was appropriate for small volumes, approximately 15 mL min<sup>-1</sup> in the measurements reported here.

The gas loop is made entirely of flexible tubing to increase the modularity of the system. Proper selection of tubing is essential, especially the portion of tubing driven by the pump, to minimize both gas permeability and total system volume. We found Viton tubing (1.6 mm internal diameter, 3.2 mm outer diameter, Cole-Parmer Instrument Company) to be best suited for this purpose; conventional silicone tubing was unacceptable due to its high gas permeability. Portions of tubing are joined with barbed polypropylene luer-lock fittings. The algal chamber consists of a 1.8 mL glass sampling vial with a small silicone rubber septum cap mounted on a ring stand with a small three-prong clamp. Two 16-gauge syringe needles, one each for gas input and output, are inserted through the rubber septum, with the output needle inserted only a short distance into the chamber to ensure that it remains above the liquid surface and does not express liquid from the chamber. A short (1-2 cm) length of snugly-fitting transparent tubing is

slipped over the gas input needle to extend it below the liquid surface for adequate bubbling. A gas-impermeable epoxy (J-B Weld, J-B Weld Company) is applied completely over the septum to minimize leakage with the external air. An identically prepared second glass vial is placed downstream in a cold water or ice bath to act as a small-volume condensation trap, as infrared absorption by water vapor can artificially elevate measurements by the IRGA. The condensation trap vial contains 1 mL coarse silica gel desiccant to absorb water vapor and further reduce its the influence on the [CO<sub>2</sub>] measurements as well as minimizing total system headspace.

To measure photosynthetic electron flow a pulse-amplitude modulated (PAM) fluorometer (Heinz Walz GmbH, Effeltrich, Germany) is incorporated into the system by mounting a 1 cm diameter fiber optic probe directly below the sampling vial. Measurements are taken through the flat glass without a significant loss of signal strength and can be automated via the PAM fluorometer software.

### Software

 $CO_2$  and  $O_2$  concentration data are collected digitally via a LabPro C410 interface and LoggerPro software (Vernier Software and Technology, Beaverton, OR). Data points are collected every 15 seconds. PAM fluorometer manipulation and data collection are accomplished via WinControl (Heinz Walz GmbH, Effeltrich, Germany).

## Gas Flux Protocol

Microalgae cultures are centrifuged and resuspended by vortex mixing natural filtered seawater or artificial seawater, buffered with Tris to pH 7.5-8.0. Three 1 mL aliquots of homogenous algal culture are taken per replicate, one each for the experimental sample to be measured, chlorophyll quantification and cell counts. Cell

count aliquots are fixed with one drop 37% formaldehyde and refrigerated for later counting via haemocytometer. 1 mL of the algal suspension is added to the experimental sampling vial and bubbled with ambient air in the dark for 30-45 minutes before beginning measurements, which was determined to be sufficient to equilibrate the dissolved inorganic carbon (DIC) and O<sub>2</sub> concentrations in the gas and liquid phases with the ambient air. This phase also provides the dark acclimation necessary for PAM fluorometry. It is essential that the assay solution be initially equilibrated with atmospheric CO<sub>2</sub> by bubbling with ambient air before closing the loop; without this step, an alkaline buffered medium will absorb CO<sub>2</sub> from the headspace, confounding photosynthetic carbon uptake measurements. Dark-adapted PAM measurements are taken just before beginning gas exchange measurements to obtain initial fluorescence data (F<sub>0</sub>, F<sub>m</sub>) and quantum yield (F<sub>v</sub> / F<sub>m</sub>, F<sub>v</sub> = [F<sub>m</sub> - F<sub>0</sub>] / F<sub>m</sub>).

Measurements begin with turning on the light source and closing the pump loop to isolate the system from the external air. Typically a period of approximately 10 minutes is required before a linear photosynthetic decrease in  $CO_2$  is observed due to liquid/gas phase equilibration lag; these initial 10 minutes are discarded from the dataset. Illumination continues until a satisfactory linear decline is observed, typically an additional 15 minutes. The light source is then turned off and data collection continues until a linear increase of  $CO_2$  due to respiration is achieved, typically within an additional 30 minutes. The non-destructive nature of data acquisition allows for post-experiment collection of the algal cells for further analysis, for example, chlorophyll measurements or  ${}^{13}C/{}^{14}C$  labeling.

# **Calculations**

Rates of carbon fixation can be determined from the rate of removal of  $CO_2$  from the apparatus's headspace. The main inorganic carbon pools in the apparatus are  $CO_2$  in the headspace and  $CO_2$ ,  $HCO_3^-$ , and  $CO_3^{2^-}$  in solution. As proton exchange reactions are extremely rapid,  $HCO_3^-$  and  $CO_3^{2^-}$  are always in equilibrium at time scales relevant to the experiments. Hydration kinetics are slow, however, and  $CO_2$  and  $HCO_3^-$  in solution may be out of equilibrium during photosynthesis. Gas exchange can also be slow, as we found for our apparatus, and so  $CO_2$  in the headspace may not be fully in equilibrium with  $CO_2$ in solution. The following system of differential equations describes the dynamics of  $CO_2$ in the headspace ( $C_{hs}$ ),  $CO_2$  in solution ( $[CO_2]_1$ ), and the sum of  $HCO_3^-$  and  $CO_3^{2^-}$  in solution (termed  $[HCO_3^-]_1$  as it is the most abundant form):

$$\frac{d[CO_2]_l}{dt} = \frac{k_{ex}}{V_l} \stackrel{\mathfrak{g}}{\in} C_{hs} - \frac{[CO_2]_l}{K_0} \stackrel{\mathfrak{o}}{=} - k_f [CO_2]_l + k_r b [HCO_3] - fP_C$$
(2)

$$\frac{d[HCO_3^-]}{dt} = k_f [CO_2]_l - k_r b [HCO_3^-] - (1 - f)P_C$$
(3)

These equations account for gas exchange between the headspace and solution, hydration of  $CO_2$  and dehydration of  $HCO_3^-$  in solution, and removal of  $CO_2$  and  $HCO_3^-$  by photosynthesis (for definitions of terms and their units see Table 2). By using a buffer, such as Tris in these experiments, the pH kept constant and the ratio of  $CO_2 : HCO_3^-$ :  $CO_3^{2-}$  is known. The measurement of  $CO_2$  by the IRGA in combination with this ratio of DIC constituents and the volumes of the headspace and liquid allows for the calculation

of the total inorganic carbon present, both gaseous in the headspace and dissolved in the medium.

Determination of the carbon fixation rate is greatly simplified if the dissolved inorganic carbon species (CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup>) are in equilibrium, which is not necessarily the case in dense algal suspensions (Badger et al. 1994). This can either be achieved by the natural extracellular carbonic anhydrase (CA) present in many microalgae (including *C. reinhardtii*, Colman et al. 1984) or by the addition of CA to the assay solution. In our experiments we added 1.7  $\mu$ M (125 WA units/mL) bovine CA (Sigma) to the assay solution to ensure that CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> were in equilibrium within the assay solution. When in equilibrium, CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> can be treated as a single component, dissolved inorganic carbon (DIC), allowing equations 1-3 to be simplified to:

$$\frac{dC_{hs}}{dt} = \frac{k_{ex}RT}{V_{hs}} \left( \partial DIC - C_{hs} \right) \tag{4}$$

$$\frac{dDIC}{dt} = \frac{k_{ex}}{V_l} \left( C_{hs} - \partial DIC \right) - \frac{P_C}{V_l}$$
(5)

where:

$$\partial = \stackrel{\acute{e}}{\underset{\ddot{e}}{\overset{\&}{e}}} K_0 \stackrel{\&}{\underset{\acute{e}}{\overset{@}{e}}} 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2 \stackrel{\"{o}}{\overset{@}{u}^{-1}}}{[H^+]^2 \stackrel{\div}{\overset{@}{u}}}$$
(6)

The parameter  $\alpha$  converts a DIC concentration to an equilibrium headspace CO<sub>2</sub> pressure. Due to slow gas exchange kinetics, CO<sub>2</sub> in the headspace is not necessarily in equilibrium with CO<sub>2</sub> in solution. This leads to a lag in the response of the headspace CO<sub>2</sub> to photosynthetic carbon consumption in solution, but does not alter the ability to determine the carbon fixation rate from headspace CO<sub>2</sub> removal. The photosynthetic carbon fixation rate can be obtained from the measurement period in which CO<sub>2</sub> removal from the headspace is linear during illumination. The lag in CO<sub>2</sub> drawdown after turning on the light is primarily due to the kinetics of gas exchange, but also possibly entails the progression of Rubisco activation. When a steady rate of CO<sub>2</sub> depletion in the headspace is observed, equation 4 implies that there is a constant difference ( $\Delta$ ) between the pressure of CO<sub>2</sub> in the headspace and the equilibrium pressure of the solution:

$$\mathsf{D} = \left( a D I C - C_{hs} \right) \tag{7}$$

The total C<sub>i</sub> in the system can then be expressed solely as a function of C<sub>hs</sub>:

$$C_{i} = \frac{C_{hs}}{RT} V_{hs} + DIC V_{l} = \frac{C_{hs}}{RT} V_{hs} + \frac{C_{hs} + \mathsf{D}}{\vartheta} V_{l}$$
(8)

The rate of carbon fixation is equal to the rate of  $C_i$  removal from the system, which can be determined by taking the derivative of equation 8 with respect to time:

$$P_{C} = \frac{dC_{i}}{dt} = \mathop{\mathbb{C}}\limits_{\Theta} \frac{V_{hs}}{RT} + \frac{V_{i} \stackrel{\circ}{\ominus} dC_{hs}}{\partial \stackrel{\circ}{\ominus} dt}$$
(9)

The rate of removal of  $CO_2$  from the headspace is obtained from the IRGA data as shown in Figure 2. The volume of the head space (V<sub>hs</sub>) must be precisely calculated using the length and internal diameter of the flexible tubing in addition to the known internal volumes of the IRGA, O<sub>2</sub> electrode, water trap and any drying columns used and the solution volume (V<sub>1</sub>).  $\alpha$  is determined from the measured pH of the buffered solution and the carbon system equilibrium constants can be calculated from formulas in the literature (DOE 1994; Lueker et al. 2000) The respiration rate can be calculated using equation 9, but with the rate of CO<sub>2</sub> increase in the dark substituted for the removal rate in the light. Essentially all of the  $O_2$  in the system is in the headspace, and so photosynthetic  $O_2$  production can be determined directly from the increase in  $O_2$  in the headspace as:

$$P_O = \frac{V_{hs}}{RT} \frac{dO_2}{dt} \tag{10}$$

### Method validation protocol

Algal cultures of both marine (Symbiodinium type D1a A001) and freshwater (*Chlamydomonas reinhardtii* type G137) algal cultures were analyzed to compare accuracy of the apparatus in both seawater and freshwater media. Both algal types were grown under the same conditions as those used for the IRGA gas exchange protocol. Photosynthetic oxygen evolution was measured utilizing membrane inlet mass spectrometry (MIMS) (Table 1). To describe the system briefly, at the base of a small (approx. 2 mL) chamber gases are drawn by vacuum across a Teflon film into a mass spectrometer (QMS 220 M2, Pfeiffer Vacuum GmbH), where they are ionized by a filament. The ions then a pass through a quadruple mass filter to select the ions of interest, which are then detected as currents in a Faraday cup amplified by a secondary electron multiplier (see Beckmann et al. 2009 for further technical details). 1 mL Symbiodinium culture grown in ASP-8A medium was concentrated and resuspended in DIC-free artificial seawater at 26°C and pH 8.0 in a 20 mM Tris buffer, followed by the addition of 2 mM bicarbonate and 20  $\mu$ M acetazolamide, a membrane-impermeable carbonic anhydrase inhibitor. Cells were illuminated at 100  $\mu$ mol quanta m<sup>-2</sup> s<sup>-1</sup> until a linear signal of mass 32 (molecular oxygen) was obtained by the mass spectrometer logging software (Quadera, Pfeiffer Vacuum GmbH). Linear rates of O<sub>2</sub> evolution and consumption, averaged over repeated 3-4 minutes of illumination and darkness, were used to calculate photosynthetic  $O_2$  flux.

To further confirm that carbon fixation values obtained by the infrared gas analyzer were accurate and within the expected range, parallel measurements of carbon fixation were taken using a stable isotope uptake technique. Symbiodinium cells were centrifuged and resuspended in filtered, autoclaved natural seawater labeled with 220µM  $H^{13}CO_3$  (99 atom percent). As the autoclaved seawater had a lower DIC concentration (1.7 mM) than natural seawater, this amounted to 13% rather than 10% enrichment, for a total of 1.92 mM HCO<sub>3</sub>. Cell aliquots were placed in 2 mL sampling vials on a shaker table in the light. After 30 minutes illumination at 100 µmol quanta m<sup>-2</sup> s<sup>-1</sup> samples were centrifuged and the supernatant poured off and frozen. MIMS was then used to determine  $^{13}$ C uptake by quantifying the total HCO<sub>3</sub> remaining in the supernatant. 700  $\mu$ L of the labeled, thawed seawater supernatant was added to the MIMS chamber and background readings of  ${}^{12}$ CO<sub>2</sub> (mass 44) and  ${}^{13}$ CO<sub>2</sub> (mass 45) obtained. The sample was then acidified by the addition of 25  $\mu$ L pH 4.5 citrate buffer to rapidly convert all DIC to CO<sub>2</sub>, which was measured by the mass spectrometer. The increase over background of both masses 44 and 45 was recorded and calibrated with additions of known aliquots of  $H^{12}CO_3$  and  $H^{13}CO_3$  to determine the [DIC] of the supernatant after photosynthetic uptake. As cell cultures were grown under natural isotope conditions, CO<sub>2</sub> released by cellular respiration during the short experimental period would presumably be almost entirely (99%)  $^{12}$ CO<sub>2</sub>. Therefore the difference between  $^{13}$ C in the sample and the stock labeled seawater was multiplied by the <sup>12</sup>C:<sup>13</sup>C ratio of the stock labeled seawater to obtain the gross carbon fixation rate.

### Assessment

The described IRGA/PAM fluorometry apparatus is capable of accurately and easily determining photosynthetic and respiratory rates of microalgae while simultaneously monitoring photosynthetic electron transport (Fig. 2.2). Calculation of gas flux in moles or by mass is possible given knowledge of the headspace and the temperature of the system. Values obtained with this system agree closely with those obtained by the MIMS and <sup>13</sup>C uptake (Table 1). Single factor analysis of variance (ANOVA) revealed no significant difference between the IRGA apparatus data and results from other methods (n = 3 for all methods,  $\alpha = 0.05$ ).

Previous measurements of *Symbiodinium* photosynthesis have varied greatly in their methods (e.g. <sup>14</sup>C labeling, O<sub>2</sub> electrodes), the use of cells in culture or in symbiosis with a wide range of invertebrate hosts, growth and experimental conditions, and in the genetic identity of the algae within the highly diverse *Symbiodinium* genus. Taking this into consideration, our results agree with several previous studies conducted under broadly similar conditions (Chang et al. 1983; Hoegh-Guldberg and Hinde 1986; Bythell et al. 1997; Davy and Cook 2001). A survey of the abundant data available for *Chlamydomonas reinhardtii* reveal rates similar to those obtained here (Table 1). *Problems and Solutions* 

Through the development of this methodology, certain difficulties were identified and required remediation to ensure accurate measurements of photosynthetic and respiratory rates. The tubing material chosen is of vital importance to minimize gas permeability and contamination of the gas stream with external air. Conventional tubing used in peristaltic pumps, such as silicone and Tygon, exhibits excessive gas permeability. We utilized Viton tubing, which is sufficiently durable in the peristaltic pump and exhibits minimal gas permeability when internal  $CO_2$  concentrations are within 250 ppm of ambient. Within these bounds, [ $CO_2$ ] drift due to leakage was less than 1 ppm min<sup>-1</sup> as measured in cell-free seawater. If measurements outside of these bounds are required, blank runs utilizing  $CO_2$ -free or high- $CO_2$  air can determine the leakage rate to correct for gas permeability. The use of other pump systems, such as small diaphragm, impeller or piston pumps with a low flow rate, are potential improvements of the apparatus design we describe here.

The sensitivity of the methodology is limited by the component (IRGA,  $O_2$  electrode, PAM fluorometer) with the lowest sensitivity. In our experience this was the  $O_2$  electrode; replacement with currently available oxygen optode systems may provide better resolution. The biomass required for accurate measurements was as low as 2 x 10<sup>6</sup> cells mL<sup>-1</sup> or 4 µg chl *a* mL<sup>-1</sup>, though greater densities may decrease the time required to obtain linear gas flux rates.

An inherent problem of IRGA instruments is the absorption of infrared light by water vapor; consequently the gas stream must be kept as dry as possible. We incorporated a glass chamber immersed in a water bath, cooled to at least  $10^{\circ}$ C below the ambient temperature, downstream of the algal chamber to serve as a condensation water trap. This also serves as a trap for water droplets inadvertently pumped out of the algal chamber before it reaches the IRGA, potentially damaging the equipment. As the IRGA utilizes an infrared-responsive photodiode, changes in gas density/pressure within the system will influence apparent [CO<sub>2</sub>]; for this reason temperature changes when the tubing is in a closed configuration should be avoided to prevent data artifacts. Additional challenges were posed by the water solubility of the head space gases. Accurate photosynthetic gas flux measurements are dependent upon maintaining equilibrium between the gas and liquid phases. In order to monitor photosynthetic rates it was necessary to ensure that the water was  $CO_2$  and  $O_2$  saturated at the beginning of the experiment via equilibration, requiring that the algae be bubbled with ambient air by opening the loop before beginning measurements. This is especially important to prevent the absorption of  $CO_2$  by the bicarbonate buffering trait of seawater, which is otherwise not distinguishable by the instrumentation from cellular and photosynthetic  $CO_2$ absorption. We found that approximately 45 minutes of bubbling 1 mL algal culture with ambient air was sufficient to avoid any initial, abiotic absorption of  $CO_2$ , and had the additional beneficial effect of standardizing the initial  $CO_2/O_2$  concentrations.

The relatively slow kinetics of CO<sub>2</sub> exchange between the gaseous phase and solution has complicated the application of IRGAs to aquatic systems. In our implementation this issue increases the time required to obtain a steady rate of CO<sub>2</sub> flux and is observed as a lag between the time when the light is turned on and when CO<sub>2</sub> depletion in the headspace begins, or conversely between when the light is turned off and when respiratory CO<sub>2</sub> concentrations begin to increase in the headspace (Fig. 2.2). The kinetics of gas exchange can be quantified empirically by a mass transfer coefficient ( $k_{ex}$ ), which relates differences in CO<sub>2</sub> concentration between the headspace and solution to CO<sub>2</sub> fluxes. By tracking absorption of CO<sub>2</sub> into DIC-free artificial seawater (buffered at pH 9), we estimated the inherent  $k_{ex}$  of the system to be  $3.0 \pm 0.3 \times 10^{-7}$  mol atm<sup>-1</sup> s<sup>-1</sup> (Fig. 2.3), which would give lag times on the order of 20 min. As this delay is too long for practical measurement of photosynthesis we sought ways to increase the mass transfer

coefficient. We first tried adding a metal frit onto the end of the vial tubing to decrease bubble size (and so increase total bubble surface area), but it was difficult to achieve sufficient pressure to drive gas through the frits. The addition of bovine CA  $(1.7 \,\mu\text{M})$ , which was originally added to equilibrate CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in solution, increased the mass transfer coefficient to  $k_{ex}$  to 1.4 x10<sup>-6</sup> mol atm<sup>-1</sup> s<sup>-1</sup> (Fig. 2.3), presumably by increasing the rate of  $CO_2$  hydration within the liquid boundary layer surrounding bubbles (Hoover and Berkshire 1969; Wanninkhof and Knox 1996) (Fig. 2.3). This increase was sufficient to reduce lag times to  $\sim 5 \text{ min}$  (e.g. Fig. 2.2), an acceptable time-scale for the uses of the apparatus described here, but additional increase of the mass transfer coefficient would improve the instrument's performance. The overall speed of the methodology may limit its utility in some applications. Pre-equilibration of the cell medium will eliminate the lengthy 30-45 minute equilibration period after the first run. The relatively inexpensive equipment (save the fluorometer) may make parallel measurements of multiple samples feasible by incorporating multiple  $IRGA/O_2$  electrode arrays while still being cheaper than other methods. The use of up to four parallel gas loops is supported by multichannel peristaltic pumps and the data collection hardware and software.

For microalgae that produce large amounts of mucilage, bubbling may cause a buildup or caking of cells at the gas/liquid interface; siliconizing the interior of the glass vial with a commercially available product (e.g. Rain-Ex<sup>TM</sup>) can mitigate this problem. For microalgae, such as *Symbiodinium*, that settle out of suspension despite agitation by bubbling, a small stir bar can be added to keep cells suspended in a constant light field. We did not notice any significant reduction in the fluorometer signal strength with the addition of a stir bar.

# Discussion

The apparatus described here provides several advantages over traditional methods of measuring photosynthesis in microalgae. Carbon flux rates are quickly, easily and directly measured with minimal sample preparation and no post-measurement processing with the additional benefit of simultaneous oxygen flux measurements and fluorometry. Determination of carbon flux rates, as described here, requires that the pH of the solution be fixed and that dissolved  $CO_2$  and  $HCO_3^-$  be in equilibrium. This greatly simplifies the calculations and reduces the number of system parameters that must be determined, but also means that carbon concentrations in the system are changing during the measurement. With the precision of the  $CO_2$  measurement instrumentation (±1 ppm) the extent of change in the carbon system can be kept small, and we did not observed any significant non-linearity in  $CO_2$  drawdown, which would be expected if the changing carbon concentrations were altering photosynthesis. As the system is software-controlled and can be automated, a complete suite of data can be obtained without user intervention once the sample is prepared and the gas loop is closed.

The measurement of oxygen evolution is fundamentally the measurement of a byproduct of photosynthesis, rather than the carbon that is fixed into sugars and other photosynthates. Inferring carbon fixation rates from oxygen evolution measurements requires the use of photosynthetic quotients, which are difficult to confirm, and may unknowingly vary with treatments and algal species. Simultaneous monitoring of oxygen evolution and carbon fixation in real time allows for the discrimination of processes that can modulate the apparent rates of carbon/oxygen flux, such as photorespiration, chlororespiration and cyclic electron transport. For example, a substantial amount of cyclic electron transport will diminish the oxygen evolution rate during illumination but can sustain a degree of  $CO_2$  fixation and respiratory  $CO_2$  evolution.

As the cells analyzed are not destructively sampled by the apparatus, further analyses of the same cell aliquots are possible, unlike conventional stable isotope wholecell combustion or extraction analyses. For example, the combination of the described methods with standard carbon isotope labeling followed by cell lysis and gas chromatography/mass spectrometry can provide a comprehensive dataset of photosynthetic performance and fixed carbon fate. The small size and relatively rapid handling time of our apparatus, including rapid data analysis, allows for the analysis of a large number of different algal samples and treatments. The handling time is comparable to that of other real-time approaches (e.g. MIMS,  $O_2$  electrodes), and while large numbers of replicate samples cannot be analyzed in parallel as with carbon isotope analyses, the per-sample costs are insignificant. Large-scale photobioreactors are have been previously described (Červený et al. 2009) and are commercially available, particularly for algal biomass production research. These systems are useful but are typically used for dense freshwater algal cultures grown at elevated  $pCO_2$ . They are also greatly more complex than our apparatus and must be devoted to the study of a single large-batch (often 0.5L to more than 10L) culture for long periods of time. Relatively few studies have measured photosynthesis of such small ( $\leq 1$ mL) volumes (Lewis and Smith 1983; Johnson and Sheldon 2007), which increases the applicability of the method. Miniaturization, in combination with increased IRGA sensitivity, seems to have improved the accuracy of the IRGA technique versus earlier works and is ideal for use with microalgae, and future improvements in miniaturization would greatly improve the

data collection and tractability of this apparatus. Custom manufacturing of the chamber to better facilitate mixing of the gas and liquid phases and miniaturization of the head space/gas loop would increase the response time of the unit.

The approach described here to determine photosynthetic rates from headspace  $CO_2$  requires that the pH be constant, which is achieved by the addition of a buffer, and that dissolved  $CO_2$  be in equilibrium with  $HCO_3^-$ , which is ensured by the addition of carbonic anhydrase. While these requirements may influence the photosynthetic performance of algae under some circumstances, they should not generally affect photosynthetic rates. Commonly employed non-toxic buffers such as Tris will not affect photosynthesis in most species, and are in fact used in the media for the two species tested here. If the effects of a particular buffer are not known, rates of photosynthesis can be measured from O<sub>2</sub> production with and without buffer to test its effect on photosynthesis since the buffer is required primarily for the analysis of the  $CO_2$  data. In principle the system can operate at any desired pH, so long as it is known and constant. In practice, however, greater sensitivity is achieved at lower pH, where the change in headspace  $pCO_2$  is greater per unit photosynthesis. The addition of CA ensures that  $CO_2$ and  $HCO_3^{-}$  are in equilibrium in the solution. Under most environmental and normal culture conditions these chemical species are in equilibrium and so the addition of CA should not alter the algae's inorganic carbon acquisition processes (e.g. carbon concentrating mechanisms) or photosynthetic performance. Only at very high cell densities or very high pH (e.g. 9-10) do photosynthetic rates approach the rates of spontaneous CO<sub>2</sub> generation from HCO<sub>3</sub><sup>-</sup> leading to disequilibrium (e.g. Miller and Colman 1980). The cell densities needed to obtain rapid measurements on the apparatus

are fairly high and could result in  $CO_2$ -HCO<sub>3</sub><sup>-</sup> disequilibrium (Badger et al. 1994), necessitating the use of CA, but maintaining conditions more similar to culture or environmental conditions.

The utility of the methodology and instrumentation presented here is wideranging. A primary advantage of this methodology is the ability to measure carbon fixation in real-time, analogous to and in combination with the oxygen evolution methods which have been in use for decades. The completely non-destructive nature of the methodology allows for its use with additional analyses of cell physiology, such as lipid or pigment content. As examples, it can (and should) be employed for rapid screening of algal strains with enhanced photosynthetic efficiencies in the pursuit of renewable biofuel sources. At the basic research level, it easily affords studies of environmental influences on photosynthesis that include those of heat, cold, salinity, acidity and nutrientdeficiency, toxic metal stresses as well as irradiance impacts and diurnal/circadian rhythms. Likewise, herbicide/algaecide effectiveness can be easily assessed as can as the extent to which cyclic and alternative electron transport pathways support physiological functionality. Even in the absence of the fluorometry component, respiratory and, particularly, fermentation activities of microbes such as yeasts and other fungi can lead to identification of strains with promise in biofuel generation. The system's dimensions can also be expanded to afford exploration of photosynthesis and respiration with larger organisms such as corals, macroalgae, or sponges. Because of the simplicity, affordability and reliability of the apparatus, all of the above can be easily adapted for laboratory courses in public schools and colleges. Lastly, an important medical application conceivably could be used to assess malignancies as part of initial and rapid biopsy

characterization of tumors that typically exhibit unusually high respiratory rates compared to benign tissues.



Figure 2.1. Diagram of the described apparatus. Gas flow direction indicated by arrows.

![](_page_54_Figure_0.jpeg)

Figure 2.2. Demonstration IRGA,  $O_2$  electrode and PAM fluorometer data from photosynthesis of *Symbiodinium* (clade C type) at 26°C and 100 µmole quanta m<sup>-2</sup> s<sup>-1</sup>. Light was turned off at the dashed line. Both raw and smoothed  $O_2$  data are shown.

![](_page_55_Figure_0.jpeg)

Figure 2.3. Effect of carbonic anhydrase (CA) on CO<sub>2</sub> mass transfer in the IRGA-based apparatus. The data show the absorption of CO<sub>2</sub> into cell- and DIC-free artificial seawater in representative runs with and without CA. The CO<sub>2</sub> measurements have been normalized to their initial values (C<sub>hs</sub>(0)) and plotted logarithmically, as the absorption should be exponential. Linear fits through the data are shown and in these examples  $k_{ex} = 2.8 \times 10^{-7}$  mol atm<sup>-1</sup> s<sup>-1</sup> without CA and 1.3 × 10<sup>-6</sup> mol atm<sup>-1</sup> s<sup>-1</sup> with CA.

Table 2.1. Comparison of C fixation and O<sub>2</sub> evolution rates obtained by the described apparatus, <sup>13</sup>C uptake and membrane inlet mass spectrometry (MIMS) methods. Data previously reported in the literature included for comparison. Single-factor ANOVA of all datasets reported here were not significant (p < 0.05). For all measurements n=3, standard error followed by p-value (where applicable) in parentheses.

Symbiodinium D1a A001				
	Apparatus	<sup>13</sup> C Uptake	Literature	
	nmol $O_2 10^6$ cells <sup>-1</sup> min <sup>-1</sup>			
Total / Gross C Fixation	3.37	3.46	3.06 (Engebretson and Muller-	
	(0.49, p = 0.087)	(0.36)	Parker 1999)	
			7.42 (McCloskey and Muscatine	
			1984)	
			2.33-7.63 (Chang et al. 1983)	
			3.61-14.58 (McBride et al. 2009)	
Net C Fixation	2.29	-	-	
	(0.2)			
C Respiration	1.09	-	-	
	(0.49)	MDAG	<b>T</b> •4	
	Apparatus			
Carrier O. Freehotter	2.72	$10^{\circ}$ cells	min	
Gross O <sub>2</sub> Evolution	2.73	2.24	2.81 (Karako-Lampert et al.	
Not O Englisting	(0.70, p = 0.033)	(0.21)	2003)	
Net O <sub>2</sub> Evolution	1.04	1.55	2.10 (Dythen et al. 1997) 2.83.3.83 (Doopo and Brion	
	(0.00, p = 0.443)	(0.17)	2.85-5.85 (Dealle and Brief)	
			1 67-10 83 (Karako-Lampert et	
			al 2005)	
O <sub>2</sub> Respiration	1.09	0.71	0.65 (Bythell et al. 1997)	
- 2 F	(0.11, p = 0.058)	(0.04)		
Chlamydomonas reinhardtii type G137				
	Apparatus		Literature	
	μmol C mg <sup>-1</sup>	Chl $a$ hr <sup>-1</sup>	$\mu$ mol O <sub>2</sub> mg <sup>-1</sup> Chl <i>a</i> hr <sup>-1</sup>	
Total / Gross Photosynthesis	174	-	-	
	(8.6)			
Net Photosynthesis	135	280 (Klein 1987)	55 (Lloyd et al. 1977)	
	(4.2)	47.4 (Levine and	90-98 (Goyal and Tolbert 1990)	
		Togasaki 1965)	180-220 (Coleman et al. 1984)	
			185 (Garbayo et al. 2005)	
Dark Respiration	38.8	-	-	
	(5.5)			

Symbol	Definition	Units
P <sub>C</sub>	photosynthetic carbon fixation rate	mol s <sup>-1</sup>
Po	photosynthetic oxygen production rate	mol s <sup>-1</sup>
C <sub>hs</sub>	CO <sub>2</sub> in the headspace	atm
$O_2$	O <sub>2</sub> in the headspace	atm
[CO <sub>2</sub> ] <sub>1</sub>	CO <sub>2</sub> in solution (liquid)	mol L <sup>-1</sup>
[HCO <sub>3</sub> <sup>-</sup> ] <sub>1</sub>	$HCO_3^- + CO_3^{2-}$ in solution	$mol L^{-1}$
DIC	dissolved inorganic carbon $(CO_2 + HCO_3^- + CO_3^{2-})$	$mol L^{-1}$
Ci	sum of all inorganic carbon species	mol
$V_{hs}$	volume of headspace	L
$\mathbf{V}_1$	volume of solution	L
k <sub>ex</sub>	mass transfer coefficient	mol s <sup>-1</sup> atm <sup>-1</sup>
$\mathbf{k}_{\mathbf{f}}$	CO <sub>2</sub> hydration rate constant	/s
k <sub>r</sub>	HCO <sub>3</sub> <sup>-</sup> dehydration rate constant	/s
R	gas constant	atm L K <sup>-1</sup> mol <sup>-1</sup>
Т	Temperature	Κ
$K_0$	Henry's law constant for CO <sub>2</sub>	$mol L^{-1} atm^{-1}$
Р	photosynthetic rate	mol s <sup>-1</sup>
F	fraction of photosynthesis supported by CO <sub>2</sub>	
В	uptake fraction of $[HCO_3^-]_1$ term that is $HCO_3^-$	

Table 2.2. Definition of terms in equations used to determine carbon fixation and oxygen production rates.

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# CHAPTER 3

# THERMAL RESPONSES OF SYMBIODINIUM PHOTOSYNTHETIC CARBON

ASSIMILATION<sup>2</sup>

<sup>&</sup>lt;sup>2</sup>Submitted to *Limnology and Oceanography*, 04/10/2013.

# Abstract

The symbiosis between hermatypic corals and their dinoflagellate endosymbionts, genus *Symbiodinium*, is based on carbon exchange. This symbiosis is disrupted by thermally-induced coral bleaching, a stress response in which the coral host expels its algal symbionts as they become physiologically impaired. The disruption of dissolved inorganic carbon (DIC) supply or the thermal inactivation of Rubisco have been proposed as sites of initial thermal damage that lead to the bleaching response. Symbiodinium possesses a highly unusual Form II ribulose bisphosphate carboxylase/oxygenase (Rubisco), which exhibits a lower  $CO_2:O_2$  specificity and may be more thermally unstable than the Form I Rubiscos of other algae and land plants. Components of the  $CO_2$ concentrating mechanism (CCM), which supplies inorganic carbon for photosynthesis, may also be temperature sensitive. Here, we examine the ability of four cultured Symbiodinium strains to acquire and fix DIC across a temperature gradient. Surprisingly, the half-saturation constant of photosynthesis with respect to DIC concentration ( $K_p$ ), an index of CCM function, declined with increasing temperature in all strains, indicating a greater potential for photosynthetic carbon acquisition at elevated temperatures. Gross photosynthesis increased with temperature, but respiration rates increased as well, leaving net photosynthesis largely unaffected by temperature. Finding no evidence for thermal inhibition of the CCM or Rubisco we conclude that these components are not likely to be the primary sites of thermal damage. Reduced photosynthetic quantum yields, a hallmark of thermal bleaching, were observed at low DIC concentrations, leaving open the possibility that reduced inorganic carbon availability is involved in bleaching.

# Introduction

The mutualism between corals and their dinoflagellate algal symbionts, genus *Symbiodinium*, is characterized by carbon exchange dynamics. Corals supply their symbionts with inorganic carbon from the water column (principally  $HCO_3^-$ ) and  $CO_2$  derived from respiration, which is photosynthetically fixed by the dinoflagellates and translocated as organic carbon to the coral. The carbon fixed by symbiotic algae can supply up to 95% of the metabolic needs of the coral (Porter and Muscatine 1977).

As algal symbionts exist within specialized vacuoles (symbiosomes) within coral cells, the animal mediates inorganic carbon flux into the coral tissue for photosynthetic carbon fixation as well as extracellular calcification (Al Moghrabi et al. 1996; Furla et al. 2000). Dissolved inorganic carbon in seawater exists primarily as  $HCO_3^-$ , with only approximately 1% present as  $CO_2$  at pH 8.1. As lipid membranes are inherently impermeable to negatively-charged  $HCO_3^-$  but are highly permeable to  $CO_2$ , carbon concentrating mechanisms (CCMs) are common among marine algae to maximize  $CO_2$  availability to Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). CCMs have been demonstrated to be present in *Symbiodinium* (Leggat et al. 1999), although the exact mechanism and identity of the components involved has not been established. Typically, free-living microalgal CCMs function by pumping  $HCO_3^-$  across the plasma and chloroplast membrane to build up a  $HCO_3^-$  pool in the chloroplast, which is then converted to  $CO_2$  by carbonic anhydrase (Moroney and Ynalvez 2007; Reinfelder 2011).

Rubisco catalyzes the primary step of carbon fixation by nearly all photosynthetic organisms and is expressed at very high concentrations in photosynthetic cells. Its high cellular abundance is due in part to inefficiencies of the enzyme that result from poor

discrimination between its small, linear, uncharged substrates, O<sub>2</sub> and CO<sub>2</sub> (Tcherkez et al. 2006). The peridinin-containing dinoflagellates, including Symbiodinium, are unique among eukaryotes in utilizing a Form II Rubisco for carbon fixation (Whitney et al. 1995). The Form I Rubiscos of land plants, algae and many prokaryotes display sequence variation but are universally characterized by a complex of eight large subunits and eight small subunits. The Form II Rubiscos of some dinoflagellates are derived from an alphaproteobacterium and exist as a homodimer of large subunits. In vitro studies of Form II Rubisco have been limited by the extreme instability of the enzyme, which shows complete inactivation in minutes at room temperature (Whitney et al. 1995; Whitney and Andrews 1998). These studies also indicate that the dinoflagellate Rubisco is especially poor at discriminating  $CO_2$  from  $O_2$ , like other Form II Rubiscos, despite the fact that illuminated coral tissue experience be extremely hyperoxic (Kuhl et al. 1995). Consequently, photorespiration rates would be very high without a CCM to maximize  $CO_2$  concentrations around the Rubisco enzyme. High temperature further reduces the CO<sub>2</sub>/O<sub>2</sub> specificity of Form I Rubisco (Brooks and Farquhar 1985), raising the possibility of thermal sensitivity of carbon fixation. The effects of high temperatures on the  $CO_2/O_2$ specificity of low-specificity Rubiscos (such as Form II) may be less pronounced, however (Tcherkez et al. 2006).

Anthropogenic  $CO_2$  release is predicted to result in lower oceanic pH and higher average sea surface temperatures over the next century (IPCC 2007). High sea surface temperature events have resulted in coral bleaching, a stress response where symbiont densities plummet as they die or are expelled by the host. There is strong evidence that disruption of photosynthesis is involved in bleaching, but the trigger for thermal damage is controversial. Hypotheses include direct damage to PSII (Warner et al. 1999) increased reactive oxygen species generation (Lesser 1997), and indirect damage to the photosynthetic apparatus resulting from decreased CO<sub>2</sub> fixation (Jones et al. 1998). These mechanisms are not mutually exclusive and may amplify one another. Work in green algal systems (Takahashi and Murata 2008) has demonstrated that Calvin cycle inhibition results in increased ROS production, inhibits the repair of PSII, and accelerates PSII degradation. Reductions in CO<sub>2</sub> fixation may result from either damage to Rubisco or reduced CO<sub>2</sub> supply. The inherent instability of Form II Rubisco and its susceptibility to photorespiration has lead to the proposal that it may be the site of initial thermal damage (Jones et al. 1998). Alternately, damage to the CCM could reduce inorganic carbon supply limiting Rubisco's ability to fix CO<sub>2</sub>. In either case, lowered rates of Calvin cycle activity could then lead to an accumulation of electrons in the photosynthetic apparatus and, ultimately, photodamage (Warner et al. 1999).

Here, we analyzed if thermal stress directly affects the amount of dissolved inorganic carbon required to saturate photosynthesis of cultured *Symbiodinium*, and whether carbon limitation, particularly at higher temperatures, results in harmful feedback effects on photosynthetic electron transport.

#### Methods

# Culture

Cultures were chosen across a broad range of *Symbiodinium* genotypic diversity and proposed thermal tolerance differences, and included the following: PK704 = S. *minutum* LaJeunesse (LaJeunesse et al. 2012); A001 = S. *'trenchi'* (*sensu* LaJeunesse et al. 2005); RT74, an undescribed member of clade B; and Mf 10.4, an undescribed member of clade A originally isolated from the Caribbean coral *Montastraea faveolata*. RT74 and PK704 are representatives of what are likely distinct species within the major B1 ITS radiation (J. Parkinson, pers. comm.), and A001 is a representative of ITS type D1a.

Symbiodinium cultures were maintained in 500 mL flasks in ASP-8A medium (Zahl and McLaughlin 1957) at 30  $\mu$ mol quanta m<sup>-2</sup> s<sup>-1</sup> with a 12 h photoperiod. The culture medium was refreshed twice weekly as well as the day before experimentation to ensure a logarithmic cell growth phase and to minimize nutrient limitation. The DIC concentration of these culture flasks was measured to be 570 ± 50  $\mu$ M using membrane inlet mass spectrometry (see below). Cultures were maintained at 26°C for the 26°C and 22°C treatments and 30°C for the 30°C and 34°C treatments for a minimum of one week prior to experimentation to allow for temperature acclimation. These temperatures were chosen to span typical natural ranges (22, 26, 30 °C) and to induce high temperature stress (34 °C). Experiments were conducted during the light phase of the acclimated photoperiod to avoid photoperiodicity effects. Cell quantity and peak diameter were measured with a cell counter (Coulter Z2, Beckman Coulter). Cells were assumed to be spherical for cell volume calculations.

Photosynthesis vs. DIC

Photosynthesis and respiration were measured in sequential light-dark phases as a function of DIC concentration using membrane inlet mass spectrometry (MIMS). The MIMS system consists of a membrane inlet chamber, which accommodates the algal suspensions, interfaced to a mass spectrometer (Pfeiffer QMS 220) for measurement of dissolved gases in the suspension. At the base of the water-jacketed chamber is a Teflon membrane through which gases diffuse into the mass spectrometer.

Cell cultures were concentrated by centrifugation and resuspended in 1 mL artificial seawater (ASW) lacking added DIC and buffered to pH 8.00 ±0.05 with 20 mM Tris. pH was measured on the total hydrogen ion scale (pH<sub>T</sub>) using thymol blue (Zhang and Byrne 1996). Background DIC concentrations in the ASW were measured to be 50  $\mu$ M using MIMS. The 1 mL cell suspension was placed in the MIMS chamber and kept in suspension with a small stir bar. Acetazolamide (50  $\mu$ M), a membrane impermeable carbonic anhydrase inhibitor, was added to avoid the confounding effects of external carbonic anhydrases on CO<sub>2</sub> uptake. A cap, featuring a narrow bore to allow DIC addition via syringe, was then placed on the MIMS chamber to minimize gas exchange.

To determine photosynthesis vs. DIC curves, photosynthetic measurements were conducted with cells exposed to alternating light/dark periods of 4-5 minutes after an initial dark adaptation period of 15 minutes (Fig. 3.1). The irradiance in the chamber was approximately 200 µmol quanta m<sup>-2</sup> s<sup>-1</sup> as measured using a  $4\pi$  photosynthetically active radiation (PAR) meter (ULM-500, Heinz Walz Gmbh.). H<sup>13</sup>CO<sub>3</sub><sup>-</sup> was incrementally added during each dark period (n = 9-10) to a final concentration at least that of ambient seawater [DIC] (2.2mM). Gas concentrations (O<sub>2</sub>, <sup>13</sup>CO<sub>2</sub>) and fluorometric measurements (see below) were made continuously during the experiments; total run time for each experiment was approximately 2 hr. Gross photosynthesis was calculated by subtracting the linear respiratory O<sub>2</sub> uptake rate in the dark from the linear O<sub>2</sub> evolution rate during the following photoperiod. A minimum of three experiments were conducted for each treatment. Gross photosynthetic rates vs. [DIC] from each individual experiment were fit to a single-ligand Michaelis-Menten saturation curve using the SigmaPlot 10 Pharmacology Ligand Bonding Module (Systat Software Inc., San Jose, CA, USA) and the calculated K<sub>p</sub> values averaged.

# Chlorophyll Fluorometry

Kinetics of chlorophyll fluorescence were measured using a Fluorescence Induction and Relaxation (FIRe) fluorometer (Satlantic LP, Halifax, NS, Canada) via a fiber optic probe, with gain adjusted so that the maximum fluorescence signal was approximately 80% of instrument saturation. The measurement protocol included both a single and multi-turnover phase. In the single turnover measurement, the sample was illuminated with actinic blue light (output peak ~450 nm) continuously for 150 µs to close all PSII reaction centers and saturate fluorescence. The relaxation of fluorescence was then monitored with pulsed light over 135 ms. The multi-turnover phase then began immediately in which continuous actinic light was applied for 100 ms to close PSII reaction centers and reduce the electron transport chain. Subsequently fluorescence relaxation was monitored for 450 ms. Two replicate measurements were made and averaged after which acquisitions were delayed for 1.5 s and then reinitiated. The data were processed using a custom Matlab script fitting the Kolber et al. model (Kolber et al. 1998) of variable fluorescence to the single turnover phase to calculate the initial fluorescence ( $F_o$ ), the maximal fluorescence during the single turnover phase ( $F_{m-ST}$ ), the effective absorption cross section of PSII ( $\sigma$ ), and the turnover time of Q<sub>A</sub> ( $\tau$ <sub>QA</sub>). The multi-turnover phase was analyzed to determine the maximal fluorescence ( $F_{m-MT}$ ), from which the effective quantum yield of PSII was calculated ( $\Phi_{PSII} = (F_{m-MT}-F_o)/F_{m-MT}$ ), and a single exponential decay was fit to the relaxation data to determine the turnover time of the plastoquinone pool ( $\tau_{PQ}$ ). The excitation pressure over photosystem II ( $Q_m$ ) was calculated as  $Q_m = 1 - ([\Delta F/F_m'] / [F_v/F_m])$  (Iglesias-Prieto et al. 2004).  $\Delta F/F_m$  values for  $Q_m$  calculations obtained by averaging across the dark period (3-4 min. duration) in between each light period to allow for short-term dark acclimation. Relative rates of electron transport (rETR) were estimated as rETR =  $E \cdot \sigma \cdot \Phi_{PSII}$ , where E is the light intensity (Kolber and Falkowski 1993).

## Results

Gross photosynthesis increased from 22 to 34 °C by 31-41% in all strains except RT74 where it decreased by 19% (Fig. 3.2). Respiration increased continuously with temperature in all strains. The increased respiration at high temperature largely offset gains in gross photosynthesis leaving net photosynthetic rates approximately constant in most strains. The largest change in net photosynthesis was a 35% increase from 22-34 °C observed in PK704, while rates in RT74 and Mf 10.4 declined slightly (28%), and rates in D1a A001 showed no changes. (Fig. 3.2a,c). Net photosynthesis to respiration (P:R) ratios declined with temperature elevation in all phylotypes except PK704 but remained above 1 at all temperatures (Fig. 3.2d).

The half-saturation constant of gross photosynthesis for DIC (K<sub>P</sub>) declined with increasing temperature in *Symbiodinium* types RT74, Mf 10.4 and D1a A001. (Fig. 3.3). In PK704, K<sub>P</sub> showed no statistically significant trend with temperature between 22 and 34 °C (p > 0.05, single-factor ANOVA), but there was some indication of a slight decline with increasing temperature. There was substantial variation in K<sub>P</sub> between cell types at low temperature (360 – 660  $\mu$ M at 22 °C), but all types converged to minimal values of

approximately 160-220  $\mu$ M at 34 °C, except PK704, which exhibited a significantly higher minimum K<sub>P</sub> (500  $\mu$ M).

The effective quantum yield of photosystem II ( $\Phi_{PSII}$ ), as calculated from multiple-turnover measurements under illumination, showed a positive response to increasing [DIC] (Fig. 3.4). DIC limitation depressed  $\Phi_{PSII}$  values of all cell types, though this response was more difficult to detect in D1a A001 where the maximal  $\Phi_{PSII}$  at high DIC was low relative to other cell types. The interaction between [DIC] and  $\Phi_{PSII}$ declined with increasing temperatures, however, and there was little to no response of  $\Phi_{PSII}$  to [DIC] at the highest temperature treatments in Symbiodinium RT74, Mf 10.4 and D1a A001 (Fig. 3.4).  $\Phi_{PSII}$  was the major governor of electron transport (rETR), such that the behavior of rETR as a function of DIC is similar to that of  $\Phi_{PSII}$ . The half-saturation constant of the relative electron transport rate exhibited a similar trend to the K<sub>p</sub> of photosynthetic oxygen evolution (Fig. 3.5). Increasing temperatures to  $34^{\circ}$ C raised the rates of rETR at low DIC, resulting in a surprising decline in K<sub>ETR</sub> to nearly 0 in some cell types. At low temperature, rETR closely tracked photosynthetic oxygen evolution as DIC was increased, but at higher temperature rETR remained high at low DIC (except in PK704) suggesting that substantial cyclic electron transport may have been induced (Fig. 3.6).

Excitation pressure on photosystem II ( $Q_m$ ) was relieved by DIC addition as expected (Fig. 3.7). The  $Q_m$  term describes the ability of illuminated PSII reaction centers to recover from photoinhibition after a very brief (< 5 min) dark acclimation (Iglesias-Prieto et al. 2004), with high values indicating slow de-excitation of PSII and photoinhibition. Baseline  $Q_m$  values appeared to vary by *Symbiodinium* type, as did the relative response of  $Q_m$  to DIC.  $Q_m$  response to DIC was generally highest at 22°C and 26°C (Fig. 3.7). At 34°C, DIC appeared to have little effect on  $Q_m$ ; the gradual increase in  $Q_m$  in Mf10.4 at 34°C is interpreted as photoinhibition developing over time.

The turnover time of the plastoquinone pool ( $\tau_{PQ}$ ) was affected by DIC availability as shown when  $\tau_{PQ}$  is plotted vs.  $\Phi_{PSII}$  (Fig. 3.8), which is itself primarily controlled by DIC in this data set (Fig. 3.4). At low  $\Phi_{PSII}$  (low DIC),  $\tau_{PQ}$  increases substantially at low temperatures, and while this effect is seen at higher temperatures the response is muted in all strains except PK704. Nonphotochemical quenching (NPQ) was strongly induced by DIC limitation in all types except D1a A001, but quickly declined as DIC saturated photosynthesis (Fig. 3.9). The time scales of the light-dark cycles (~10 min) between DIC additions are comparable to typical time scales for primary NPQ acclimation (Muller et al. 2001). Therefore, NPQ should be nearing an equilibrium value at each DIC level as is suggested by the fact that the NPQ decline mirrors increases in photosynthesis in several strains. Rather than increasing at high temperatures as expected, NPQ was unaffected by (RT 74, Mf 10.4) or decreased (D1a A001, PK 704) with increasing temperature (Fig. 3.9).

### Discussion

There is strong evidence that thermal bleaching of corals results from disruption of photosynthesis in the zooxanthellae symbionts (Lesser 1997; Warner et al. 1999; Hoegh-Guldberg et al. 2007), though the primary site of damage remains in question. One hypothesis is that high temperatures inhibit  $CO_2$  fixation, either through inactivation of Rubisco or impairment of the CCM, causing a backup in the photosynthetic electron
transport chain, which increases oxidative stress and damages photosynthetic proteins (Jones et al. 1998; Wooldridge and Done 2009). CO<sub>2</sub> is scarce in hospite as shown by the induction of the algal CCM in zooxanthellae, the high concentrations of DIC required to saturate photosynthesis in corals, and, in some cases, DIC limitation of photosynthesis at ambient inorganic carbon concentrations (Goiran et al. 1996; Leggat et al. 1999; Herfort et al. 2008). Any disturbance of inorganic carbon delivery pathways in the coral or zooxanthellae could easily result in limitation of carbon fixation rates. To investigate the potential role of the CCM and carbon fixation in thermal bleaching, we assessed the effect of temperature on photosynthetic carbon requirements in four strains of *Symbiodinium*. These strains were chosen to span a portion of the breadth of *Symbiodinium* diversity, including types from clades A (Mf 10.4), B (RT74, PK704), and D (D1a A001), but cannot be assumed to be representative of all members of their respective clades.

Rather than inhibiting carbon acquisition, temperature enhanced the capacity of *Symbiodinium* to take up inorganic carbon, including at temperatures above the observed bleaching thresholds for most reefs (29 - 31 °C; Hoegh-Guldberg 1999; Kleypas et al. 1999). At low temperatures, the DIC half-saturation constant of photosynthesis ( $K_P$ ) was high, up to 600  $\mu$ M, despite cells being grown under low DIC conditions (pH 8.5; DIC 570  $\mu$ M) to ensure that the CCM was fully induced. The high CO<sub>2</sub> requirements of Form II Rubisco may contribute to the high half-saturation constants at low temperatures, but at higher temperatures the K<sub>P</sub> declined to levels below 200  $\mu$ M, a value more similar to other microalgae (Badger et al. 1994; Rost et al. 2003). The response of the CCM was

smooth and continuous to temperature despite the shorter acclimation times at 22 °C and 34 °C, suggesting that CCM enhancement was rapid.

The mechanism responsible for the negative relationship between DIC  $K_P$  and temperature is not immediately clear. One possible explanation for the decline in  $K_m$  with temperature is a direct thermal enhancement of inorganic carbon transport enzymes or efficiency. Unfortunately, there is little literature evaluating the effects of temperature on carbon concentrating mechanisms in algae with which to compare our results or to offer more detailed insight into the factors increasing CCM efficacy at higher temperatures. The substantial cyclic electron transport (CET) or pseudocyclic electron transport (pCET)/Mehler reaction at high temperature, implied by the decoupling between rETR and photosynthesis at low DIC, could be used to increase the operation of the CCM. While not a mechanistic explanation for the response, it does offer a potential compensating energy source to support CCM activity. Strain PK704, however, does not show any evidence of pCET yet still exhibits a lower  $K_p$  at high temperature, indicating that pCET is not necessarily required for the increased DIC affinity.

A potential mechanism contributing to the thermal response of  $K_p$  is increased respiration and re-fixation of CO<sub>2</sub> driven by metabolic  $Q_{10}$  effects. The  $Q_{10}$  factor describes the relative increase in activity to a temperature increase of 10 °C. The  $Q_{10}$  of corals is approximately 2 (Porter and Muscatine 1977), meaning that the respiration rate doubles as the temperature goes up 10°C, similar to our results. This "recycled" DIC may be fixed before leaving the cell, manifesting as a reduction in the apparent  $K_p$ . That the ratio of gross photosynthesis to respiration always remained greater than 1 (Fig. 3.2d), however, suggests that even at high temperatures the  $CO_2$  made available by increased respiration is insufficient to completely account for the increased DIC affinity.

Regardless of the mechanism, the increased affinity of *Symbiodinium* for DIC with temperature suggests that neither the CCM nor Rubisco are thermally impaired at temperatures that induce bleaching. Continued exposure to 34 °C would likely negatively affect photosynthesis, but exposures were kept short in order to see primary effects of temperature stress on the CCM, rather than secondary effects related to general metabolic decline. The half-saturation constant of photosynthesis for DIC  $(K_p)$  can be taken as a fairly direct measurement of CCM performance. This parameter is correlated with overall CCM activity including carbonic anhydrase activity, HCO<sub>3</sub><sup>-</sup> uptake, and accumulation of an inorganic carbon pool in organisms ranging from cyanobacteria to green algae to diatoms (Kaplan et al. 1980; Rost et al. 2003; Vance and Spalding 2005). The consistent reduction in K<sub>p</sub> clearly shows the CCM is more active, rather than impaired, at higher temperatures (Fig. 3.3). The only scenario in which this may be misleading is if the maximal photosynthetic rate decreases at high temperature, in which case a constant CCM activity would still result in a decrease in K<sub>p</sub>. The maximal gross photosynthetic rate, however, stays roughly constant or increases slightly with increasing temperature (Fig. 3.2). If Rubisco were thermally inhibited, as has been suggested, its half-saturation constant may increase, leading to an increase in whole-cell K<sub>p</sub> unless the CCM compensates. Complete denaturing of a portion of the Rubisco protein would result in a decline in the maximal photosynthetic rate. Neither of these responses were observed, suggesting Rubisco was not thermally inhibited, though the cellular affinity for DIC offers only indirect information on Rubisco performance.

Our data contrast with those of Leggat et al. (2004), who found no effect of temperature on the K<sub>p</sub> of Symbiodinium isolated from the giant clam Tridacna gigas across a temperature range from 28 °C-37 °C. Zooxanthellae of giant clams exist within sinuses of the mantle tissue bathed in haemolymph, not endosymbiotically (within cell vacuoles). Thus the zooxanthellae of clams likely experience a very different microhabitat from those of coral, with potentially large differences in the O<sub>2</sub> and DIC concentrations, a lower pH and an increased distance from the site of calcification. Differences in experimental protocol may also explain the discrepancy. Symbiodinium cells in that study were used less than 24 hours after isolation and were temperature acclimated for 1.5 hours. This short time frame, combined with the potential stress of isolation from the host tissue, may limit the capacity of the cells to induce a physiological response to a changed temperature regime. Cells in our work were exposed to low DIC concentrations in culture and were allowed to temperature acclimate at 26 and 30 °C for a minimum of one week before experimentation, presumably sufficient time to allow metabolic acclimation and CCM induction.

Buxton et al. (2009) used a similar methodology to ours, combining MIMS and chlorophyll fluorometry, to compare DIC limitation effects on cultured *Symbiodinium* and colonies of the scleractinian coral *Pocillopora damicornis* at 26 °C and 30 °C. Though calculations of  $K_p$  were not performed, their data do show an apparent decrease in  $K_p$  for the coral holobiont at high temperature, but this was accompanied by a significant reduction in the maximal rate of photosynthesis. Their experiments on cultured *Symbiodinium* did not sufficiently resolve the response of photosynthesis to low DIC to assess thermal effects on the affinity for DIC.

Our results show that impairment of the algal CCM or Calvin cycle are not likely to be the primary site of damage leading to thermal bleaching. Inorganic carbon supply may still play a role in bleaching, however, if host carbon transport processes are interrupted at high temperature. Thermal disruption of the inorganic carbon supply could limit photosynthesis, particularly as inorganic carbon availability appears to be low in hospite (Leggat et al. 1999). Carbon limitation of photosynthesis indeed generates a backup in the electron transport chain as shown by the decreased fluorescence yields, increased excitation pressure over PSII, and longer plastoquinone turnover times at low DIC (Figs. 5, 7, 8). The enhancement of NPQ at low DIC may partially alleviate backup in the electron transport chain, but clearly is not sufficient to fully dissipate excess light absorption (Fig. 3.9). This increased electron pressure can create oxidative stress as electrons are transferred to oxygen indiscriminately, contributing to bleaching (Lesser 1997; Smith et al. 2005). Although the backup is reduced at higher temperatures, this may be due to employment of the Mehler reaction (Asada 1999), which itself creates some oxidative stress and has been implicated in the coral bleaching response (Lesser 1997). Continual inorganic carbon limitation due to disruption of the host supply mechanism could then eventually lead to bleaching and breakdown of PSII, a hallmark of thermal bleaching (Warner et al. 1996).

More generally, our extensive characterization of photosynthetic properties in several *Symbiodinium* phylotypes sheds light on the functional diversity of this genus. Previous work on *Symbiodinium* functional diversity, focusing on photo-physiology and thermal sensitivity (Robison and Warner 2006; McCabe Reynolds et al. 2008; Hennige et al. 2009) has emphasized the variability of physiological parameters within this group.

We too found that the absolute value of many parameters varied substantially between phylotype; for example, K<sub>p</sub> varied between 250 and 630 µM at 22 °C and respiration rates varied 2-4-fold at any given temperature (Figs. 2, 3). For many traits one phylotype stood out from the others, in particular PK704, which had anomalously high rates of photosynthesis, high K<sub>p</sub> values, and large sensitivities of excitation pressure over PSII, plastoquinone turnover time, and NPQ to DIC (Figs. 2, 3, 7, 8, 9). Despite these differences, the photosynthetic properties of all types exhibited similar trends in response to changes in temperature and DIC, indicating a generally consistent behavior of the CCM relative to other photosynthetic systems within the *Symbiodinium* genus. As temperature increased, the P:R ratio,  $K_p$ , and  $K_{ETR}$  decreased, while gross photosynthesis and respiration increased in most or all phylotypes (Figs. 2, 3, 4). Similarly, as DIC increased most or all phylotypes showed increased  $\Phi_{PSII}$ , rETR, and decreased excitation pressure over PSII, plastoquinone turnover time, and NPQ (Figs. 7, 8, 9). Different Symbiodinium phylotypes may be more or less susceptible to inorganic carbon limitation in hospite, but our data suggests that increased temperature will improve inorganic carbon acquisition capabilities rather than impair them.

#### Conclusion

Inorganic carbon acquisition and supply to the symbionts is central to the coral symbiosis and is the base of the coral reef food web. While it has been suggested that disruption of inorganic carbon supply or breakdown of Rubisco may be the initial site of damage that leads to thermal bleaching, *Symbiodinium* inorganic carbon acquisition was not impaired at elevated temperatures. Extrapolation of results obtained on cultured

*Symbiodinium* to those in hospite must certainly be done with caution, but the uniformly positive effects of high temperature on inorganic carbon acquisition by four distinct strains of *Symbiodinium* strongly suggests this response is common throughout the genus. Carbon limitation of photosynthesis induced electron pressure on the electron transport chain, which could damage the photosynthetic apparatus if the condition is sustained. Consequently, impairment of the carbon delivery system via the host remains a potential mechanism by which high temperature may ultimately lead to bleaching, a subject that deserves further investigation.



Figure 3.1. Example plot of  $O_2$  evolution and  $\phi_{PSII}$  with increasing dissolved inorganic carbon (DIC) in *Symbiodinium* type RT74 at 22°C. Shaded areas indicate darkness, unshaded areas an irradiance of 200 µmol quanta m<sup>-2</sup> s<sup>-1</sup>. DIC was added during each dark period. Slopes from each linear portion of the data were used to calculate P<sub>net</sub> and R.



Figure 3.2. Maximum rates of  $O_2$  evolution and consumption of *Symbiodinium* cultures normalized to total cell volume. a) Maximum net photosynthetic rate; b) maximum respiration rate; c) maximum gross photosynthetic rate; d) the ratio of maximal net photosynthesis to maximum respiration. n = 3-6, standard errors shown. Points slightly offset for clarity.



Figure 3.3. The half-saturation constant of photosynthesis for DIC ( $K_p$ ) of four cultured *Symbiodinium* types across a temperature gradient. Data calculated from gross photosynthetic oxygen evolution. n = 3-6, standard errors shown. Points slightly offset for clarity.



Figure 3.4. Effective quantum yield ( $\phi_{PSII}$ ) versus dissolved inorganic carbon (DIC) concentration of *Symbiodinium* cultures: a) RT74; b) Mf10.4; c) D1a A001; d) PK704. Curves are fit using a Michaelis-Menten model.



Figure 3.5. The half-saturation constant of the relative electron transport rate (rETR) of cultured *Symbiodinium*. n = 3-6, standard errors shown. Points slightly offset for clarity.



Figure 3.6. Relative electron transport rate (rETR) of photosynthesis of four cultured *Symbiodinium* strains: a) RT74; b) Mf10.4; c) D1a A001; d) PK704. Curves are fit using a Michaelis-Menten model.



Figure 3.7. Excitation pressure over PSII,  $Q_m = 1 - ([\Delta F/F_m'] / [F_v/F_m])$  for *Symbiodinium* cultures: a) RT74; b) Mf10.4; c) D1a A001; d) PK704.



Figure 3.8. Effective quantum yield of photosystem II ( $\phi_{PSII}$ ) against the turnover rate of the plastoquinone pool ( $\tau_{PSII}$ ) for *Symbiodinium* cultures: a) RT74; b) Mf10.4; c) D1a A001; d) PK704.



Figure 3.9. Non-photochemical quenching, NPQ =  $(F_m - F_m') / F_m'$  of *Symbiodinium* cultures: a) RT74; b) Mf10.4; c) D1a A001; d) PK704.

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# CHAPTER 4

# TERMINAL ALTERNATIVE OXIDASE ENHANCEMENT BY THERMAL STRESS IN THE DINOFLAGELLATE SYMBIODINIUM<sup>3</sup>

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<sup>&</sup>lt;sup>3</sup> To be submitted to *Coral Reefs*.

# Abstract

An alternative terminal electron acceptor, mitochondrial alternative oxidase (AOX), which competes for electrons with the conventional cytochrome c oxidase (COX), is universal in higher plants and is represented in nearly every algal taxon save the cryptophytes. AOX has been hypothesized to function as a means of reducing oxidative stress, as well as a potential mechanism for ameliorating several other physiological stressors. Here, the presence of an active AOX in cultured Symbiodinium was detected through the response of oxygen consumption to the AOX inhibitor salicylhydroxamic acid (SHAM) with and without the COX inhibitor cyanide and corroborated with analysis of publicly available transcriptome data. Cyanide-insensitive, SHAM-sensitive AOX activity was found to be capable of accounting for a large portion (25-45%) of Symbiodinium dark respiration. The potential for changes in AOX capacity to play a compensatory role in mediating thermal stress was supported by AOX inhibitor assays after acclimation to high (32 °C) and low (18 °C) temperature conditions in cultured Symbiodinium. Maximum capacity of the AOX pathway was found to increase by 80-85% under both high and low temperatures. The physiological implications for the presence of an alternative oxidase in the coral/algal symbiosis and its potential role in the response to many forms of biotic and abiotic stress, particularly oxidative stress, oxygen reperfusion and nutrient limitation, are discussed.

# Introduction

Dinoflagellates of the genus *Symbiodinium* primarily exist within the cells of scleractinian corals, an obligate mutualism that drives the coral reef ecosystem via photosynthesis. Coral symbionts, also known as zooxanthellae, are capable of providing over 95% of the metabolic needs of the host by translocating photosynthetic products, primarily glycerol, to their animal hosts. Zooxanthellae photosynthesis not only provides the host with up to 95% of its metabolic needs, but also with the oxygen necessary to promote maximum calcification (Colombo-Pallotta et al. 2010). The coral reef environment, however, is ideal for the formation of toxic reactive oxygen species (ROS), with high temperatures and irradiance, including in the ultraviolet. ROS stress has been implicated as a primary mechanism of coral bleaching (Lesser 1996, 1997; Richier et al. 2005). It has generally been assumed that the algal photosynthetic electron transport chain is responsible for ROS production. However, the mitochondrial electron transport chain is also capable of ROS production, and may be an important source of ROS stress at the organismal or ecosystem scale (Maxwell et al. 1999).

The primary terminal oxidase for aerobic respiration in eukaryotes is the mitochondrial cytochrome c oxidase (COX). This enzyme, also known as Complex IV, spans the inner mitochondrial membrane and, together with Complexes I-III, forms the mitochondrial electron transport chain (mETC). COX catalyzes the reduction of  $O_2$  to  $H_2O$  via the oxidation of reduced cytochrome c and facilitates formation of proton gradients to drive ATP synthase. This enzyme is highly conserved among aerobes and is strongly inhibited by cyanide (CN) (Henry et al. 1974). The observation that respiratory  $O_2$  consumption in some organisms is not completely inhibited by CN lead to the

discovery of another mitochondrial oxidase, the alternative oxidase (AOX). AOX is an iron-containing protein homodimer bound to the matrix side of the inner mitochondrial membrane (Rasmusson et al. 1990). AOX diverts electron flow between Complexes II and III, reducing O<sub>2</sub> to H<sub>2</sub>O via the oxidation of reduced ubiquinone (UQH<sub>2</sub>) (Moore and Siedow 1991). This reaction short circuits the mETR, dissipating much of the redox energy as heat and so reducing ATP yield (McDonald 2008). AOX activity is distinguished experimentally by its insensitivity to cyanide and other COX inhibitors (e.g. azide, carbon monoxide) but is sensitive to salicylhydroxamic acid (SHAM) (Meeuse 1975).

The presence of a nuclear-encoded alternative oxidase is apparently universal in plants (McDonald et al. 2002; Finnegan et al. 2003), and has been described in most eukaryotic taxa (McDonald and Vanlerberghe 2006). Its function in thermogenesis in some plants, primarily the Araceae, was first described by Lamarck (Lamarck 1778). In this system, AOX activity is upregulated in the inflorescence, producing heat rather than chemical energy in order to volatize chemical attractants for pollinators (Meeuse 1975). Its ubiquity, however, demands that there is a broader function beyond the niche case of thermogenesis (see (Finnegan et al. 2004) for a review).

Attention has been focused on AOX as a means of ameliorating oxidative stress, particularly in preventing the generation of ROS. In addition to ROS production from the photosynthetic electron transport chain (e.g. water-water cycle/Mehler reaction), mitochondria are a major source of ROS in all eukaryotes (Maxwell et al. 1999). ROS are produced by autooxidation of mETC components, including ubiquinone (Maxwell et al. 1999). As AOX functions by oxidizing the mitochondrial ubiquinone pool, its potential to modulate ROS production has been a focus of study (Millenaar et al. 1998; Maxwell et al. 1999). Maxwell et al. (1999) demonstrated in transgenic tobacco plants a negative relationship between enhanced expression of the mitochondrial AOX protein and ROS in the plant tissue.

The alternative respiratory component was originally thought to only function as an "overflow valve", draining electrons from the ubiquinone pool when the COX pathway was saturated (Bahr and Bonner Jr 1973). However, it has been shown that AOX can compete with COX for electrons and so is active continuously to regulate and smooth electron flow through the mETC in order to avoid over-reduction of intermediates and generation of ROS (Robinson et al. 1995). AOX has been shown in plants to be inducible by many biotic and abiotic stressors which restrict electron flow to COX and/or result in higher ROS production, such as cold stress (Vanlerberghe and McIntosh 1992; Fiorani et al. 2005), viral infection (Lennon et al. 1997) and herbivory (Zhang et al. 2012); the coral mutualism may have similar capability to use AOX to mediate multiple stressors. Here, we present evidence for the presence of an active AOX in cultured Symbiodinium strain C1 152 by assessing the effects of the inhibitor SHAM on  $O_2$  consumption in the dark in the presence and absence of cyanide and analysis of publicly available transcriptome data. The potential for AOX to participate significantly in response to thermal stress was determined by AOX capacity assays on cultures acclimated to highand low-temperature stress. The AOX mechanism was found to be unexpectedly capable of accounting for up to 45% of dark respiration in *Symbiodinium* cultures and, moreover, was upregulated by thermal stress.

# Methods

# Algal culture

Symbiodinium strain C1 152 was maintained in ASP-8A medium (Zahl and McLaughlin 1957) at 26 °C at 30 µmol quanta cm<sup>-2</sup> s<sup>-1</sup> with a 12 hr photoperiod. Cultures were refreshed with new media every 3-4 days and the day before experimentation to maintain logarithmic growth phase. Cultures were acclimated to experimental temperatures for at least four days before experimentation. Cultures used in the high-temperature (32 °C) experiments were first acclimated to 30 °C to avoid potential direct damage. Experiments were conducted during the acclimated photoperiod light phase to minimize photoperiodicity effects.

#### Respirometry

 $O_2$  consumption rates were measured by membrane inlet mass spectrometry (MIMS). In this system, a high vacuum draws gases across a Teflon membrane from 1 mL liquid sample in a water-jacketed chamber. These gases are ionized by a filament and the constitutive masses determined by a quadropole mass spectrometer (Pfeiffer QMS 220). For a thorough review of the instrumentation and method, see (Beckmann et al. 2009). *Symbiodinium* cultures were concentrated by centrifugation and resuspended in artificial seawater (Morel et al. 1979) buffered to  $8.05 \pm 0.05$  with Tris. As we only wished to determine the effects on mitochondrial processes without interference from potential light enhanced oxygen uptake processes (e.g. Mehler reaction, photorespiration, etc.), cells were kept in the dark for at least 30 minutes before and for the duration of the inhibitor experiments. Measurements were taken as quickly as possible to minimize side effects or substrate limitation caused bythe inhibitors. After each inhibitor addition the  $O_2$ 

signal was allowed to stabilize for 1 min before measuring the linear O<sub>2</sub> uptake rate for 3-4 min. Cyanide (CN) was dissolved in water at 100 mM NaCN. As SHAM is very poorly soluble in water, SHAM was dissolved in DMSO at a concentration of 1 M. Negative control experiments showed no effect of DMSO on O<sub>2</sub> consumption when DMSO was less than 10% of the total solution. Attempts to replicate these data with another known AOX inhibitor, n-propyl gallate, were inconclusive due to abiotic redox reactions between the propyl gallate and the artificial seawater medium, causing its neutralization (data not shown).

To determine the maximum capacity of the AOX system ( $V_{alt}$ ), SHAM additions were made in the presence of 0.5 mM NaCN, based on the protocols described in (Moller et al. 1988). The fraction of oxygen consumption remaining in the presence of CN was termed  $V_{CN}$  and residual  $O_2$  consumption that was insensitive to both CN and SHAM was designated  $V_{res}$ .  $V_{alt}$  was calculated as the fraction of oxygen consumption that was sensitive to SHAM in the presence of CN.

$$V_{alt} = V_{CN} - V_{res}$$

Consumption of oxygen by the MIMS instrumentation was accounted for using the consumption rate of argon, which is biologically inert but has similar physical properties as  $O_2$ . Respiration rates were normalized to the initial linear oxygen uptake rate in the absence of inhibitors. Statistical analyses of  $V_{alt}$  values were conducted in Microsoft Excel.

# Transcriptome analysis

Previously published transcriptome annotations of *Symbiodinium* strains Mf1.05b and K8 (Bayer et al. 2012) in the GenBank short read archive were searched for

alternative oxidase sequences and candidates identified. AOX mRNA sequences were collected from organisms with previously described AOX activity chosen and relevant phylogenetic relationships to *Symbiodinium*(accession numbers): *Glycine max* (soybean, NM\_001249237) and *Zea mays* (AY059646) to represent the Viridiplantae, the apicomplexan human parasite *Trypanosoma brucei brucei* (Tb10.6k15.3640 – 3662536) and the marine parasitic dinoflagellate *Perkinsus marinus* (gi|294955973). *T. brucei brucei* is included both as a representative of the apicomplexans, sister to the dinoflagellates, as well as for its remarkable use of AOX as the dominant terminal oxidase in some phases of its life cycle (Chaudhuri et al. 2006). These sequences were aligned with the *Symbiodinium* AOX candidate sequences using ClustalW (Geneious 5.5.0, Biomatters Ltd.) and the *Symbiodinium* transcript (mf105\_c20291) with the maximum identity determined. This putative *Symbiodinium* AOX amino acid sequence, together with the sequences from the other species, was translated and the translation aligned via ClustalW.

#### Results

#### Inhibition of respiration by CN and SHAM

To determine whether *Symbiodinium* has a functioning AOX, *Symbiodinium* type C1 152 cells in culture were treated with CN, which inhibits COX but not AOX, and their  $O_2$  consumption measured. CN was unable to fully inhibit *Symbiodinium*  $O_2$  consumption (Fig. 1). As CN was added at progressively higher concentrations, respiration initially decreased rapidly, but declining to only 50% of the initial rate when 250  $\mu$ M CN was applied. Further increases in CN concentration had no significantly greater effect on  $O_2$ 

consumption. In subsequent experiments, a standard concentration of 0.5 mM CN was used to ensure full inhibition of the cytochrome oxidase.

Addition of the AOX inhibitor SHAM in the presence of CN almost completely arrested the  $O_2$  consumption rate, suggesting AOX was almost completely inactivated together with COX inhibition (Fig. 2). In order to estimate further the capacity of AOXmediated  $O_2$  consumption in the dark relative to that of total  $O_2$  uptake, serial additions of SHAM were made with and without the prior addition of CN (Fig. 3). In the presence of CN, the effect of SHAM on  $O_2$  consumption was maximal at 20-25 mM, and any remaining  $O_2$  consumption at this point was considered the residual rate ( $V_{res}$ ), insensitive to both CN and SHAM.  $V_{alt}$ , the maximum estimated capacity of the alternative oxidase, was 25% of the total respiratory rate at 26 °C. Without CN, SHAM exhibited a small additional inhibitory effect at concentrations of 60mM and greater (Fig. 3); the high concentration effects can be attributed to unspecific inhibition of the COX pathway by SHAM (Moller et al. 1988).

#### Effect of thermal stress on AOX capacity

Symbiodinium cultures were exposed to high (30 °C) and low (18 °C) temperature stress for 5 days to determine the effects of thermal stress on the maximum capacity of AOX ( $V_{alt}$ ). Cyanide addition in the absence of SHAM inhibited  $O_2$  consumption by 50% at 26 °C, while inhibiting  $O_2$  consumption by only 33-36% after acclimation to 18 °C and 30 °C (Fig. 4). The maximum capacity for  $O_2$  consumption (and, by extension, mitochondrial electron transport) by AOX was estimated to be 45% of the total under thermal stress and 25% under the control temperature (Fig. 5).  $V_{alt}$  values under both low and high thermal stress were significantly higher than at the control temperature 26 °C (p < 0.05) but not significantly different from one another (Fig. 5). The half-saturation constant of SHAM in the presence of cyanide was apparently higher at 18 °C, perhaps due to changes in cell membrane permeability (Fig. 4). Importantly,  $V_{res}$  was not affected by temperature (Fig. 4).

#### Identification of an AOX transcript from Symbiodinium

Previously published transcriptomes of two *Symbiodinium* cultures were searched for homologs of AOX. A sequence (mf105\_c20291) from *Symbiodinium* Mf1.05b, a member of the B clade, was translated and identified as being strongly homologous to those of other widely divergent species confirmed to possess AOX (Fig. 6). This sequence contains the universal AOX iron-binding domains and putative ubiquinolbinding domain (McDonald 2008; Albury et al. 2010) and shows high similarity the AOX of the Viridiplantae, the parasitic marine dinoflagellate *Perkinsus marinus* and the apicomplexan human parasite *Trypanosoma brucei brucei*.

#### Discussion

Physiological studies of corals and metabolic estimates of reef productivity have traditionally been conducted by measuring oxygen consumption and production (Odum and Odum 1955; Porter et al. 1984; Anthony et al. 2008). *Symbiodinium* provides the energetic and structural foundation of the coral reef ecosystem, and so any process that is a significant portion of their metabolic activity, particularly under stress conditions, is of deserves resolution. Previous studies of aquatic systems using the stable isotope discrimination technique have indicated widespread and significant activity of the AOX pathway in marine systems (Luz et al. 2002). Any significant, widespread alternative

oxidase activity in zooxanthellae, taken across an entire reef, would represent a potentially enormous diversion of energy to diminish the production of ROS. Many potential functions have been proposed for AOX in plants and other organisms, and their potential relevance in the coral symbiosis heretofore has not been considered.

# Presence of an active AOX in Symbiodinium

Inhibition of dark O<sub>2</sub> uptake by the selective AOX inhibitor SHAM in the presence of sufficient cyanide to completely inhibit cytochrome c oxidase (COX) is strongly indicative of alternative oxidase activity. Cyanide was only capable of inhibiting respiration of Symbiodinium C1 152 by 50%, even at very high concentrations (Fig. 1), but O<sub>2</sub> uptake was further inhibited by the addition of SHAM (Fig. 2). There was some residual O<sub>2</sub> consumption (25%) insensitive to either CN or SHAM. A similar fraction of residual respiration has been observed in other microalgae (Eriksen and Lewitus 1999), and may be due to the collective activity of peroxidases and other oxygen-consuming enzymes (Moller et al. 1988). To date, evidence of AOX activity in photosynthetic dinoflagellates has been limited, having only been assayed in one species, Amphidinium *carterae*, in a single study (Eriksen and Lewitus 1999) and potential genes identified in another (Butterfield et al. 2012). Like Symbiodinium, a significant portion of respiration in Amphidinium carterae, 40%, was insensitive to cyanide but inhibited by SHAM, indicating substantial AOX capacity. The presence of a Symbiodinium transcript with strong similarities to the previously identified AOX protein of higher plants and fellow alveolate Trypanosoma brucei brucei, including the putative quinone binding site and iron-binding ligands (Fig. 6), is further evidence of the presence of AOX in Symbiodinium. The assayed culture and analyzed transcriptomes are from genetically

distinct *Symbiodinium* strains, but the presence of AOX in related organisms (e.g. the dinoflagellate *Perkinsus marinus* and the apicomplexan *T. brucei brucei*) as well as its general ubiquity among photoautotrophs strongly supports its widespread presence in *Symbiodinium*.

As electron flow can be instantaneously redirected to AOX when COX activity is impaired, the inhibition of oxygen consumption by SHAM in the presence of CN cannot fully resolve the activity of the enzyme in the absence of inhibitors (Day et al. 1996). Instead, the measure of V<sub>alt</sub> estimates only the capacity of AOX but is likely proportional to the amount of AOX protein present (McDonald et al. 2002). *Symbiodinium* cultures were subjected to multiple days under stress conditions to up-regulate any potential stress response processes, including induction of AOX. In both high- and low-temperature conditions we noted a significant increase of 85% in alternative oxidase capacity (Fig. 5). The AOX protein shares features with the plastid terminal oxidase (PTOX) involved in chlororespiration, and so it is conceivable that some of the SHAM response we note is due to PTOX inhibition. PTOX activity is generally low compared to that of COX, however, and has been shown to be relatively insensitive to SHAM (Cournac et al. 2002). *Potential roles of AOX in corals* 

One of AOX's major proposed functions is to reduce ROS generation by intercepting electron flow through the mETC. This function may be especially important in corals since ROS contributes to high-temperature coral bleaching (Lesser 1996, 1997; Richier et al. 2005). Sustained temperatures of only 1-2 °C above the typical thermal maximum can lead to progressive thermal inhibition of symbiont photosynthesis followed by photodamage and expulsion of the symbionts (Lesser 1997; Hoegh-Guldberg 1999; Warner et al. 1999). Reactive oxygen species derived from the symbionts have been demonstrated to trigger symbiont expulsion and bleaching. Oxidative stress has also been shown to increase at low temperatures in plants (Watanabe et al. 2008), and AOX-antisense *Nicotiana tabacum* plants under low temperature conditions exhibit higher H<sub>2</sub>O<sub>2</sub> production (Zhang et al. 2009). Coral reef distribution is thermally limited to areas with average sea surface temperature minima above approximately 18 °C (Kleypas et al. 1999). Low-temperature stress events can induce photoinhibition in *Symbiodinium* and prove lethal to the host, though the cellular mechanisms involved have not been precisely determined (Kemp et al. 2011). Although AOX is utilized in plant thermogenesis, and in some species, such as the skunk cabbage *Symplocarpus foetidus*, can raise tissues 35 °C over ambient temperatures (Knutson 1974), this would seem highly unlikely to be at play in corals due to differences in thermal conductance between air and water.

Injury due to re-introduction of oxygen, known as oxygen reperfusion injury, is well-documented in animals and plants (Van Toai and Bolles 1991; Pilcher et al. 2012). Under hypoxic or anoxic conditions, reduced cytochrome oxidase activity can lead to an overreduced ubiquinone pool and mETC, promoting autooxidation of mitochondrial components. AOX may serve to divert electron flow and mitigate  $O_2$  reperfusion injury during frequent transitions between low and high internal  $O_2$  conditions. Coral tissues experience large diurnal changes in internal oxygen concentrations due to photosynthetic oxygen evolution. Over the course of a day,  $O_2$  concentrations can swing from 250% saturation in strong illumination to less than 2%  $O_2$  saturation in darkness (Kuhl et al. 1995). High oxygen concentrations are essential for maximal calcification (Colombo-Pallotta et al. 2010) and respiratory H<sup>+</sup> translocation (Jokiel 2011). Complete anoxia has been measured in the diffusive boundary layer around some corals and extremes in O<sub>2</sub> saturation in the light and dark would be exacerbated in low water flow conditions with larger diffusive boundary layers (Shashar et al. 1993). Low oxygen conditions in darkness could be expected to be more severe in warmer conditions due to higher holobiont respiration rates. The roots of plants must also contend with periods of anoxia, especially when submerged in water. Soybean roots have been shown to progressively increase transcription of the ROS scavenger superoxide dismutase while under anoxia; roots anoxic for 5 hours (or incubated with the antioxidant ascorbate) suffered less ROS damage than those anoxic for only 1 hour (Van Toai and Bolles 1991). Lower photosynthetic rates have been shown in cyanobacterial mats after darkness-induced hypoxia (Schreiber et al. 2002), and coral photosynthesis is lower in the morning than in the afternoon even at equivalent irradiance levels (Levy et al. 2004).

Coral reefs thrive in oligotrophic environments, with low nitrogen and phosphorous serving to limit both micro- and macroalgal growth (Muscatine and Porter 1977). The coral/algal mutualism must therefore have the capacity to operate under limited nutrient availability. Furthermore, host-mediated nutrient limitation has been proposed as a mechanism for controlling symbionts populations (Yellowlees et al. 2008). Phosphatase induction by low P conditions has been demonstrated in cultured *Symbiodinium* and in zooxanthellae of multiple coral species, indicating that zooxanthellae *in hospite* are prone to P limitation (Jackson et al. 1989; Annis and Cook 2002; Godinot et al. 2013). Increased AOX activity under P limitation has been documented in the bean *Phaseolus* (Rychter and Mikulska 1990). As electron flow through AOX reduces mitochondrial proton gradients and therefore generates less ATP than the conventional mETC, it can function to maintain electron flow through the ubiquinone pool even when P limitation negatively impacts oxidative phosphorylation. If this flow is constricted, over-reduction of the ubiquinone pool would increase ROS production (Parsons et al. 1999). AOX may serve to maintain mitochondrial electron flow in *Symbiodinium* under P limitation.

There has been investigation of the role of AOX in plant tissues which produce cyanide or cyanogenic glycosides in vivo as either a byproduct of ethylene synthesis or as a defense against herbivory (Gleadow and Woodrow 2002). Though cyanide exposure is not of concern for corals save for destructive fishing practices (Jones and Hoegh-Guldberg 1999), they face a similar stress of cytochrome oxidase inhibition from hydrogen sulfide exposure during heavy sedimentation. Weber et al. (2012) found that coral death after nutrient-loaded sedimentation was mediated by the microbial community and primarily driven by low pH (7.0) and  $O_2$  at the coral surface; subsequent H<sub>2</sub>S production, primarily from degraded coral tissue and mucus, accelerated coral decline. Corals exposed to a combined anoxia, low pH and 10  $\mu$ M H<sub>2</sub>S treatment were able to recover only to exposure less than 24 h. AOX activity in the symbionts alone would seem unlikely to be sufficient to convey any resistance to  $H_2S$ , but intriguingly, an AOX gene has been identified from the genome of the ecologically dominant scleractinian coral Acropora millepora (McDonald et al. 2009). The activity of this host pathway in the coral holobiont has yet to be assayed.

# Conclusion

Here we document the existence of an alternative oxygen-consuming mechanism which is capable of accounting for a large fraction of the oxygen consumption of the algal symbionts but, based on its location in the mETC, can only contribute as an electron acceptor for glycolysis. As this enzyme is also found in representatives of nearly all other eukaryotic lineages, its presence in *Symbiodinium* is unsurprising, but its involvement in a marine photosynthetic mutualism raises intriguing questions as to its possible function *in hospite*. This pathway must be considered in future metabolic analyses of corals if more detailed and accurate models of coral metabolism and physiology are to be developed. Quantitative estimates of AOX activity data must be taken cautiously, however, as characterization of AOX activity from inhibitor titrations have experimental limitations for their accurate interpretation (Day et al. 1996; McDonald et al. 2002). Further investigations utilizing an oxygen isotope fractionation technique, which determines AOX activity based on the differential <sup>16</sup>O/<sup>18</sup>O fractionation rates of the COX and AOX enzymes, would greatly increase our understanding of AOX function in *Symbiodinium* and its role in the coral/algal symbiosis.


Figure 4.1. Response of *Symbiodinium* C1 152 respiration to cyanide.  $n \ge 3$ , standard errors shown.



Figure 4.2. Sample response of *Symbiodinium* C1 152 dark O<sub>2</sub> consumption rate to cyanide (CN) followed by salicylhydroxamic acid (SHAM) at 26°C. Increases in O<sub>2</sub> signal upon addition of SHAM are due to changes in Teflon membrane gas permeability due to DMSO solvent.



Figure 4.3. Response of cultured *Symbiodinium* strain C1 152 dark respiration to salicylhydroxamic acid (SHAM) with and without the presence of 0.5 mM cyanide (CN) at 26°C. Data normalized to the O<sub>2</sub> consumption rate in the absence of inhibitors. Standard errors shown,  $n \ge 3$ .



Figure 4.4. Effects of salicylhydroxamic acid (SHAM) on O<sub>2</sub> consumption in the presence and absence of 0.5mM cyanide (CN) in cultured *Symbiodinium* strain C1 152.



Figure 4.5. SHAM-sensitive  $O_2$  consumption in the presence of cyanide ( $V_{alt}$ ) of *Symbiodinium* strain C1 152 in culture. Standard errors shown,  $n \ge 6$ . Bars with different letters are significantly different (single-factor ANOVA, p < 0.05).

Symbiodinium strain Mf1.05b Perkinsus marinus Trypanosoma brucei brucei Glycine max Zea mays	1	MIHHMRSLRRMVHCN-WIKPVMDEAENERMHLMTFMELSNQRWHQRVLVV 50 MNRHLRSLRKMERDFGWIHTLLEEAENERMHLMIALSLMKPGPLLRALVL MLRHLSSLRYMTRDKGWINTLLVEAENERMHLMTFIELRQPGLPLRVSII MLLHCKSLRRFEHSGGWFKALLEEAENERMHLMTFMEVAKPKWYERALVI MLLHLRSLRRFEHSGGWIRALLEEAENERMHLMTFMEVAKPKWYERALVL * *:***::: *:. ************************	
Symbiodinium strain Mf1.05b Perkinsus marinus Trypanosoma brucei brucei Glycine max Zea mays	51	CGQGAFFAAYTLFYVLTPRIAHRFVGYLEEEAVHTYTEMLKMVDEG 10 GAQGAFFTFYGLAYALSPNYAHRFVGYLEEEAVLTYTCLLQSVDKGERTY ITQAIMYLFLLVAYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEG TVQGVFFNAYFLGYLLSPKFAHRMFGYLEEEAIHSYTEFLKELDKG AVQGVFFNAYFLGYLISPKFAHRVVGYLEEEAIHSYTEYLKDLEAG *:.:: :: :: *.: *.: **::*******	0
Symbiodinium strain Mf1.05b Perkinsus marinus Trypanosoma brucei brucei Glycine max Zea mays	101	KIKNVAAPQIPIKYWNMPSDATLRDVILVVRADEADH 15 IVLEPYFTLGQIPEFALQQAPFVAQDYYQLPSSATLRDVFACMRADESHH RLRPTKN-DVPEVARVYWNLSKNATFRDLINVIRADEAEH NIENVPAPAIAIDYWQLPPGSTLRDVVMVVRADEAHH KIENVPAPAIAIDYWQLPADATLKDVVVVRSDEAHH .:.:: :* ::: *:. :.:*:*:*: *:	0

Figure 4.6. Alignment of alternative oxidase amino acid sequences of *Symbiodinium* strain Mf1.05b (mf105\_c20291), marine dinoflagellate *Perkinsus marinus* (gi|294955973), apicomplexan *Trypanosoma brucei brucei* (Tb10.6k15.3640 - 3662536), *Glycine max* (soybean, NM\_001249237), and *Zea mays* (maize, AY059646). The quinone-binding domain, as per Albury et al. 2010, is shaded and iron-biding ligands as per McDonald 2008 indicated by arrows. Agreement of *Symbiodinium* sequence to the others marked by asterisks (full agreement) or dots (partial agreement).

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# CHAPTER 5

## DISCUSSION

#### Carbon cycling within coral tissues

The ability of Form II Rubisco to fix carbon in a hyperoxic microenvironment suggests that carbon supply is facilitated by transmembrane dissolved inorganic carbon (DIC) transporters and carbonic anhydrases, both of which are known to exist in the host. The amount of respired CO<sub>2</sub>, either from the host or the symbiont, that is re-fixed by the Calvin cycle is unknown. If substantial, carbon recycling would provide a metabolic sink for excess light absorption and relieve excitation pressure from the photosystems. Physiological experiments to quantify this effect would clarify the interplay of carbon exchange between symbiont and host. These values would also inform models of symbiont population dynamics, particularly under stressful conditions where carbon availability may be critical in the stabilization or breakdown of symbiosis. Photorespiration rates within the host have also been difficult to measure but would also greatly add to our understanding of carbon dynamics in corals.

The relationship between temperature and [DIC] response was fairly consistent across all *Symbiodinium* phylotypes tested in Chapter 3. K<sub>m</sub> values differed, however, particularly at lower temperatures (Fig. 3.4). Given the considerable phylogenetic and functional diversity of *Symbiodinium* (LaJeunesse et al. 2004), it is notable that DIC

affinity increased with most, but not all, culture types. The potential ecological function of differences in DIC affinity between *Symbiodinium* genotypes is intriguing, particularly in regards to coevolution with the host and competition between zooxanthellae types in the coral tissue.

Differential effects of *Symbiodinium* clades across different coral hosts are still largely unknown (Littman et al. 2010). Several studies have investigated thermal responses of C1 and D types in Pacific acroporids (Abrego et al. 2008), but it is unclear whether or not the differences found exist at the cladal or subcladal level, especially given the wide diversity found within clade C. Rigorous physiological studies within clades and within subcladal complexes (e.g. C1, C3), corroborated with detailed phylogenetic trees, are needed to determine the phylogenetic depth of physiological function.

#### Inorganic carbon supply as a determinant of zooxanthellae populations

There are previous reports that corals are carbon limited at ambient DIC conditions. Al Moghrabi et al. (1996) demonstrated that freshly isolated zooxanthellae of *Galaxea fascicularis* were indiscriminate in their use of  $CO_2$  and  $HCO_3^-$ , potentially meaning that they are more responsive to changes in respiratory  $CO_2$  supply. Goiran et al. (1996) compared the DIC K<sub>m</sub> of microcolonies of the scleractinian *Galaxea fascicularis*, to that of freshly isolated zooxanthellae and cultured *Symbiodinium*. They measured a lower K<sub>m</sub> in the freshly isolated zooxanthellae (72  $\mu$ M) than the same strain maintained in culture (181  $\mu$ M). This would imply that the *Symbiodinium* carbon concentrating mechanism (CCM) is induced *in hospite*. Interestingly, the K<sub>m</sub> of the intact colonies was

much higher (413  $\mu$ M), potentially due in part to competition for DIC from calcification. Marubini and Thake (2012) documented an increase in calcification of the ecologically significant *Porites porites* of over 60% upon adding 2mM DIC to seawater. The addition of nitrate or ammonia to seawater, however, reduced the calcification rate of the controls, presumably due to competition for carbon from the zooxanthellae, which multiplied with nutrient addition. Weis (1993) reported that photosynthesis of the non-calcifying symbiotic anthozoan *Aiptasia pulchella* exhibited a DIC K<sub>m</sub> of 2.6 mM and was only saturated by greater than 6 mM DIC.

Thermal effects on carbon availability and carbon concentrating mechanisms may be a major factor determining seasonal coral productivity, calcification and symbiont population dynamics. In the Florida Keys, which experience a relatively wide seasonal range of temperatures (typically 20-31°C), long-term seasonal monitoring of coral physiology has documented a predictable reduction in coral tissue biomass (as ash-free dry weight) and symbiont density in the summer (Fitt et al. 2000). These periods correspond to simultaneously higher rates of calcification, which would diminish the inorganic carbon available to the symbionts. This establishes a conflict where high zooxanthellae populations with high DIC affinity would seem to be incompatible with significant calcification rates due to DIC competition. A reinforcing relationship between DIC affinity and zooxanthellae density may be established where DIC supply from the host (if assumed to be constant year-round) may supply either a large number of lowaffinity symbionts or a low number of high-affinity symbionts.

To test this concept, *Symbiodinium* cultures were grown under high- and low-DIC culture conditions to determine whether CCM induction is responsive to DIC supply

(Figure 5.1). High DIC growth conditions resulted in a higher DIC half-saturation constant in three of the four Symbiodinium strains, demonstrating the inducibility of the carbon concentrating mechanism. These results were compared to freshly isolated zooxanthellae from the scleractinian coral *Porites astreoides*. Despite the coral being acclimated to a fairly low temperature (24 °C), which in culture conditions resulted in generally higher K<sub>m</sub> values (Figure 3.5), the freshly isolated zooxanthellae (FIZ) halfsaturation constants were extremely low, indicating that the zooxanthellae are carbonlimited in symbiosis. The significantly higher DIC K<sub>m</sub> values at lower temperatures (22°C) raises the question of whether carbon affinity may be a factor during the winter months, reducing both productivity and oxygen evolution, necessitating higher symbiont densities. Competition for DIC between zooxanthellae could also increase during warm conditions, provided that the algal photosystems are not compromised by thermal bleaching conditions. This conflict could be resolved by a reduction in zoooxanthellae densities (and therefore DIC demand), increasing the availability of DIC for calcification while still providing sufficient  $O_2$ . Corals expel a portion of their symbiont population daily. Warming temperatures and correspondingly greater competition for DIC may result in a ratcheting down of the algal population to match the DIC available to support photosynthetic performance and prevent DIC limitation. Organic carbon is translocated from the algae to the coral host primarily as glycerol, and experimental induction of glycerol release in *Symbiodinium* cultures via osmotic stress strongly depressed Symbiodinium growth rates but did not impair photosynthetic rates and simultaneously increased Rubisco expression (Suescún-Bolívar et al. 2012). Reduced mitotic indices and increased Rubisco expression are both indicative of carbon stress on the algae.

Calcification is metabolically demanding, and sustaining maximal calcification rates combined with the thermal increase in host respiration ( $Q_{10}$  effect) (Jokiel and Coles 1977) may cause the coral host to run a metabolic deficit during the summer months corresponding to the observed reduction in tissue biomass. Many coral species spawn in late summer, and so must allocate resources over the summer months to gamete production, further exacerbating the metabolic deficit. Although there are many practical difficulties in assays of Form II Rubisco activity (Whitney et al. 1995), determination of Rubisco expression by zooxanthellae in hospite on a seasonal basis, particularly in consideration of its abundance and metabolic expense, would greatly improve our knowledge of coral reef function.

That the ability of coral symbionts to acquire inorganic carbon, as measured by the  $K_m$ , is affected by temperature provides a mechanism for seasonal changes in the inorganic and organic carbon exchange between the two partners. A lower algal DIC  $K_m$ during the warmer months, coupled with the well-documented increase in calcification rates, would imply a dramatically increased inorganic carbon demand of the holobiont during warm periods. Algal inorganic carbon demand is no doubt modulated by the host organism and additional investigations with whole corals and FIZ are required to determine whether the changes in algal physiology described here increase the inorganic carbon demand of the holobiont on a seasonal basis. Thermal effects on the algal CCM, coupled with host DIC demand, may provide a mechanism for the observed seasonal change in coral symbiont densities. The impact of ocean acidification and the resulting change in  $CO_2$ :  $HCO_3^-$  ratio may also physiological function of corals in the future. In addition to a reduced photosynthetic electron transport rate, impaired inorganic carbon supply lowers the effective quantum yield and induces nonphotochemical quenching in cultured *Symbiodinium*, effects that are indicative of photoinhibition and potential bleaching in whole corals. The role of insufficient carbon supply in coral bleaching, whether due to biotic or abiotic stressors, deserves further investigation.

#### The role of alternative oxidases in symbiosis

Alternative oxidase (AOX) is also important in the metabolism of many eukaryotic parasites of mammals, including the apicomplexan human parasites Trypanosoma brucei brucei (African sleeping sickness), Toxoplasma gondii (toxoplasmosis) and *Cryptosporidium hominis* (cryptosporidiosis), and may be the primary or sole terminal oxidase in some life cycle stages. The kinetoplastid Trypanosoma brucei brucei, the causative agent of African sleeping sickness, utilizes this AOX system in its bloodstream form to aerobically metabolize glucose, obtained from the host bloodstream, to pyruvate without utilizing a typical cytochrome c oxidase (COX) (Chaudhuri et al. 2006). In this system, T. brucei catabolizes glucose to two molecules of pyruvate, yielding two molecules ATP per glucose. This is facilitated by the AOX accepting electrons via the ubiquinone pool from the oxidation of glycerol 3-phosphate to dihydroxyacetone phosphate, which is salvaged by conversion to glyeraldehyde 3phosphate and catabolized to pyruvate. AOX activity in plants is stimulated primarily by pyruvate as an allosteric activator but also by glyoxylate, 2-oxoglutarate and oxaloacetate (Millar et al. 1996). SHAM addition inhibits this recycling of dihydroxyacetone phosphate (DHAP), resulting in glycerol formation in T. brucei and a 50% reduction in ATP generation (Chaudhuri et al. 2006). The role of the AOX in the Symbiodinium

glycerol cycle (Falkowski and Raven 2007) and potential interactions between AOX activity and glycerol production deserves greater investigation given the prominence of glycerol in the coral mutualism as the primary means of metabolic export to the coral host.



Figure 5.1. Dissolved inorganic carbon (DIC) half-saturation constants under high- and low-DIC culture conditions of *Symbiodinium* in culture and in freshly isolated zooxanthellae (FIZ). Four cultured *Symbiodinium* strains grown are compared to FIZ of the scleractinian coral *Porites astreoides* at 26 °C. Experiments performed as described in Chapter 3. Strains marked with an asterisk are significantly different (p < 0.05, singlefactor ANOVA).

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