Biological Responses of Juvenile *Tridacna maxima* (Mollusca:Bivalvia) to Increased pCO_2 and Ocean Acidification

By

Charley G. Waters

A Thesis submitted in partial fulfillment
of the requirements for the degree of
Master in Environmental Studies
The Evergreen State College
October 29, 2008

This Thesis for the Master of Environmental Studies Degree

By

Charley G. Waters

Has been approved for

The Evergreen State College

By

_____

Erik V. Thuesen, Ph. D.

Member of the Faculty, Zoology

_____

Timothy Quinn, Ph. D.

Member of the Faculty, Conservation Biology

_____

Grace Sparks, Ph. D.

Professor, Seattle Central Community College

_____

Date

Table of Contents

List of Figures

List of Tables

Acknowledgements

Thanks are due to E. V. Thuesen, Ph. D., T. Quinn, Ph. D., G. Sparks, Ph. D., C. Barlow, Ph. D., R. Peroutka for her unselfish dedication to the project and demonstrated competence in scanning electron microscopy, J. Freeman, J. Warmack, J. Williford, F. Goetz, P. Robinson and all of the Scientific Instruction Technicians, LabStores and Lab Receiving personnel at The Evergreen State College for invaluable support along the way, M. M. Bachtold and L. D'Andrea, Ph. D., for unwavering encouragement over the years, M. Gutowska for critical research design input, and T. Rose for extensive editorial support. A generous Foundation Activity Grant from The Evergreen State College Foundation contributed to this study.

Abstract

Biological Responses of Juvenile *Tridacna maxima* (Mollusca:Bivalvia) to Increased pCO_2 and Ocean Acidification

Charley G. Waters

Anthropogenic acidification of oceanic surface waters is expected to increase ~0.4 units from current pH levels of ~8.1 during this century. The effects of increased ocean acidification on biological processes vital to marine organisms are not well understood. This paper describes a controlled experiment designed to measure biological responses of juvenile *Tridacna maxima* clams to increased levels of atmospheric CO_2 partial pressure (pCO_2) and concomitant increases in ocean acidification. Over a 13-week period, four *T. maxima* populations (n = 30 each) were incubated in carbonate chemistry conditions manipulated by pCO_2 concentrations approximating glacial (180 ppmv), present (380 ppmv) and projected (560 ppmv and 840 ppmv) levels of atmospheric CO_2 per International Panel on Climate Change (IPCC) IS92a scenario. Net differences in mean shell lengths and heights significantly declined between pCO_2 180 ppmv and pCO_2 840 ppmv ($P < 0.05$). Approximately 11% of individual *T. maxima* specimens exhibited negative shell growth (dissolution) in pCO_2 180 ppmv conditions compared to > 60% in pCO_2 conditions of 840 ppmv. The mean net weight of all specimens varied significantly within populations ($P < 0.05$), but not between populations ($P > 0.05$), indicating an uncoupling of processes that contribute to shell precipitation or dissolution from normal mantle growth as pCO_2 increases. Cumulative results indicate differences in biological responses of *T. maxima* occurred at pCO_2 levels well below thresholds previously considered detrimental to other marine organisms in similar conditions. Results further indicate that tridacnid clams may be more susceptible to ocean

acidification than corals. Monitoring the health of *Tridacna* spp. may prove useful in helping predict the effects of increasing pCO_2 on coral reef ecosystems.

1.0 Introduction

The oceanic carbonate chemistry system mediates a two-way distribution of carbon species between two of Earth's great reservoirs of carbon; the atmosphere and deep ocean sediments. Since the industrial revolution, anthropogenic behaviors such as fossil fuel emissions, deforestation and cement manufacturing are raising levels of atmospheric carbon dioxide (CO_2) at exponential rates, thereby interfering with the ocean's capacity to mediate a gradual transition of carbon from one reservoir to another (The Royal Society, 2005). The scope of anthropogenic impacts on atmospheric conditions is numbing. Current accumulation rates of atmospheric CO_2 exceed rates dating back 650,000 years (IPCC, 2007), and even at 2005 levels of atmospheric CO_2, it is anticipated that returning ocean chemistry to what it was barely 200 years ago may take tens of thousands of years (The Royal Society, 2005).

Marine scientists are closely monitoring levels of atmospheric CO_2 as part of a well understood chain of chemical reactions between CO_2 partial pressure (pCO_2) and seawater. These reactions predictably alter oceanic carbonate chemistry by increasing hydrogen ion (H^+) concentrations, thereby reducing pH in a process colloquially termed ocean acidification (Doney, 2008). Research initiatives directed at assessing specific effects of ocean acidification on marine organisms as a result of elevated pCO_2 are scarce. This paper describes a controlled experiment designed to measure biological responses of juvenile *Tridacna maxima* clams to levels of acidification that correspond to past, present and expected levels of atmospheric pCO_2. Study results may advance our understanding of how atmospheric conditions might affect marine organisms with characteristics similar to *T. maxima*. This study

begins with a summary description of biological attributes of *T. maxima* and their autotrophic symbionts.

1.1. Biology of *Tridacna maxima* Clams

Tridacna clams are a genus of *Tridacnidae* (Mollusca:Bivalvia) that thrive in photic zones of equatorial waters ranging from Eastern Africa to the central Pacific. Of all ten species, *Tridacna maxima* are the most geographically wide-ranging (Heslinga et al. 1990; Ellis, 1998). Optimal water conditions for *Tridacna* clams include a temperature range of 25ºC to 30ºC, salinity from 32 to 35 parts per thousand (‰), and pH levels from 8.1 to pH 8.5 (Heslinga et al. 1990; Ellis, 1998). Commonly referred to as “giant clams”, *Tridacna* clams range in size from approximately 15 cm to 137 cm in length and can weigh up to 250 kg (Rosewater, 1965; Heslinga et al. 1990; Knop, 1996; Ellis, 1998). Shell sizes for *T. maxima* typically range from 30 cm to 40 cm (Knop, 1996). The iridescent pigmentation of *T. maxima* (Figure 1) may serve as protection from potentially damaging ultraviolet light or perhaps too much light in general (Knop, 1996).

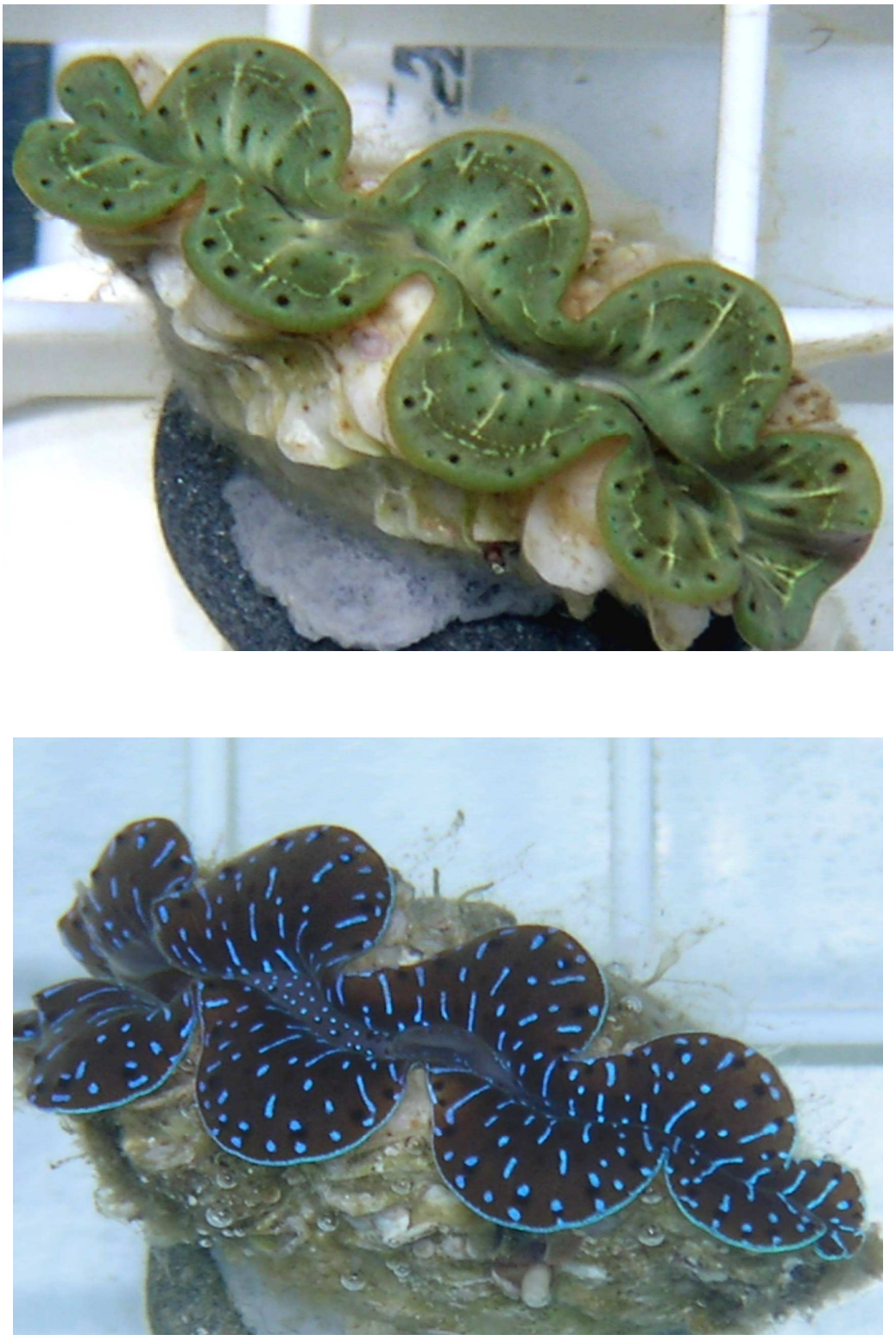

Figure 1: Examples of variable pigmentation and iridophore regimens in juvenile *Tridacna maxima* clams ~4 cm in length. Source: C. Waters.

As protandric hermaphrodites, *Tridacna maxima* rely on pheromonal communication to initiate broadcast spawning of fecundate zygotes during different breeding seasons. Environmental triggers to spawning include tide, lunar cycle and temperature (Fitt and Trench, 1981; Heslinga et al. 1990; Ellis, 1998). Natural predators of *Tridacna* spp. consist of crabs, lobsters, fish, various boring snails, octopi, eagle rays and humans (Heslinga and Fitt, 1987; Heslinga et al. 1990; Ellis, 1998). *Tridacna maxima* have three sources of nutrient uptake; filter feeding of phytoplankton, principally *Isochrysis galbana* and its congeners (Heslinga and Fitt, 1987), soft-tissue (mantle) absorption of dissolved nutrients from the water column (Fankboner, 1971), and photosynthates from a symbiotic association with *Symbiodinium microadriaticum*, autotrophic dinoflagellates (Heslinga and Fitt, 1981, Ellis, 1998). *Tridacna* spp. are the only known species of Bivalvia to engage in symbiotic associations solely with *S. microadriaticum* (Taylor, 1969), commonly referred to as zooxanthellae (Fitt and Trench, 1981). Life expectancy among *Tridacna* clams is not well studied although Heslinga et al. (1990) suggest ranges between several decades for most tridacnid species and perhaps > 100 years for the largest of the species, *Tridacna gigas*.

1.2. Symbiotic Association Between *Tridacna* spp. and *Symbiodinium microadriaticum*

Symbiodinium microadriaticum zooxanthellae are not inherited by larval clams (Belda-Baille et al. 1999; Hirose et al. 2005). Instead, within 2 to 3 days of fertilization, veliger larvae begin ingesting (but not digesting) and storing zooxanthellae in their stomach (Fitt and Trench, 1981). Following larval metamorphoses, zooxanthellal packets are relocated via a complex network of epithelial tubes and haemal fluids to extra-cellular tertiary locations of the

hypertrophied tissue (Figure 2) where they undergo mitosis (Trench et al. 1981; Heslinga and Fitt, 1987; Heslinga, 1990; Norton et al. 1992).

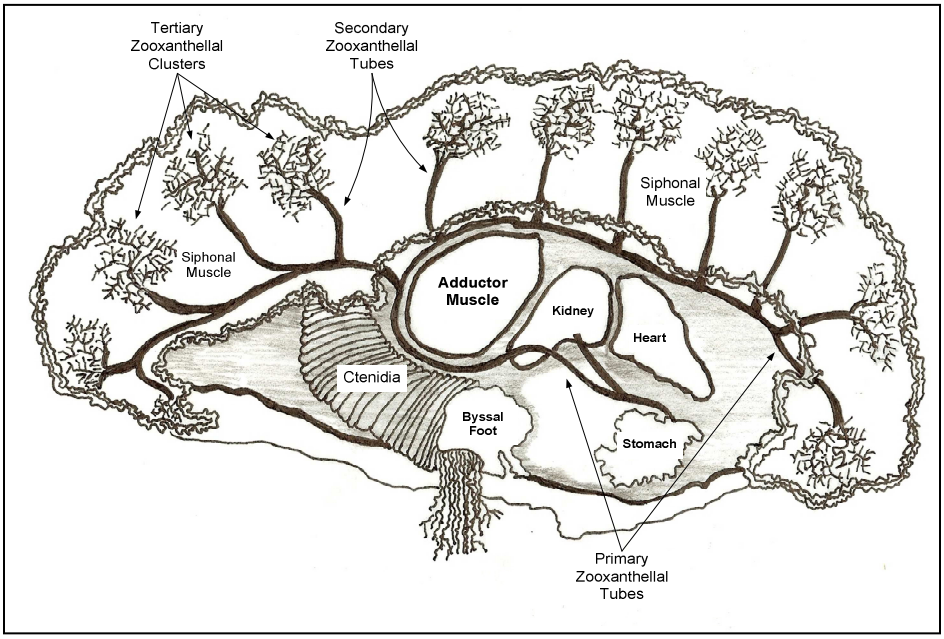

Figure 2: Medial view of basic *Tridacna maxima* anatomy shows epithelial tubes branching from the stomach to primary and secondary zooxanthellal tubes leading to tertiary clusters in the hypertrophied tissue. Source: C. Waters, redrawn from Norton et al. (1992).

Specific photosynthates generated by zooxanthellae for *Tridacna* sp. hosts include varying degrees of respiratory carbon, complex sugars such as glycerol and glucose, amino acids such as alanine, and small amounts of oxygen (O_2) (Fankboner, 1971; Trench et al. 1981; Heslinga and Fitt, 1987; Paracer and Ahmadjian, 2000; Hirose et al. 2005). In exchange, tridacnid hosts provide zooxanthellae safe haven from prey, a homeostatic environmental (Cowen, 1988), nutrient salts, CO_2, and nitrogenous wastes that fuel zooxanthellal photosynthetic productivity (Fankboner, 1971). The overall exchange of elements and nutrients is summarized in Figure 3.

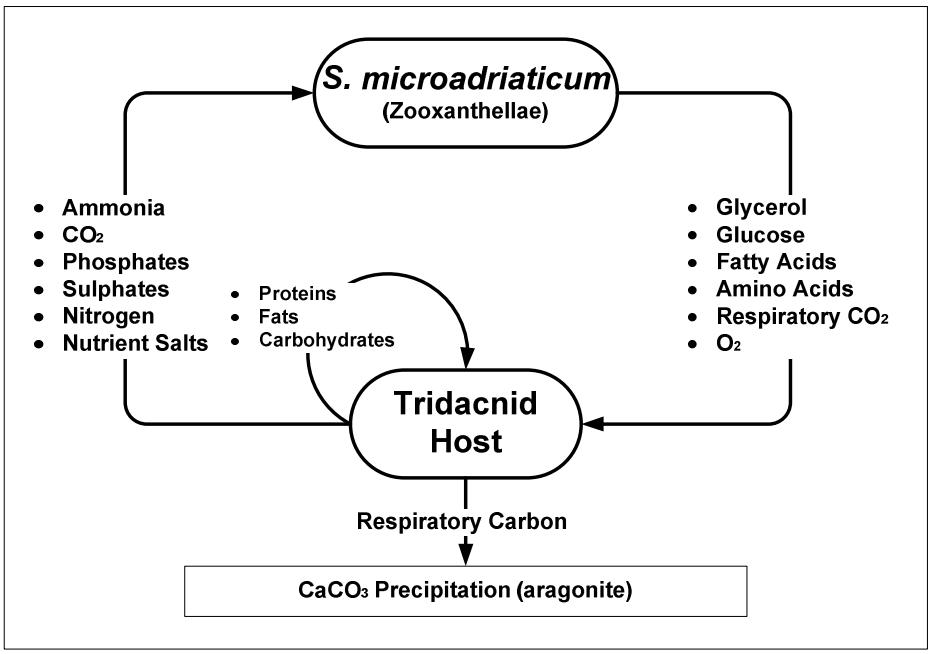

Figure 3: Elements and nutrients exchanged between *Symbiodinium microadriaticum* symbionts and *Tridacna maxima* hosts. The precise volumes, rates, frequency and conditions of exchange of nutrients and elements between organisms are not fully understood. Source: C. Waters.

The relationship between zooxanthellal photosynthetic output and host metabolic activities of *Tridacna maxima* and other marine symbionts is elegant yet extremely complex. Cumulative photosynthates generated by zooxanthellae support multiple metabolic, anabolic and catabolic activities of the host, including mucus production, byssal development, nutrient digestion and $CaCO_3$ precipitation (shell formation) (Goreau et al. 1973; Yonge, 1980; Trench et al. 1981). For example, under favorable conditions, zooxanthellae may provide up to 100% of the respiratory carbon required by the host (Fisher, 1985; Heslinga and Fitt, 1987), and > 50% of the host's nutritional requirements (Hirose et al. 2005). Experimental evidence suggests that growth rates for juvenile *Tridacna* spp. increase when zooxanthellae begin migrating across the alimentary tract of developing siphonal tissue, and that hosts void of zooxanthellae do not survive beyond three weeks (Fitt and Trench, 1981).

The exact volumes and rates of zooxanthellal contributions of photosynthates to *Tridacna maxima* are difficult to quantify because multiple factors have been shown to influence zooxanthellal photosynthetic activity. Such factors include nutrient saturation levels of nitrogen (N) and phosphorus (P), ammonia (NH_3), community metabolic output, light and seasonal conditions, availability and source of carbon species, and salinity (Amariyanto and Hoegh-Guldberg, 1997; McConnaughey et al. 2000; Medakovic et al. 1997; Legget and Yellowlees, 1999; Ringwood and Keppler, 2002). Steady rates and volumes of zooxanthellal photosynthetic activity are particularly significant to *T. maxima* because absent or inconsistent sources of photosynthetic output may jeopardize $CaCO_3$ precipitation.

Calcium carbonate precipitation describes a mechanism organisms use to create minerals from surrounding reservoirs of elements. Calcification refers to the

creation of hard tissue structures from elements in the calcite group of carbonates. Calcifying marine organisms precipitate $CaCO_3$ structures for skeletal support, protection, and as a reserve source of carbonate (CO_3^{2-}) to offset naturally occurring spikes in carbonic acid (H_2CO_3) (Lindinger et al. 1984; Lowenstam and Weiner, 1989). Calcite species used to precipitate $CaCO_3$ structures include calcite, aragonite, High-magnesium calcite and others, or some combination thereof. The chemistry between carbonate species is similar but differences in structure and symmetry tend to make aragonite more soluble than calcite (The Royal Society, 2005). Calcification can only occur when surrounding waters are supersaturated (≥ 100%) with the required carbonate and trace elements such as strontium (Sr) and molybdenum (Mo) (Knop, 1996); undersaturated levels result in dissolution (Gattuso et al. 1998). *Tridacna maxima* shells primarily consist of aragonite with trace amounts of calcite (Lowenstam and Weiner, 1989).

Evidence supporting the importance of $CaCO_3$ precipitation in the life history of *Tridacna* spp. is compelling. Approximately 12 hours after fertilization, *Tridacna* zygotes pass through a gastrula stage of embryonic development to hatch as trochofore larvae. A shell gland at the base of the trochofore begins precipitating $CaCO_3$ shells (Heslinga et al. 1990). The soft tissue of a hatched trochofore is completely surrounded by shell within two days of fertilization, thereby signifying the veliger stage of development (Heslinga et al. 1990; Ellis, 1998).

The relationship between zooxanthellal photosynthesis and calcification of marine organisms remains elusive and subject to much debate. Early research suggested that zooxanthellae per se may not be clearly linked to $CaCO_3$ precipitation in corals, yet precipitation rates declined in relation to zooxanthellal loss (Goreau, 1959). More recently, it has been suggested that symbiotic photosynthetic output may account for ~90% of the primary productivity in reef environments that

contribute to $CaCO_3$ precipitation (Cowen, 1988). A rigorous study of the relationship between photosynthesis and calcification by McConnaughey et al. (2000) concluded that photosynthetic rates and calcification of reef corals generally correlated over the course of the day, and virtually ceased at night even when levels of aragonite were supersaturated. Research findings by Legget et al. (1999) reinforce the potential role of light in the calcification process. This team found *Symbiodinium microadriaticum* in *Tridacna* sp*.* to exhibit light-activated intra-cellular carbonic anhydrase activity, suggesting a carbon concentrating mechanism that may facilitate calcification. In contrast, Woolridge (2008) suggests that reduced pH rather than sunlight interferes with urease enzyme activity, the enzyme known to facilitate $CaCO_3$ precipitation.

Other research findings indicate an *inverse* relationship between photosynthesis and hermatypic scleractinia corals (Hoegh-Guldberg and Smith, 1989; Langdon and Atkinson, 2005). An inverse relationship between photosynthesis and calcification is corroborated by Marubini and Atkinson (1999), who found that a combination of light and nutrient availability affected carbon partitioning between photosynthesis and calcification in corals. Marubini and Davies (1996) suggest that inverse relationships between photosynthesis and calcification may be explained by rapid, large-scale absorption of diffusion-limited CO_2 by zooxanthellal populations for photosynthesis, thereby reducing reserves of inorganic carbon ordinarily available for calcification.

An exhaustive discussion of variables affecting calcification is beyond the scope of this study. The temptation to transfer knowledge gained from coral calcification responses to photosynthesis to *Tridacna maxima* should be mitigated by the fact that *Tridacna* spp. select different species of inorganic carbon from seawater. For example, symbionts in *Tridacna gigas* specimens have been shown to select CO_2 as a primary source of carbon as opposed to symbionts in corals that

select HCO_3^- (Legget et al. 2000). Yet to be fully explained is whether either species of inorganic carbon is used for host calcification, symbiont photosynthesis, or perhaps a combination of the two species depending on prevailing pH conditions (Legget et al. 2000).

In summary, the relationship between zooxanthellal photosynthesis and $CaCO_3$ precipitation depends on the hosting organism, life history stage, zooxanthellal population density, and multiple environmental variables including the availability of inorganic carbon. The extent to which increasing pCO_2 and declining oceanic pH influence calcification and photosynthesis in complex marine organisms with symbiotic associations is unknown. Exploring the relationship between elevated levels of pCO_2 and biogeochemical processes such as calcification and photosynthesis begins with a review of basic oceanic carbonate chemistry.

1.3. Overview of Oceanic Carbonate Chemistry and Ocean Acidification

Anthropogenic contributions to ocean acidification begin with elevated levels of atmospheric CO_2 as a result of fossil fuel emissions, deforestation and, more recently, cement manufacturing (Kleypas et al. 2005). It is impossible to categorically attribute current conditions of atmospheric CO_2 to anthropogenic activities, but the evidence is compelling (Houghton, 2005). For example, current concentrations of atmospheric CO_2 are > 380 ppmv (Doney, 2008), a 38% increase from ~280 ppmv levels at the beginning of the Industrial Revolution (IPCC, 2007; Kleypas, 2008). The last time-frame for an increase of 100 ppmv (from inter-glacial ages of ~180 ppmv to ~280 ppmv) is estimated to be between 640,000 and 800,000 years (IPCC, 2007; Doney, 2008). If current rates of increase continue under IPCC's "business as usual" scenario IS92a, atmospheric concentrations of CO_2 are expected to reach ~560 ppmv sometime in the middle of this century and ~840 ppmv by the close of the

millennium (Orr, 2005; IPCC, 2007) (Table 1). The result may be a decline of ~0.4 units in oceanic surface pH from current averages of ~8.1 to ~7.7 (Caldeira and Wicket, 2003), lower than it has been in more than 20 million years (Feely et al. 2004).

Table 1: Past, present and projected atmospheric and oceanic parameters for chemical and physical factors based on 2001 IPCC IS92a "business as usual" scenarios. Source: IPCC as modified by Feely et al. (2001) and Kleypas et al. (2005).

PARAMETER	UNIT	GLACIAL	PRE- IND.	PRESENT	2 X CO_2	3 X CO_2
Temperature	ºC	15.7	19	19.7	20.7	22.7
Salinity	‰	35.5	34.5	34.5	34.5	34.5
Total Alkalinity (T_A)	µequiv kg^{-1}	2356	2287	2287	2287	2287
CO_2	µatm (ppmv)	180	280	380	560	840
Carbonic Acid (H_2CO_3)	µmol kg^{-1}	7	9	13	18	25
Bicarbonate (HCO_3^-)	µmol kg^{-1}	1666	1739	1827	1925	2004
Carbonate (CO_3^{2-})	µmol kg^{-1}	279	222	186	146	115
Hydrogen (H^+)	µmol kg^{-1}	4.79 x 10^{-03}	6.92 x 10^{-03}	8.92 x 10^{-03}	1.23 x 10^{-2}	1.74 x 10^{-02}
Aragonite	Ω_{arag}	4.26	3.44	2.9	2.29	1.81
Calcite	Ω_{calc}	6.63	5.32	4.46	3.52	2.77
Dissolved Organic Carbon (DIC)	µmol kg^{-1}	1952	1970	2026	2090	2144
Total pH	pH_T	8.32	8.16	8.05	7.91	7.76

The relationship between atmospheric CO_2 and seawater is partially explained by four primary reactions:

$$CO_{2(aq)} + H_2O_{(l)} \leftrightarrow \underset{\text{Carbonic Acid}}{H_2CO_{3(aq)}} \quad (1)$$

$$H_2CO_{3(aq)} \leftrightarrow H^+_{(aq)} + \underset{\text{Bicarbonate}}{HCO_3^-{}_{(aq)}} \quad (2)$$

$$HCO_3^- \leftrightarrow H^+_{(aq)} + \underset{\text{Carbonate}}{CO_3^{2-}{}_{(aq)}} \quad (3)$$

$$pCO_{2(aq)} + H_2O_{(l)} + CO_3^{2-}{}_{(aq)} \leftrightarrow 2HCO_3^-{}_{(aq)} \quad (4)$$

Atmospheric CO_2 follows Henry's Law by mixing with seawater (pCO_2) across the air-sea interface to form carbonic acid (H_2CO_3) (1). H_2CO_3 rapidly dissociates to form bicarbonate (HCO_3^-) (2), and dissociates again at a slower reaction rate to form carbonate (CO_3^{2-}) (3), thereby increasing concentrations of H^+ ions on a logarithmic scale and reducing pH. In addition to reduced pH, reactions between pCO_2 and seawater increase concentrations of HCO_3^- and decreases concentrations of CO_3^{2-} (Open University, 1994; Kleypas et al. 2005; Herfort et al. 2008). For example, atmospheric conditions of 560 ppmv are expected to reduce CO_3^{2-} concentrations by 30%, and increase H^+ ion concentrations ranging from ~60% (Haugan, 2004) to between 100% and 150% (Orr, 2005). A further reduction in CO_3^{2-} ions occurs as pCO_2 reacts with H_2O and CO_3^2 to form $2HCO_3^-$ molecules, thereby compounding already reduced concentrations of CO_3^{2-} (4) (Haugan, 2004).

Two additional equations that contribute to understanding ocean acidification in a larger framework of carbonate chemistry are equilibrium constants for HCO_3^- and CO_3^{2-} in terms of H^+ ion concentrations in solution, and alkalinity of seawater.

Reaction rates between equations (2) and (3) contribute to a relatively stable oceanic pH of ~8.1. The reactions are said to be at equilibrium when equation (3) is complete as represented by equation (5), where *K* is the equilibrium constant and represents the ratio between HCO_3^- and CO_3^{2-} to "buffer" H^+ ion concentrations; as the ratio (*K*) increases, pH declines.

$$(H^+) = K \frac{[HCO_3^-]}{[CO_3^{2-}]} \quad (5)$$

In addition to elevated levels of atmospheric CO_2, alkalinity is known to affect levels of pH differently depending on chemical conditions and measurement methods (Kelypas et al. 2005; Dickson et al. 2007). Alkalinity generally describes the concentration of negative charge in a solution that reacts with H^+ ions. Concentrations of HCO_3^- and CO_3^{2-} in seawater are closely related to alkalinity since these ions are orders of magnitude more abundant than conjugate bases and weak acids capable of accepting protons. Carbonate alkalinity (CA) is expressed as:

$$CA = 2[CO_3^{2-}] + [HCO_3^-] \quad (6)$$

The carbonate ion in equation (6) is multiplied by 2 because there are 2 units of negative charge.

Technically, elements other than carbonate and bicarbonate contribute to the total alkalinity (T_A) of the Earth's oceans. Equation (7) describes total alkalinity, rigorously defined by Dickson (1981) as "the number of moles of hydrogen ions equivalent to the excess of proton acceptors…over proton donors," where ellipses

represent such minor acid or base species that they can be omitted, and $[H^+]_F$ represents the free concentration of hydrogen (Dickson et al. 2007).

$$T_A = [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] + [HPO_4^{2-}] \quad (7)$$
$$+ 2[PO_4^{3-}] + [Si(OH)_3^-] + [NH_3] + [HS^-] + \ldots$$
$$- [H^+]_F - [HSO_4^-] - [HF] - [H_3PO_4] - \ldots$$

As stated, the precise relationship between photosynthesis and calcification on the ocean's carbonate chemistry system remains elusive. Equation (8) shows how carbon dioxide and water react to fuel photosynthetic activity (light energy), and how carbon is oxidized through metabolic respiration.

$$CO_{2(g)} + H_2O \underset{\text{Respiration}}{\overset{\text{Photosynthesis}}{\rightleftharpoons}} (CH_2O) + O_{2(g)} \quad (8)$$

$$2HCO_3^- + Ca^{2+} \leftrightarrow CaCO_3 + CO_2 + H_2O \quad (9)$$

($CaCO_3$ Precipitation: $\rightarrow$; $CaCO_3$ Dissolution: $\leftarrow$)

Photosynthesis and respiration are pathways for carbon to enter and exit organisms. These processes, referred to as organic carbon metabolism, influence the rate at which $CaCO_3$ is formed (9).

Calcium carbonate precipitation and dissolution is termed inorganic carbon metabolism; precipitation occurs as reactions move to the right, and dissolution occurs as reactions move to the left (9). Ocean acidification can negatively affect this

reaction regardless of direction. Under normal seawater conditions, Ca^{2+} is not considered limiting to $CaCO_3$ precipitation in corals, strongly suggesting that precipitation rates are influenced primarily by changes in concentrations of CO_3^{2-} (Kleypas et al. 2005; Gazeau et al. 2007). As reaction (3) demonstrates, $CaCO_3$ precipitation is predicted to decline in proportion to expected reductions in CO_3^{2-} ion concentrations as levels of pCO_2 continue rising.

In terms of dissolution, saturation horizons describe depth boundaries (lysocline) at which $CaCO_3$ begins dissolving because surrounding waters lack sufficient concentrations of CO_3^{2-} ions at equilibrium with Ca^{2+} ions. The current saturation horizon for aragonite, the predominant element of *Tridacna* shells, is between 0.5 and 2.5 km from the surface, while the saturation horizon for calcite is between 1.5 and 5 km (The Royal Society, 2005). Figure 4 illustrates CO_3^{2-} curves by depth for aragonite and calcite for a station in the Atlantic. Dots represent CO_3^{2-} concentrations; CO_3^{2-} concentrations to the right of the curve support calcification, while concentrations left of the curve cause $CaCO_3$ to dissolve.

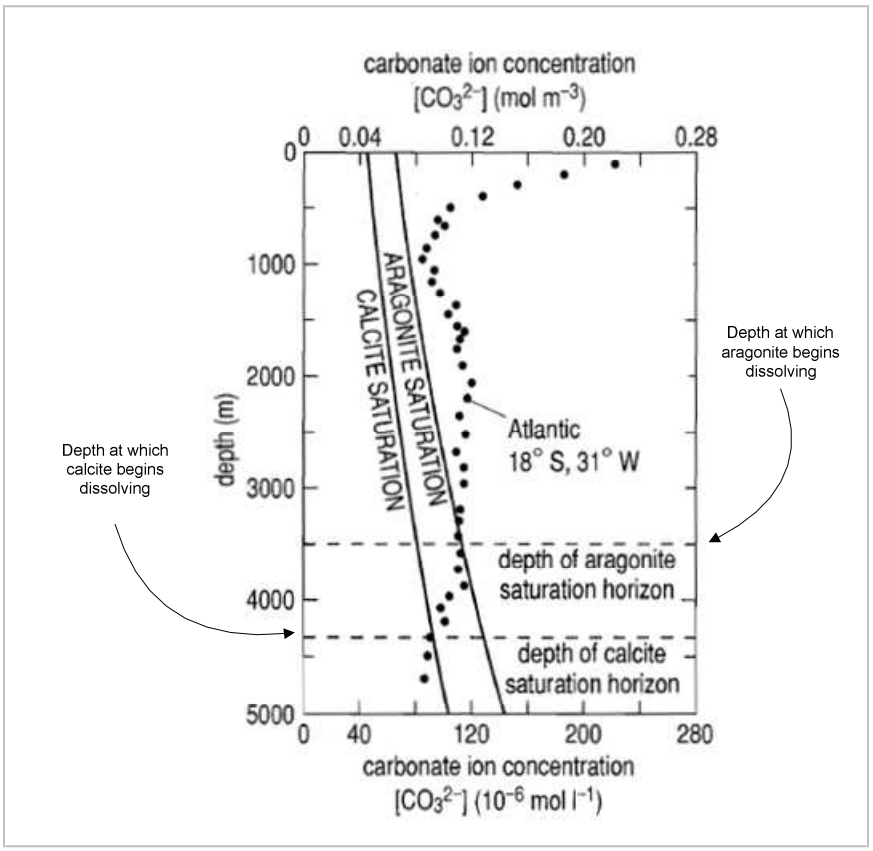

Figure 4: Calcium carbonate concentration curves for aragonite and calcite by depth at a station in the Atlantic. Source: Marine Biogeochemical Cycles, Elsevier, The Open University, 2005, modified by C. Waters.

Differences in horizon depths occur because aragonite is ~50% more soluble in seawater than calcite (Mucci, 1993). Rising saturation horizons may have two significant effects on ocean biogeochemical processes. First, all marine calcifiers must reside above respective saturation horizons for $CaCO_3$ precipitation to occur (The Royal Society, 2005). As saturation horizons rise, marine calcifiers will only survive in waters closer to the surface if they can rapidly adapt to rising saturation horizons. Second, premature dissolution of $CaCO_3$ structures from dead organisms is likely to interfere with normal sequestration of carbon to deep ocean sediments, thereby altering the ocean's role in the global carbon cycle.

Understanding how atmospheric conditions affect ionic equilibria of seawater is fundamental to investigating the biological responses of marine organisms to ocean acidification. The following section briefly investigates the potential ramifications of ocean acidification on ecological processes, economic systems, and social and political activities.

1.4. Ecological, Economic, Social and Political Implications of Ocean Acidification

Previous sections summarized the relationship between increased oceanic acidification and biogeochemical processes such as photosynthesis and calcification that are critical to marine calcifiers engaged in symbiotic associations. This section describes how ocean acidification may be the first in a series of cascading events that influence ecological systems, monetary and resource economies, and social and political activities.

Ocean acidification may affect multiple biological levels from molecules to populations that combine to support ecological systems. For example, many organisms beyond the realm of marine environments may be vulnerable to the

effects of elevated CO_2 in terms of disturbed internal acid-base balance, oxygen transport and metabolic processes that influence species growth, survival and reproduction (Seibel and Fabry, 2003). In addition, ocean acidification coupled with stressors such as water column stratification and decreased upwelling in response to warmer temperatures and climate change may adversely affect species diversity and abundance (Kleypas et al. 2005).

The impact of ocean acidification on the growth and survival of planktonic organisms is particularly salient because planktonic organisms form the base of the human food web (Kleypas et al. 2005; The Royal Society, 2005). Pteropods are an example of planktonic organisms known to be particularly vulnerable to reduced pH conditions (Orr, et al. 2005). This is important because pteropods are at the base of a food web involving salmon, herring, mackerel, cod and baleen whales (Knutzen, 1981; Fabry, 2005; Orr, et al. 2005). If ocean acidification decreases the fitness of marine calcifiers such as pteropods, corals, molluscs and oysters, only species capable of shifting latitudinal distributions or depth ranges may be favored for survival (Seibel and Fabry, 2003).

By default, community economies that rely on the well-being of marine environments may be adversely affected by ocean acidification. For example, increased ocean acidification has been shown to affect certain species of fish (Pörtner et al. 2004). Although there is insufficient information to conclude ocean acidification will affect commercial fish stocks, with global revenues of US $78 billion, any reductions to existing fish stocks may be economically significant. Coral reef degradation as a result of ocean acidification may have adverse effects on commercial fish species (The Royal Society, 2005), further exacerbating direct threats to fish stocks such as over-harvesting or Allee effects. Coral reefs also have

indirect economic benefits since they support world-wide subsistence food gathering by many millions of people (The Royal Society, 2005).

Coastal economies supported by tourism may be economically challenged if coral reef ecosystems become compromised as a result of ocean acidification. 'Reef interested' tourism accounts for 68% of Queensland Australia's total gross regional product, or AUS$1.4 billion dollars (Hoegh-Guldberg and Hoegh-Guldberg, 2004). In 2000, the World Resources Institute estimated that benefits from Caribbean coral reefs in terms of fisheries, dive tourism and shoreline protection were between US$3.1 billion and US$4.6 billion. These values do not account for indirect monetary contributions coral reefs make to stabilizing coastlines or creating favorable conditions for other ecosystems such as mangrove forests and sea grass beds (Hoegh-Guldberg and Hoegh-Guldberg, 2004; The Royal Society, 2005).

Social and political implications of ocean acidification are likely to parallel social and political implications of global warming. In general, society appears more focused on removing excess CO_2 from the atmosphere than curtailing its production (Libes, 2005). The scientific community is justifiably frustrated with the rapid rate of information diffusion but slow social acceptance of scientific fact. Yet, the scientific community has had immeasurable effects in positively influencing the world's social values with respect to the environment. For example, an overwhelming percentage of today's "reduce, reuse and recycle" consumer behavior is a result of rigorous science, cogent explanations and persistent attempts to inform social and political policy. Continual streams of consistently accurate educational material from scientists may continue to positively influence social values and political decisions as much as economic interests. In time, ocean acidification will become a priority item on the world's enviro-political agenda. For example, on July 9, 2008, the United States House Senate Subcommittee on Energy and Environment of the Committee

on Science and Technology passed H.R. 4174: The Federal Ocean Acidification Research and Monitoring Act (FOARAM), a topic completely foreign to politicians just a few years ago.

1.5. Biological Responses of Marine Organisms to Ocean Acidification

Research pioneers investigating the effects of oceanic acidification on marine life controlled seawater pH using acid-base solutions such as hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH). Published results were compelling; a broad spectrum of marine organisms incubated in pH conditions exhibited adverse biological responses. Experimental organisms included corals (Marubini and Atkinson, 1999; Langdon et al. 2003; Langdon and Atkinson, 2005), pteropods (Orr et al. 2005), foraminifera (Wolf-Gladrow et al. 1999), coccolithophorids (Riebesell et al. 2000; Zondervan et al. 2001), algae (Gao et al. 1993; Kuffner, 2007), fish and squid (Pörtner et al. 2004), copepods and sea urchins (Kurihara and Shirayama, 2005), bacteria and nematodes (Takeuchi et al. 1997) and mollusks (Chalebrese, 1966; Bamber, 1990)

An alternative method for controlling seawater pH involves manipulating seawater carbonate chemistry using CO_2 partial pressure (pCO_2) (Burnett 1997). The differences in chemistry between acid-base control of seawater pH and pCO_2 have been explained by Langdon and Atkinson (2005), and the benefits of using pCO_2 to manipulate pH in studies investigating the effects of ocean acidification on marine organisms have been explained by Fabry et al. (2008). A series of research initiatives aimed at quantifying calcification responses of marine organisms exposed to oceanic pH levels controlled by pCO_2 concentrations has recently emerged. Shell length growth rates of *Mytilus edulis* mussels incubated for 44 days in pH conditions ranging from pH 6.7 to pH 8.1 were shown to decline as pCO_2 increased (Berge et

al. 2006). Calcification rates of *M. edulis* mussels and *Crassostrea gigas* oysters exposed to multiple pCO_2 treatments in 2 hour incubation periods declined linearly as pH declined (Gazeau et al. 2007).

Other research initiatives have examined the effects of ocean acidification on calcification and other processes. Leclercq et al. (2002) has reported on the effects of elevated pCO_2 on primary productivity, respiration and calcification of a coral reef community. Iglesias-Rodriguez et al. (2008) recently reported that calcification and respiration rates in coccolithophores increased as levels of pCO_2 increased. Intra- and extra-cellular acid-base parameters, metabolic rates and overall specimen growth of *Mytilus galloprovincialis* mussels have been observed in normocapnia and hypercapnia conditions (Michaelidis et al. 2005). Results following 3 months of incubation revealed a slower rate of shell growth and a corresponding slower rate of soft-tissue growth of *M. galloprovincialis* in hypercapnic conditions. Bibby et al. (2008) suggest that *Mytilus edulis* mussel responses to ocean acidification may include impaired haemocytes and associated cellular signaling pathways. Their results indicate that signaling pathways dependent on specific calcium thresholds may be compromised as $CaCO_3$ dissolution increases in response to reduced oceanic pH.

Research initiatives aimed at quantifying biological responses of marine calcifiers such as corals engaged in symbiotic associations exposed to levels of acidification controlled by increased pCO_2 remain rare (Leclerq, 2002). To date, biological responses of molluscs engaged in symbiotic associations exposed to ocean acidification controlled by increased pCO_2 are unknown.

1.6. Biological Responses of *Tridacna maxima* to Ocean Acidification

This project examines the biological responses on *Tridacna maxima* to increased pCO_2 conditions per IPCC IS92a projections outlined in Table 1: 1) calcification rates measured as shell length, height and percentages of populations exhibiting change in shell precipitation will decline as pCO_2 increases; 2) calcium [Ca^{2+}] ion concentrations will increase in mantle tissue as pCO_2 increases; 3) qualitative differences in aragonite shell crystals will be visible as pCO_2 increases; 4) mantle and shell growth rates measured by Condition Index (CI) ratios and net weight change will differ as pCO_2 increases; 5) population density of zooxanthellae will respond to increases in pCO_2; 6) concentrations of chlorophyll *a* [Chl *a*] per zooxanthellae will respond to increases in pCO_2.

2.0 Materials and Methods

2.1. Clam Specimens

One hundred twenty *Tridacna maxima* clams cultured in the Republic of Vanuatu were shipped via overnight express to the research laboratory at The Evergreen State College in Olympia, Washington, USA. Shell lengths ranging from ~18 mm to ~49 mm indicated an estimated age range of 6 to 12 months (Heslinga et al. 1990). Specimens arrived in individual plastic bags ~8 cm^3 filled with source seawater. Individual bags were set afloat in aquaria described below for >1 hour to facilitate temperature acclimation of specimens. After manually removing visible commensal or potentially parasitic organisms, clam specimens were randomly assigned to four indoor 110-l glass aquaria. Each specimen was fastened by epoxy to individual pebbles marked with unique location identifiers and placed in permanent

positions at the interstices of 1-cm plastic mesh no less than 2 cm away from their nearest neighbor in each aquarium.

2.2. Aquaria

The chemical and physical water parameters in each aquarium were designed to match typical seawater of Oceania over a 13-week incubation period. Artificial seawater (ASW) in all aquaria consisted of Tropic Marin® Pro-Reef sea salt mixed with deionized water to an optimal salinity of 34.5 psu. Stock ASW was diluted with a Marine Biological Laboratory (MBL) seawater recipe (Bidwell & Spotte 1985) but excluded sodium bicarbonate ($NaHCO_3^-$) and was mixed to 34.5 psu. This carbonate-free artificial seawater (CFASW) was used to offset artificially high total alkalinity (T_A) levels in the commercial ASW mix that could skew pCO_2 values. Over the course of the experiment, elevated T_A levels were adjusted downward by exchanging tank water with CFASW, while low T_A levels were adjusted upward by infusions of commercial ASW.

Each aquarium was fitted with a dedicated Rena® Filstar xP2 water filtration canister containing mechanical and biological media to circulate aquarium water at ~280 L/h. A Rio® 180 circulation power head augmented surface water circulation. Finnex® (HMT-150) electronic heaters maintained aquaria temperatures at 27.5º C (± 1.0). Lighting for each tank consisted of 250 Watt (19000 lumen) MINIHQI250 Pendent/Retrofit Krystal Star metal halide lights with a 11000K spectral composition for an 11:11 light and dark photoperiod punctuated by 1 h of indirect exposure to 60 Watt “dawn” and “dusk” compact fluorescent lighting provided by Aqualight™. White foam core light barriers separated each tank to eliminate light spill-over from adjacent aquaria lights.

Every evening, 5 ml of DT's® Premium Reef blend live marine phytoplankton was added to each tank, and an additional 5 ml of PhycoPure™ phytoplankton-zooxanthellae mix was added on alternate evenings. Once per week, each tank received 5 ml of Seachem™ Reef Complete to help maintain optimal calcium (Ca^{2+}), magnesium (Mg) and strontium (Sr) levels.

2.3. Carbonate Chemistry

Each tank was fitted with a CO_2 bubbling system consisting of 5 lb. gas bottles of commercially available CO_2, Milwaukee MA957 CO_2 gas regulators connected to submerged membrane CO_2 reactors, check valves and Aqua Medic (GmbH) pH controllers. The pH probes dedicated to each controller were recalibrated every other day using Markson LabSales buffers (pH 4.00, pH 7.00 and pH 10.00 NIST). The pH controllers were programmed to control tank-specific pH set points reflecting projected levels of pCO_2. Hysteresis ranges maintained pH levels at ± 0.05 margins. Chemical and physical conditions in each tank are shown in Table 2. For this experiment, all pH values were rounded to the nearest 0.1 per IPCC estimates with the exception of pH condition 7.76, which was rounded to pH 7.70 to create even intervals between all pH treatments. A mixture of N_2 (79%) and oxygen O_2 (21%) gases were bubbled into the tank with the lowest pCO_2 to reach a pH level of 8.30 (± 0.05) since ambient pCO_2 tended to reduce pH well below the targeted 8.30 (± 0.05) control threshold. When the N_2/O_2 mixture failed to raise pH to desired levels, 0.4 M sodium hydroxide (NaOH) was added, typically in 0.5 ml increments that never exceeded 40.0 ml/d. Final chemical and physical conditions in each aquarium are shown in Figure 1.

2.4. Data Collection

Values for pH, temperature and salinity were recorded at least twice daily for the duration of the experiment. Observed pH values were collected from Aqua Medic pH micro-processors. An Oakton® Ion 6 pH/Ion/ºC meter was used to collect temperature values, and a YSI 3100 conductivity meter (Yellow Springs Instrument Co., Inc.) was used to measure salinity psu. Values for dissolved oxygen (DO %), nitrate (NO_3^-), ammonia (NH_3), Ca^{2+} and T_A were collected on a weekly basis. DO was measured using a YSI 85 meter. Nitrate and ammonia levels were determined with over-the-counter dip test kits. Qualitative data (color matching) test strips were used to ensure experimental seawater calcium (Ca^{2+}) ranged from 400 mg l-1 to 450 mg l-1. Frequent ASW exchanges in significant volumes precluded the significance of measuring Ca^{2+} using atomic absorption spectrometry.

Total alkalinity values were collected weekly by titrating tank-specific water with standardized hydrochloric acid (HCl). Acidimetric titration data were copied to the United States Geological Survey (USGS) on-line Alkalinity Calculator (Rev 2.20). The fixed endpoint method was used to determine total hydroxide (OH^- mg l^{-1}), CO_3^{2-} mg l^{-1}, and HCO_3^- mg l^{-1} concentrations. USGS calculations were input to a custom Excel spreadsheet that calculated $2[CO_3^{2-}] + [HCO_3^-]$. Total alkalinity output values, daily mean salinity, temperature and pH (seawater scale) values were entered in the CO2sys_macro_PC.xls program (Lewis and Wallace, 1998) distributed by the Carbon Dioxide Information and Analysis Center (CDIAC) to calculate tank-specific pCO_2 levels and other elemental data. Output values were used in determining whether high-carbonate ASW, CFASW (MBL), or a 325:75 mixture (T_A = 2200.8 µmol/kg) of the two was added or exchanged in tanks to attain or maintain alkalinity thresholds needed to achieve targeted pH values at the chosen pCO_2.

After a 3-week acclimation period at pCO_2 180 ppmv in all tanks, pCO_2 levels in 3 tanks were adjusted upward over a 3-day period to achieve chosen experimental pCO_2 levels for each tank. On a weekly basis, all *T. maxima* specimens were removed from respective aquaria, cleaned of algal and parasitic growth, pat-dried, and measured for shell length, height and weight. Cen-tech electronic digital calipers (accuracy: 0.02 mm) were used to collect shell length and height values. Shell length measurements were taken at the maximum lateral distance that touched both caliper arms. Shell height followed the same protocol except in a vertical direction from the umbo to the farthest (highest) mantle edge.

A combination of methods for calculating condition indices introduced by Lawrence and Scott (1982) and Rebelo et al. (2005), was used to determine the condition index (CI) of each specimen based on dry tissue weight (24 h/60ºC) and shell volume (CI = dry weight (g) x 100/internal cavity volume ml). Dry tissue and shell weights were measured using a Mettler Toledo AB204-S balance. Tissue [Ca^{2+}] of whole animals was measured using a Perkin Elmer Elan, model DRC-e Atomic Absorption spectrophotometer.

To measure zooxanthellal population density per specimen, the mantle tissue of each specimen was separated from other organs, blotted dry and weighed. Mantle tissue was placed in a 5 ml homogenizer containing 3 ml of artificial seawater (ASW) and ground in a pestle mortar 50 times. Homogenate was centrifuged in a test tube at 1150 rpm for 5 minutes. Supernatant was drained leaving only zooxanthellal pellets. Pellets were immersed in 3 ml of ASW and shaken vigorously. Sample solution was immediately transferred via pipette to a 0.1 mm hemocytometer for cell counts. Cell counts were multiplied by sample volume to determine zooxanthellal density per ml. Four replicate counts of zooxanthellae were made for each specimen.

Chlorophyll *a* was measured at 664 nm and 647 nm with a Hewlett Packard 3458 diode array spectrophotometer.

2.5. Scanning Electron Microscopy Imaging Protocol

Tridacna maxima shells were mechanically cleaned of all soft tissue. Leading shell edges were fractured and labeled. Every effort was made to collect images from the same relative location of a cross-section of leading shell edges where precipitation is expected to be recent. Each shell was placed in 35% hydrogen peroxide solution for 40 minutes to digest organic material on the surface of the shell. Shell edges were air dried, sputter coated with gold palladium, mounted in 12 mm outside diameter Pelco mounting tabs and placed in a Jeol JSM-6480LV Scanning Electron Microscope with a Control User Interface, Version 7.06 ©2004 Jeol Techniques, Ltd., for image collection.

2.6. Statistical Analysis

Statistical significance for all tests was determined at $P < 0.05$. Intra-treatment differences were evaluated using Student's t-test, paired two samples for means. Differences across treatments were evaluated using ANOVA, single factor analysis, followed by Tukey's HSD. Reported error (±) represents Standard Error of the Mean (SEM). Linear regression was used to correlate morphometric parameters.

3.0 Results

3.1. Chemical and Physical Conditions

Tridacna maxima were successfully incubated for 13 weeks in laboratory aquaria containing experimental seawater with chemical and physical characteristics

listed in Table 2. Mean target values for pCO_2 deviated less than 1% from target values for pCO_2 in 3 aquaria. Mean pCO_2 values for the fourth aquarium were 340 ppmv versus the target value of 380 ppmv. However, the mean seawater pH level for the tank with 340 ppmv pCO_2 was 8.09, very close to the pH target value of 8.10 (± 0.05) for the duration of the experiment. As pCO_2 increased, pH levels declined. Total alkalinity (T_A) gradually declined as pCO_2 increased and, as expected, concentrations of HCO_3 increased and CO_3^{2-} declined as pCO_2 increased. All aquaria were supersaturated with aragonite although Ω_{ar} declined to 1.43 in highest pCO_2 conditions. All other chemical and physical parameters across experimental aquaria were similar for the duration of the experiment. Overall, *T. maxima* mortality over the 13-week period was < 4%. One specimen died in pCO_2 conditions of 180 ppmv, 3 in pCO_2 380 ppmv, 1 in pCO_2 560 ppmv and zero in pCO_2 840 ppmv.

Table 2: Chemical and physical parameters for each tank of experimental artificial seawater (ASW) used to incubate *Tridacna maxima* specimens for a 13-week period.

The first set of numbers represents mean values for each control parameter. The second set of numbers represent standard error (±) values for each parameter, and the third set of numbers represent sample size. 1) Input parameters for CO2SYS.xls program (K1, K2 from Mehrbach et al. 1973, refit by Dickson & Millero, 1987) based on weekly data collection inputs. 2) T_A values from CO2SYS output converted to meq/kg. 3) CO2SYS output based on inputs (1) above. 4). Weekly Ca^{2+} ion concentration values are based on Atomic Absorption analysis of ASW. 5) Mean values based on daily a.m., mid-day and p.m. collections. 6) Values based on data collected on a weekly basis.

CONDITION PARAMETERS (UNITS)	GLACIAL 180 PPMV (PH 8.30)	CURRENT 380 PPMV (PH 8.10)	2 X PRE-IND. 560 PPMV (PH 7.90)	3 X PRE-IND. 840 PPMV (PH 7.70)
Salinity (‰)[1]	34.5, ± 0.1, 16	34.7, ± 0.2, 14	34.8, ± 0.1, 14	34.2, ± 0.2, 13
Temperature (ºC)[1]	27.5, ± 0.1, 16	27.6, ± 0.1, 14	27.6, ± 0.2, 14	27.7, ± 0.3, 13
T_A (µmol/kgSW)[1]	2180, ± 41, 16	2155, ± 20, 14	2108, ± 45, 14	1983, ± 47, 13
pH (Seawater)[1]	8.3, ± 0.0, 16	8.1, ± 0.0, 14	7.9, ± 0.0, 14	7.7, ± 0.0, 13
T_A (meq/kg)[2]	2.23, ± 0.04, 16	2.20, ± 0.02, 14	2.16, ± 0.04, 14	2.03, ± 0.04, 13

Condition Parameters (Units)	Glacial 180 ppmv (pH 8.30)	Current 380 ppmv (pH 8.10)	2 X Pre-Ind. 560 ppmv (pH 7.90)	3 X Pre-Ind. 840 ppmv (pH 7.70)
pCO_2 (µatm/ppmv)[3]	180, ± 5, 16	340, ± 5, 14	564, ± 13, 14	849, ± 23, 13
HCO_3^- (mmol/kgSW)[3]	1391, ± 28, 16	1598, ± 18, 14	1719, ± 38, 14	1720, ± 38, 13
CO_3^{2-} (mmol/kgSW)[3]	317, ± 7, 16	223, ± 2, 14	156, ± 4, 14	104, ± 5, 13
Ω Ar[3]	4.6, ± 0.1, 16	3.6, ± 0.0, 14	2.2, ± 0.1, 14	1.4, ± 0.1, 13
Ca^{2+} (mg l^{-1}) (AA)[4]	411, ± 7, 15	438, ± 5, 14	444, ± 2, 15	435, ± 4, 15
pH[5]	8.3, ± 0.0, 210	8.1, ± 0.0, 210	7.9, ± 0.0, 210	7.7, ± 0.1, 210
Salinity (‰)[5]	34.4, ± 0.1, 210	34.4, ± 0.4, 210	34.9, ± 0.4, 210	34.3, ± 0.3, 210
Temperature (ºC)[5]	27.5, ± 0.1, 210	27.6, ± 0.1, 210	27.4, ± 0.5, 10	27.6, ± 0.3, 210
DO (%)[6]	71.4, ± 1.6, 12	77.9, ± 1.3, 14	77.7, ± 3.3, 13	76.2, ± 5.5, 13
Ca^{2+} (mg l^{-1})[6]	448, ± 3, 12	447, ± 3, 12	453, ± 10, 12	450, ± 7, 12
Nitrate (NO_3^-)[6]	0.0, ± N/A, 12	0.0, ± N/A, 12	0.0, ± N/A, 12	0.0, ± N/A, 12

Condition Parameters (Units)	Glacial 180 ppmv (pH 8.30)	Current 380 ppmv (pH 8.10)	2 X Pre-Ind. 560 ppmv (pH 7.90)	3 X Pre-Ind. 840 ppmv (pH 7.70)
Ammonia (NH_3) (ppmv)[6]	0.0, ± N/A, 12	0.0, ± N/A, 12	0.0, ± N/A, 12	0.0, ± N/A, 12

3.2. Calcification Response: Shell Length and Height

Differences in shell length and height of *Tridacna maxima* specimens between experimental aquaria at the start of the experiment were not statistically significant (ANOVA, $P > 0.05$). However this changed by the end of the experiment. Mean differences in shell length remained unchanged in aquaria with low pCO_2 conditions (Student's paired t-test, $P < 0.05$) (Fig. 5a). However, shell lengths declined significantly between pCO_2 conditions of 180 ppmv and pCO_2 840 ppmv over the 13-week period (ANOVA, $P < 0.05$, Tukey's post-hoc analysis).

Differences in shell height within each experimental condition over the 13-week period were more pronounced (Student's paired t-test, $P < 0.05$) (Fig. 5b). Mean specimen shell heights in the three lowest pCO_2 conditions increased, while mean shell heights of specimens in pCO_2 conditions of 840 ppmv decreased. Mean differences in shell height between populations incubated in the three lowest pCO_2 conditions were not significantly different, but were significantly different in pCO_2 conditions of 840 ppmv (ANOVA, $P < 0.05$, Tukey's post hoc analysis).

(a) (b)

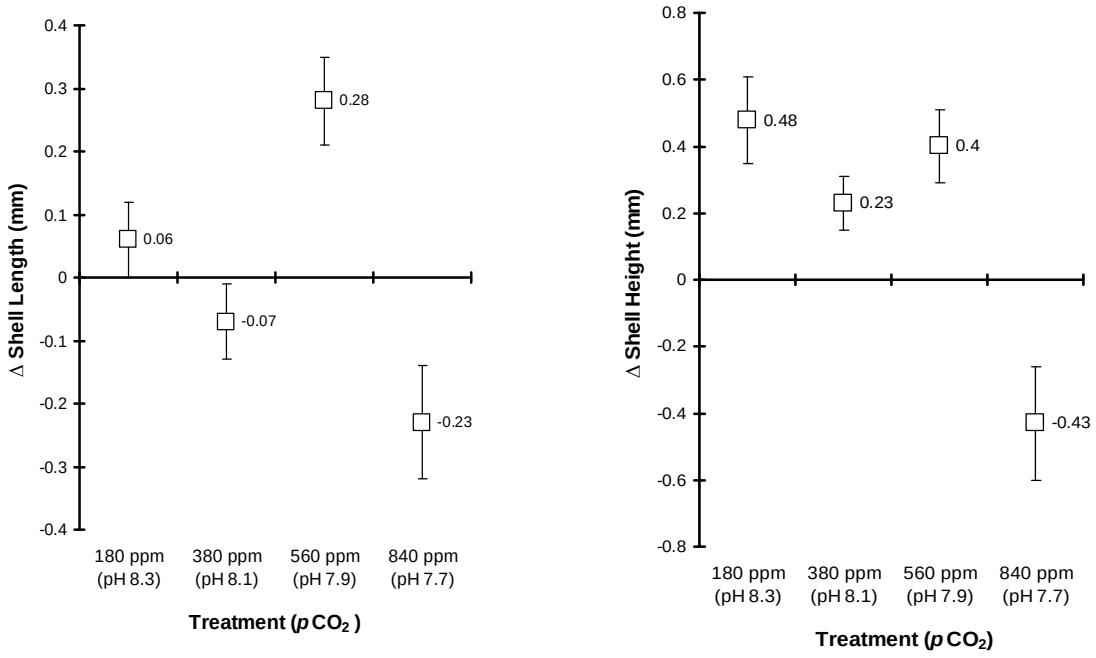

Figure 5: Mean differences in calcification response rates in terms of (a) shell length and (b) shell height of juvenile *Tridacna maxima* clams within populations by pCO_2 treatment over a 13-week period. Differences within populations were not statistically significant (Student's paired t-test, $P > 0.05$,) but were significant between populations (ANOVA, $P < 0.05$, Tukey's post hoc analysis). Y-error bars represent standard error values (±).

Shell length and height revealed striking differences in individual shell growth patterns between pCO_2 conditions. Over half of the specimens incubated in the lowest pCO_2 condition of 180 ppmv exhibited shell growth in terms of both shell length and height (Fig. 6). In contrast, at the highest pCO_2 conditions of 840 ppmv, over 60% of individual specimens exhibited decreases in both shell length and height. Individual specimens growing in either one or both dimensions by pCO_2 condition were 88.9% in 180 ppmv, 92.3% in 380 ppmv and 86.2% in 560 ppmv. These growth patterns contrasted sharply with those specimens in pCO_2 conditions of 840 ppmv where only 39.3% of specimens exhibited shell growth in either dimension. There was no significant correlation between shell length and shell height at the start of the experiment and end of the experiment.

There was substantial individual variation of growth patterns in response to pCO_2 conditions. All tanks had individuals that increased and decreased in size. However, the patterns between the tanks at the highest and lowest pCO_2 are striking. In the tank with the lowest pCO_2, over half of the individuals increased in both height and length. In the tank with the highest pCO_2, over 60% of the individuals decreased in both height and length. At pCO_2 of 180 ppmv, 340 ppmv and 560 ppmv, 88.9%, 92.3% and 86.2% of the individuals, respectively, exhibited growth in either or both of the parameters. This contrasted greatly with clams at a pCO_2 of 840 ppmv, where only 39.3% of the clams exhibited shell growth in one or both dimensions.

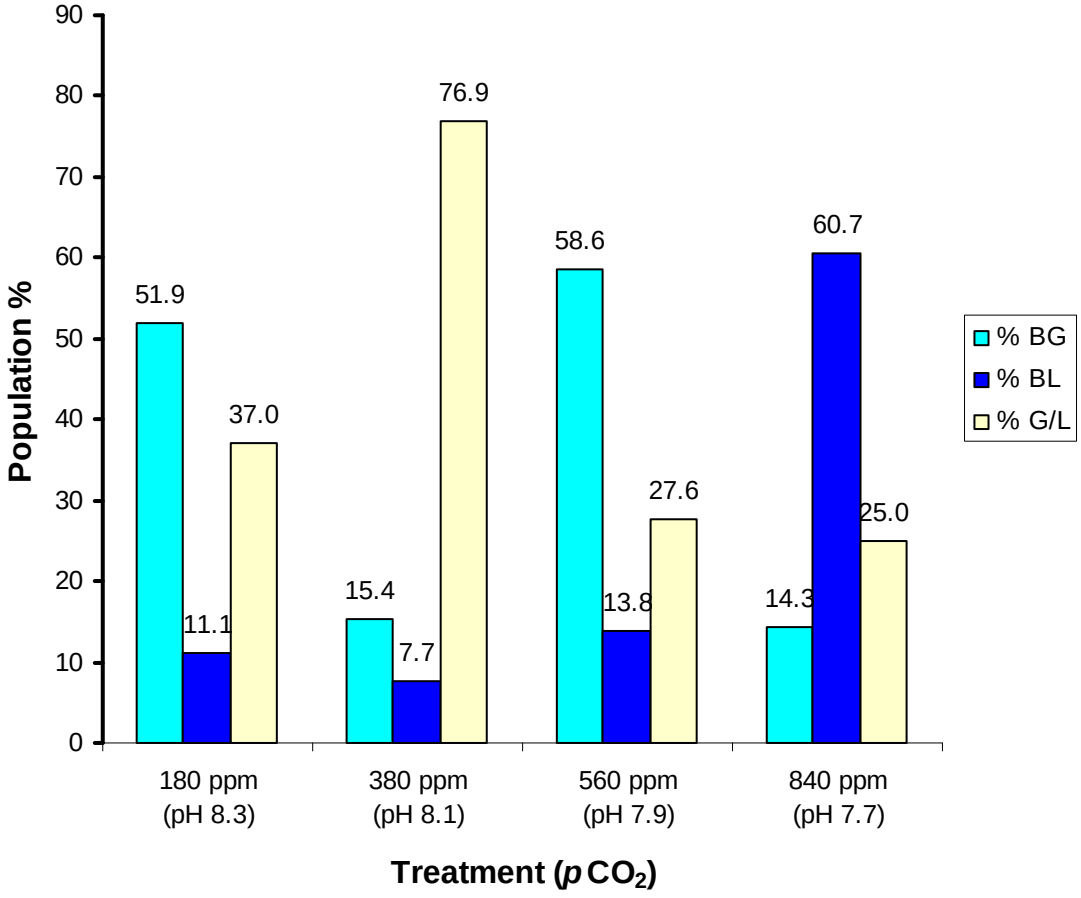

Figure 6: Population percentages of *Tridacna maxima* specimens that gained in both length and height (BG), lost in both length and height (BL), and gained in one dimension but lost in the other (G/L) by pCO_2 treatment over a 13-week period.

3.3. Calcification Response: Calcium (Ca^{2+}) Concentrations in Soft Tissue

Concentrations of Ca^{2+} ions in dry *T. maxima* soft tissue after a 13 -week incubation period in pCO_2 conditions of 180 ppmv, 380 ppmv and 540 ppmv were 63.8 mg/g (± 12.3, n = 15), 57.8 mg/g (± 7.7, n = 15) and 61.1 mg/g (± 12.5, n = 15), respectively, averaging 62.2 mg/g (Figure 7). Differences in pCO_2 concentrations between these populations were not statistically significant (ANOVA, $P > 0.05$). The mean concentration of Ca^{2+} ions in the highest pCO_2 condition of 840 ppmv was ~35% higher at 82.4 mg/g (± 8.0, n = 15) dry tissue weight, but this was not statistically different from the lower pCO_2 conditions (ANOVA, $P > 0.05$).

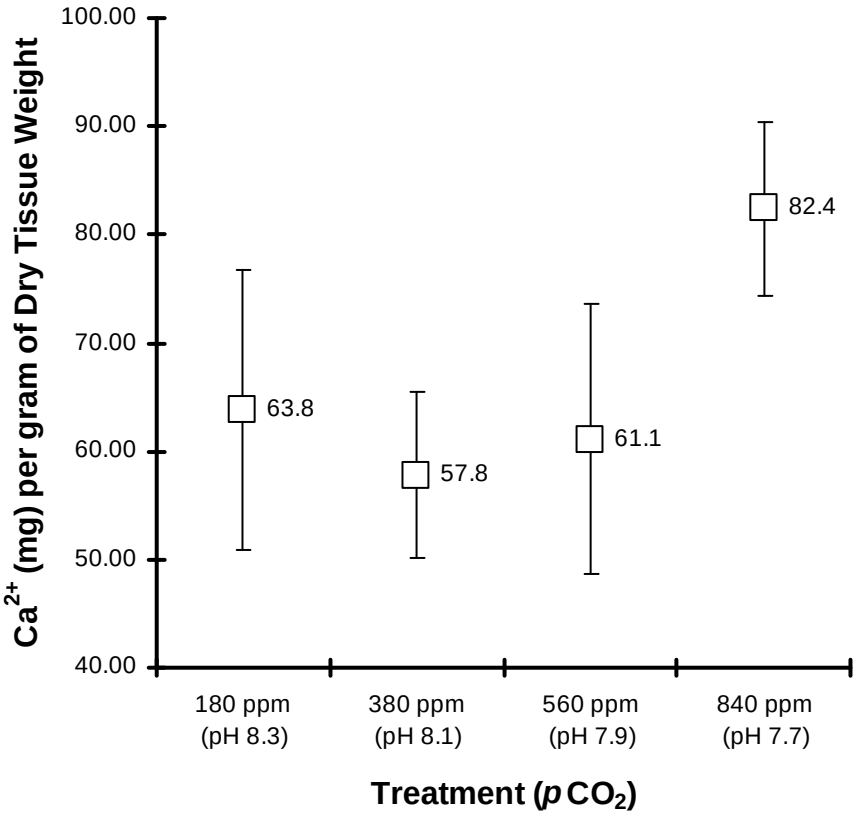

Figure 7: Mean differences in Ca^{2+} ion concentrations per gram of dry tissue weight of *Tridacna maxima* mantle tissue by pCO_2 treatment at the end of a 13-week incubation period were not significantly different (ANOVA, $P > 0.05$). Y-error bars represent standard error values (±).

3.4. Calcification Response: Aragonite Shell Crystal Conditions

Qualitative comparisons between scanning electron microscope images suggest conditions of individual aragonite crystals differ as pCO_2 increases (Figure 8). Individual shell crystals in pCO_2 treatments of 180 ppmv appear well defined by crisp edges, while individual crystals from shells incubated in higher pCO_2 conditions appear rounded and corroded.

(a) (b)

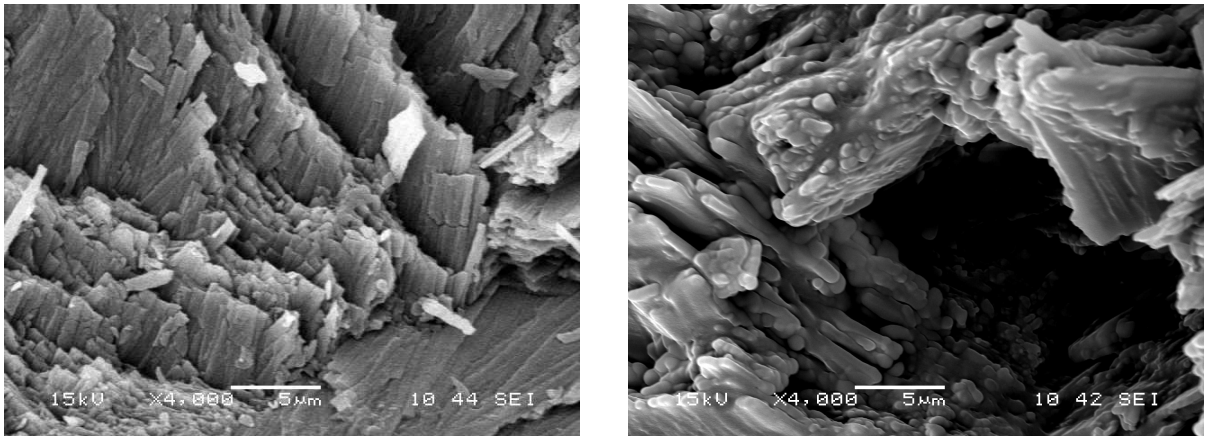

Figure 8: Qualitative differences in the condition of *T. maxima* shell aragonite crystals in pCO_2 180 ppmv (a) and pCO_2 840 ppmv (b). Images were taken from leading mantle shell locations where most recent shell extrusion is expected. Source: R. Peroutka

3.5. Calcification and Soft Tissue Response: Growth Ratios and Weights

Within each aquarium, mean differences in net weight were significantly different from introduction through week 13 (Student's paired t-test, $P < 0.05$) (Figure 9). The mean net differences in weight gain of specimens in pCO_2 380 ppmv conditions were significantly different from specimens incubated in pCO_2 180 ppmv and 840 ppmv (ANOVA, $P < 0.05$, Tukey's post hoc analysis). All specimens in the lowest and highest pCO_2 conditions exhibited net weight gain over the 13-week incubation period. Five of the 27 clams in pCO_2 conditions of 380 ppmv did not increase in net weight, and 1 of the 29 clams in pCO_2 conditions of 560 ppmv did not increase in weight. Ratios between dry tissue weight and shell weight between all pCO_2 conditions were not significant (ANOVA, $P > 0.05$) (Figure 10). After a 13-week incubation period, the differences in ratios between dry tissue weight and shell volume (Condition Index) values between all pCO_2 treatments were not significant (ANOVA, $P > 0.05$) (Figure 11).

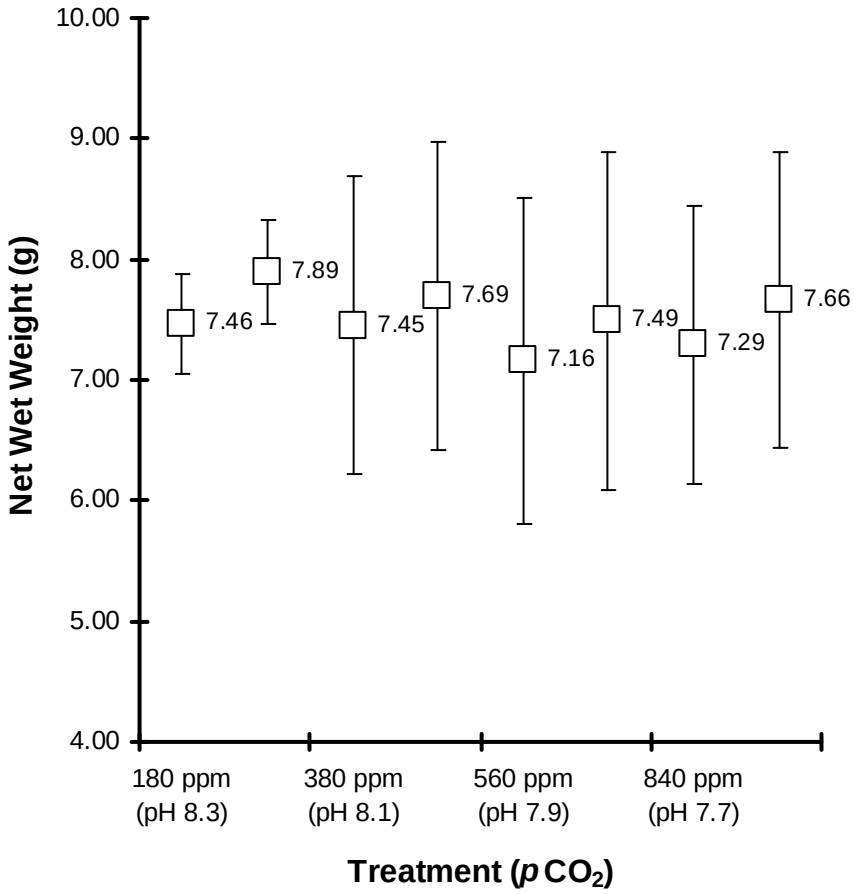

Figure 9: Differences in mean net weight (g) response of *Tridacna maxima* specimens to increased pCO_2 at introduction (□) and at 13 weeks (■) were significant within treatment populations (Student's paired t-test, $P < 0.05$). Differences in mean net weight gain in pCO_2 380 ppmv were statistically significant between pCO_2 treatments of 180 ppmv and 840 ppmv (ANOVA, $P < 0.05$, Tukey's post hoc analysis). Y-error bars represent standard error values (±).

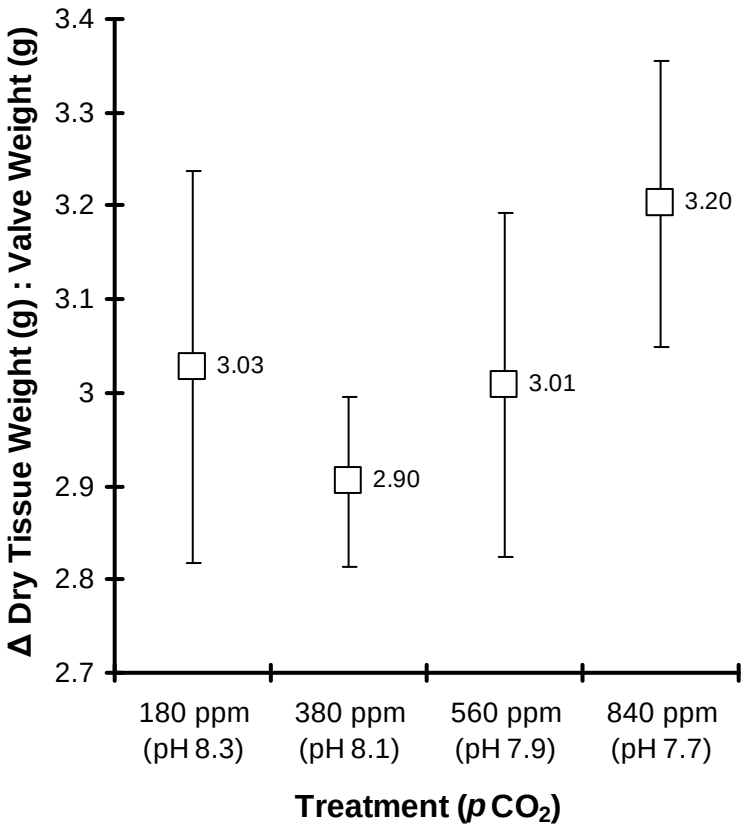

Figure 10: Mean dry soft-tissue to shell weight response ratios of *Tridacna maxima* to decreased pH conditions over a 13-week period were not significantly different (ANOVA, $P > 0.05$). Y-error bars represent standard error values (±).

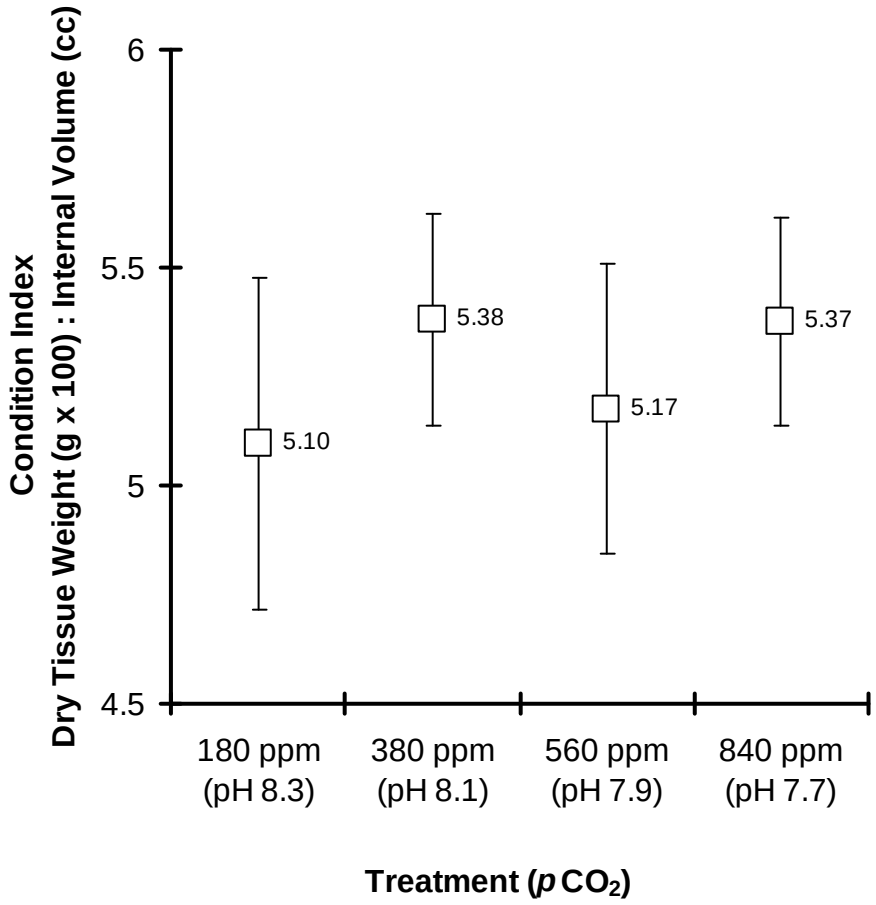

Figure 11: Differences in Condition Index (CI) responses of *Tridacna maxima* specimens to different pCO_2 conditions over a 13-week period were not statistically significant ($P > 0.05$). Index values were derived from dry tissue weight (g) and shell volume (cc^3). Y-error bars represent standard error values (±).

3.6. Symbiotic Association: Zooxanthellae Population Density

Mean net differences in zooxanthellal population density per gram of wet mantle tissue over a 13-week period were not statistically significant (ANOVA, $P > 0.05$, Tukey's ad hoc analysis). The highest concentrations of zooxanthellae were 17.48×10^6 and 18.00×10^6 in pCO_2 conditions of 180 ppmv and 560 ppmv, respectively (Figure 12).

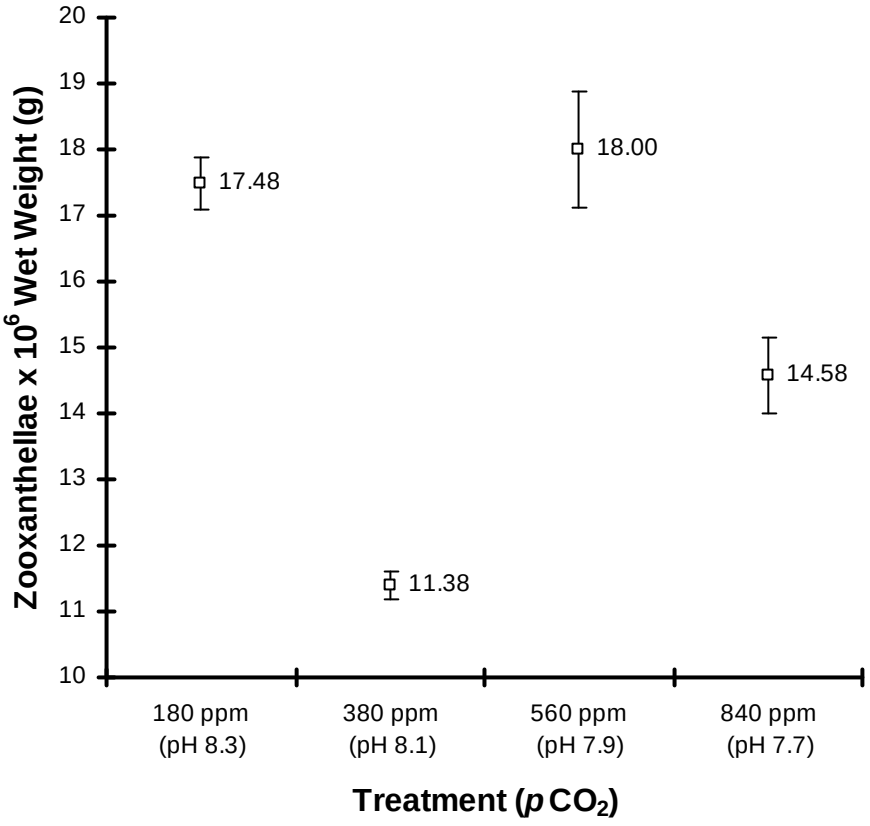

Figure 12: Mean counts of zooxanthellal population density per gram of *Tridacna maxima* mantle tissue by pCO_2 treatment after a 13-week incubation period. Differences in population densities across pCO_2 treatments were not statistically significant. Y-error bars represent standard error values (±).

3.7. Symbiotic Association: Chlorophyll *a* Density per Algal Cell

The concentration of chlorophyll *a* [chl *a*] per zooxanthella cell in the pCO_2 treatment of 180 ppmv was 22.84 X 10^{12} (± 2.75 x 10^{12}), significantly different from concentrations zooxanthella cells incubated in pCO_2 conditions of 840 ppmv 11.52^{12} (± 1.27 x 10^{12}) (ANOVA, $P < 0.05$, Tukey's post hoc analysis) (Figure 13). Concentrations of chl *a* per zooxanthella cell in pCO_2 conditions of 380 ppmv and 560 ppmv were 16.63 x 10^{12} (± 1.30 x 10^{12}) and 16.43 x 10^{12} (± 2.59 x 10^{12}). Differences in the mean concentration of chl *a* per zooxanthella cell were statistically significant between pCO_2 180 ppmv and all other treatments (ANOVA, $P < 0.05$, Tukey's post hoc analysis).

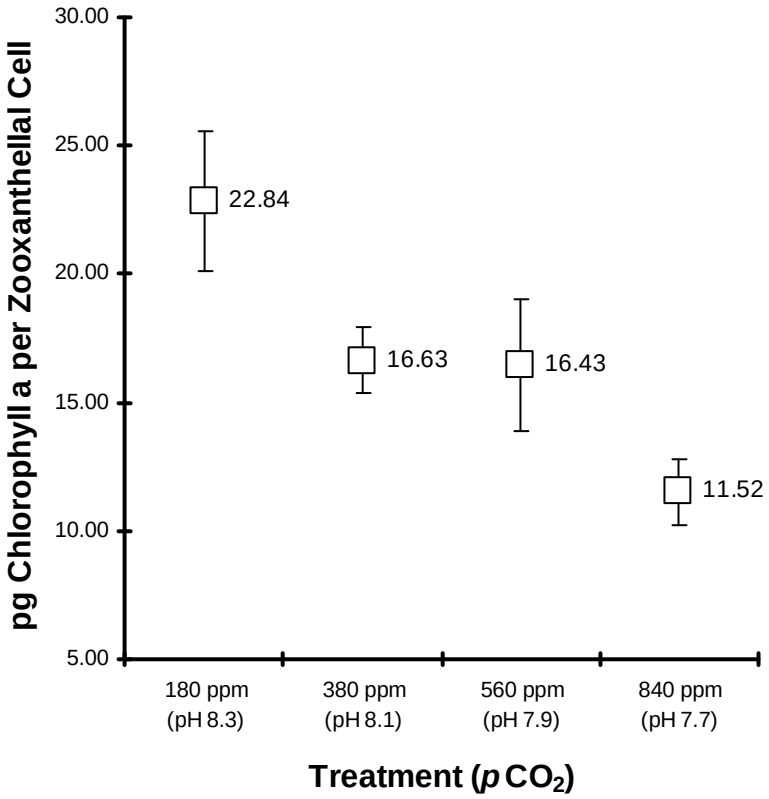

Figure 13: Chlorophyll *a* content in mantle tissue of *Tridacna maxima* expressed as pg chl *a* per zooxanthella by pCO_2 treatment over a 13-week period. Concentrations of chl *a* in pCO2 conditions of 180 ppmv were significantly different from all other treatments (ANOVA, $P < 0.05$, Tukey's post hoc analysis). Y-error bars represent standard error values (±).

4.0 Discussion

Results of this experiment indicate that juvenile *Tridacna maxima* specimens incubated in pCO_2 conditions of 840 ppmv over a 13-week period exhibited negative biological responses in terms of shell growth, population percentages exhibiting net shell growth, soft-tissue accumulation of un-precipitated Ca^{2+} and the condition of aragonite crystals in their shells. Experimental results also indicate that soft-tissue development continued even though shell precipitation declined. Although it appears that elevated pCO_2 did not effect the symbiotic association between *T. maxima* and zooxanthellae population densities, there was a significant decline in the concentration of chlorophyll *a* per zooxanthellal cell as pCO_2 increased. In general, we may tentatively predict that *T. maxima* clams will begin exhibiting negative biological responses when oceanic levels of pCO_2 exceed ~563 ppmv (pH ~7.90).

4.1. Materials and Methods

Biological responses of *Tridacna maxima* specimens incubated in pCO_2 conditions of 840 ppmv were particularly acute. From a methodology perspective, this is significant for two reasons. First, the initial experimental design specified a control of pCO_2 180 ppmv and variable treatments of pCO_2 380 ppmv and pCO_2 pH 560 ppmv; without adding a pCO_2 treatment of pCO_2 840 ppmv, little would be known about extended pCO_2 tolerance thresholds of *T. maxima*. Second, observing results from a broader spectrum of pCO_2 intervals and concomitant pH values helped identify more specific response tolerance thresholds (cf Gazeau et al. 2007). Inherent to identifying more specific tolerance thresholds is the ability to begin classifying organisms as bio-indicators of certain environmental conditions.

Several datasets from *T. maxima* specimens incubated in pCO_2 conditions of 380 ppmv appear anomalous. For example, a comparison of datasets for shell length

and height reveals a parallel pattern; calcification rates fluctuate between pCO_2 180 ppmv and pCO_2 560 ppmv before dropping sharply between pCO_2 560 ppmv and pCO_2 840 ppmv. The initial decrease in differences from pCO_2 180 ppmv to pCO_2 380 ppmv in both metrics remains a mystery. One possible explanation may be the inadvertent introduction of error in my initial size frequency distribution calculations for specimens in pCO_2 380 ppmv conditions.

4.2. Chemical and Physical Experimental Conditions

Variation in experimental carbonate chemistry parameters used in other studies of bivalves make comparisons between this experiment and other ocean acidification research initiatives challenging. Table 3 shows carbonate chemistry parameters based in the IPCC IS92a “business as usual” scenario compared to parameters used for this experiment and parameters used in other investigations of bivalves to increasing levels of pCO_2.

Table 3: Comparison between present oceanic carbonate chemistry parameters per IPCC estimates before the end of this century, this experiment, and other studies of bivalve responses to increasing pCO_2, where N/A = Not Applicable, and (*) = not reported in published parameters but calculated by entering three of four reported parameters into the CO2sys_macro_PC.xls program (Lewis and Wallace, 1998). In each table cell, upper and lower values reflect upper and lower boundaries of pCO_2 concentrations, respectively.

	pCO_2 (ppmv)	**pH**	**HCO_3^-** (µmol kg^{-1})	**CO_3^{2-}** (µmol kg^{-1})	**T_A** (µEqiv kg^{-1})	Ω_{arag}	*Summary Results*
IPCC	180 840	8.32 7.76	1666 2004	279 115	2356 2287	4.26 1.81	N/A
Tridacna maxima	180 ± 5 849 ± 5	8.29 ± 0.05 7.72 ± 0.05	1391 ± 28 1720 ± 38	317 ± 7 104 ± 5	2180 ± 41 1983 ± 47	4.6 ± 0.1 1.4 ± 0.1	Shell dissolution, steady mantle development, increased soft-tissue concentrations of Ca^{2+}, inconclusive change to zooxanthellal population density, and decreased chl *a* per algal cell were observed at pCO_2 between 563 and 840 ppmv.
Crassostrea gigas (Kurihara et al. 2007)	348 2268	8.21 ± 0.08 7.42 ± 0.02	1506 1825	161.4 26.4	1964 ± 0 1964 ± 0	3.00 0.68	Impaired larval morphology and inhibited shell mineralization were observed at pCO_2 2268 ppmv.
Mytilus galloprovincialis) (Michaelidis et al. 2005)	1079 5026	8.05 ± 0.02 7.3 ± 0.0	2890 ± 380 2340 ± 130	254* 373*	6427* 4950*	3.85* 0.57*	Reduced shell length, changed acid-base variables in haemolymph (HCO_3^- accumulation), and reduced O_2 consumption were observed at pCO_2 between 1079 and 5026 ppmv.

	pCO_2 (ppmv)	pH	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	T_A (µEqiv kg^{-1})	Ω_{arag}	*Summary Results*
Mytilus edulis	421	8.13	1916*	189	2421	3.4	Reduced calcification rates were
Crassostrea gigas	2351	7.46	2015*	53	2546	1.0	observed at 740 ppmv.
(Gazeau et al. 2007)							
Crassostrea gigas	698	8.07	2237*	170	2740	3.1	Shell dissolution was observed at
(Gazeau et al. 2007)	2774	7.55	2562*	60	2736	1.1	1800 ppmv.

Explaining the disparity between experimental conditions shown in Table 3 is difficult because oceanic pH values as a result of increased pCO_2 values are influenced by multiple biotic and abiotic factors (Seibel and Fabry, 2003). In addition, not all research initiatives report conditions known to affect observed pCO_2 values. Any effort to establish experimental standards for parameters in ocean acidification investigations is mitigated by known variations in responses of species and species habitats. However, reporting all measured parameters affecting experimental carbonate chemistry conditions would aid in evaluating similarities and differences in variables and responses between research initiatives.

Carbonate chemistry for this experiment over a 13 week period closely mimicked oceanic conditions predicted by IPCC's "business as usual" IS92a scenario with the exception of IPCC estimates for current levels of pCO_2. The pCO_2 levels for this experiment 340.5 ppmv were ~10% below levels estimated by the IPCC assuming normal air-sea fluctuations (Gloor, 2003), yet well within the expected oceanic pH value of 8.10 (± 0.05) at 8.09.

4.3. Calcification Responses (Shell Growth)

Tridacna maxima calcification responses to increased pCO_2 differed from results of other research initiatives measuring responses of molluscs incubated in high pCO_2 conditions. For example, Michaelidis et al. (2005) observed that growth rates of *Mytilus galloprovincialis* decline between normocapnic (pH 8.05 ± 0.02) and hypercapnic (pH 7.3 ± 0.03) treatments. Conversely, *T. maxima* shell lengths in this experiment exhibited negative growth ($CaCO_3$ dissolution) between pH 7.9 and pH 7.72. *T. maxima* responses also differed from experimental observations of *Mytilus edulis* mussels incubated in elevated pCO_2 conditions (Berge et al. 2006). *M. edulis* mussels incubated in pH treatments ranging from pH 6.7 to pH 8.1 exhibited virtually

no growth of shell length at pH 6.7, and no significant difference in growth between pH 7.4 and pH 7.6. In contrast, the mean change in shell lengths of *T. maxima* was ~70% lower at pCO_2 840 ppmv than pCO_2 380. Results of this experiment indicate that differences in *T. maxima* growth in terms of shell length occurred at pCO_2 levels well below thresholds considered detrimental to *M. galloprovincialis* and *M. edulis* specimens in similar conditions. This result may indicate that bivalves with more pronounced periostracum such as *M. galloprovinicalis* and *M. edulis* living in naturally fluctuating pCO_2 conditions may be more tolerant to ocean acidification than *T. maxima*.

The significance of dissolution responses in terms of *Tridacna maxima* shell length and height is reinforced by a sharp increase in the number of individuals incubated in high pCO_2 conditions that exhibited shell size reductions in both length and height. Studies conducted on *Mytilus edulis* by Berge et al. (2006) revealed adverse effects on shell growth between pH 7.4 and pH 7.1. In contrast, *Tridacna maxima* specimens in this experiment exhibited negative effects between pH 7.7 and pH 7.9, well above pH levels that affected *M. edulis* specimens.

In addition to serving as skeletal support or protection from predators, calcium carbonate ($CaCO_3$) structures are also a source of carbonate (CO_3^{2-}) that can be used to offset naturally occurring spikes in carbonic acid (H_2CO_3) (Lindinger et al. 1984; Lowenstam and Weiner, 1989). As a result, concentrations of Ca^{2+} in specimen tissue would be expected to increase as pCO_2 increases. Concentrations of Ca^{2+} ions in the mantle tissue of *Tridacna maxima* varied slightly between pCO_2 180 ppmv and pCO_2 560 ppmv before rising to their highest levels at pCO_2 > 560 ppmv, perhaps indicative of $CaCO_3$ dissolution in response to hypercapnic acidification of the seawater.

Shell growth responses of *T. maxima* to increased ocean acidification may be attributable to one or more mechanisms known to affect calcification. For example, a decline in $CaCO_3$ precipitation – or increase in $CaCO_3$ dissolution – is expected as levels of CO_3^{2-} decline. Calcium carbonate ($CaCO_3$) precipitation rates in corals decline as pCO_2 increases (Kleypas et al. 2005), and $CaCO_3$ precipitation of marine calcifiers is CO_3^{2-} limited, not Ca^{2+} limited (Gattuso et al. 1998; Gazeau et al. 2007). Alternative mechanisms known to mediate the calcification process include zooxanthellal enzyme proteins such as carbonic anhydrase (CA) and urease (Yellowlees et al. 1993; Woolridge, 2008). In addition, competition between host and symbionts for inorganic carbon also mediates calcification rates (Marubini and Davies, 1996).

Scanning electron microscopy has been used to view structural changes to shell crystallization patterns of *Tridacna gigas* incubated in elevated levels of ammonium and phosphate (Belda et al. 1993). Precise delineation between recent and prior shell growth for this experiment was not established, rendering the existing methodology non-repeatable. However, captured images strongly hint at the feasibility of investigating changes in aragonite shell conditions in response to elevated pCO_2 similar to changes in conditions in response to elevated nutrients (Belda et al. 1993). Aragonite crystal conditions in high and low pCO_2 for this experiment suggest at least two areas of future research. First, crystalline lattice uniformity may contribute to shell density and rate of development, which may be reduced in high pCO_2 conditions. Because *Tridacna* spp. and other molluscs are prey to boring organisms, compromised shell density or calcification rates may adversely affect species fitness (Langdon and Atkinson, 2005). Second, several types of protein are embedded within crystalline lattice-work of shells (Lowenstam and Weiner, 1989). Identifying shell protein types and volumes may be used to

measure differences in protein synthesis rates as pCO_2 increases. The similarity of images between *T. Gigas* and *T. maxima* indicate that introducing x-ray diffractometry to the methodology and refining techniques for distinguishing crystallization patterns at topical shell locations before and during treatments may yield similar results.

4.4. Calcification / Soft-Tissue Response

Condition Index (CI) comparisons in this study indicate that the soft-tissue of *Tridacna maxima* continued growing or stayed the same at higher pCO_2, even though shells either stopped growing or dissolved. This result contrasts with results observed by Michaelidis et al. (2005), who found that shell growth goes hand in hand with decreased soft tissue weight for *M. galloprovincialis* in elevated pCO_2 conditions. The potential significance of this result relates to the observed relationship between increased mean net specimen weights and declining shell size as pCO_2 increased. Mean net increases in weight, in conjunction with declines in mean net shell size and weight, indicate an uncoupling between photosynthetic activity and shell growth, not uncommon among photosymbionts in larger temporal scales (Woolridge, 2008). However, the value of these data is mitigated by the fact that ratios between tissue and shell growth often fluctuate during different life-history phases (Green et al. 2004) making definite conclusions hard to reach.

4.5. Symbiotic Association

The densities of zooxanthellae in this study are within the range of those observed previously for *Tridacna gigas* (Fitt et al. 1995). Results of this study indicate there is no detectable effect of increasing pCO_2 on zooxanthellae density between *T. maxima* populations as pCO_2 increased. One explanation for the initial

decline is that as levels of pCO_2 increased, fewer zooxanthellae were required to generate the same volumes of photosynthates. *Tridacna maxima* hosts may regulate zooxanthellal density in optimal cost-benefit ratios in terms of energy expenditure versus photosynthate infusion. Energy conserved by the host may be used to support other activities such as protein synthesis (Hand 1991), respiration, reproduction and calcification (Seibel and Fabry, 2004; Hoegh-Guldberg, 2007).

Chlorophyll *a* per cell in the present study was similar, but ~3-7 times higher than previously reported for *T. maxima* (Jantzen et al. 2008). *T. gigas* incubated in high nutrient conditions showed increased concentrations of zooxanthellae, but chl *a* concentration per zooxanthella cell declined (Belda et al. 1993). These results are similar to observations of chl *a* concentrations in zooxanthellae of *Tridacna maxima* reported here. Higher pCO_2 values resulted in reduced concentrations of chl *a*, perhaps indicating that *T. maxima* can not fully regulate the delivery of CO_2 to zooxanthellal symbionts. Zooxanthellae may regulate concentrations of chl *a* based on net photosynthetic productivity; the higher the pCO_2, the less chl *a* per algal cell is needed to maintain net photosynthetic productivity.

4.6. Conclusions

The presence of zooxanthellae symbionts in *Tridacna maxima* complicates direct comparisons between biological responses of *Mytilus galloprovinciallis*, *Mytilus edulis*, *Crassostrea gigas* and *T. maxima* in elevated pCO_2 conditions. For example, results of this experiment indicate that negative shell growth of *T. maxima* occurred at pCO_2 levels well below thresholds known to negatively affect previously studied bivalves. This response may be attributable to zooxanthellal carbonic anhydrase enzyme activity that mediates shell precipitation in normal pCO_2 conditions, but may become inhibited as pCO_2 increases. In addition, the presence of photosymbionts

may explain the continued development of soft-tissue as pCO_2 levels increase, a response not observed in other mollusc bivalves in similar experimental conditions.

Comparing calcium carbonate precipitation responses of corals in reduced pH conditions to shell growth responses of tridacnid clams is also challenging for several reasons. First, *T. gigas* hosts have been shown to select CO_2 as a primary source of carbon in contrast to coral symbionts that select HCO_3^- (Legget, et al. 2000). Second, photosynthetic organism preference for HCO_3 (Leclerq, 2002) implies that hosts may prefer CO_2 while symbionts favor HCO_3. Yet to be fully explained is whether either species of selected inorganic carbon is used for host calcification, symbiont photosynthesis, or perhaps a combination of the two carbon species depending on prevailing pH conditions (Legget et al. 2000). Third, while corals have been shown to share symbiotic associations with multiple zooxanthellal species, *Tridacnid* clams are the only known species of Bivalvia to engage in symbiotic associations solely with *S. microadriaticum* (Taylor, 1969). As a result, unlike corals, *Tridacnid* clams are not capable of selecting or managing zooxanthellal clades as a way of temporarily adapting to fluctuating environmental conditions. Fourth, while coral symbionts maintain intra-cellular positions, *S. microadriaticum* in *Tridacna maxima* are inter-cellular and perhaps more vulnerable to internal acid-base conditions of the host. Fifth, comparing biological responses between *Tridacna* spp. and corals is particularly challenging because no research investigating the effects of ocean acidification as a result of elevated pCO_2 on individual species of hermatypic scleractinian corals has been conducted.

The degree to which increased pCO_2 and ocean acidification affects biological processes vital to marine calcifiers appears critical for inclusion in near- and long-term research objectives. Biological responses of marine calcifiers to ocean acidification may adversely affect the oceanic carbonate chemistry system, deep

ocean carbon sequestration, primary productivity, the global food web and healthy marine ecosystems. The unique physical and biological attributes of juvenile *Tridacna maxima* and their symbiotic partners can offer comparably unique insights into the biological responses of other marine calcifiers to ocean acidification. Results of this study appear to indicate that tridacnid clams may be more susceptible to ocean acidification than corals, and monitoring the health of *Tridacnidae* may prove useful in helping predict the effects of increasing pCO_2 on coral reef ecosystems.

Bibliography

Ambariyanto, Hoegh-Guldberg O, (1999) Net uptake of dissolved free amino acids by the giant clam *Tridacna maxima*: alternative sources of energy and nitrogen? Coral Reefs 18: 91-96

Bamber RN, (1990) The effects of acidic seawater on three species of lamellibranch mollusc. J. Exp. Mar. Biol. Ecol. 143: 181-191

Belda-Baille CA, Sison M, Silvestre V, Villamor K, Monje V, Gomez ED, Baille BK, (1999) Evidence for changing symbiotic algae in juvenile tridacnids. J. Exp. Mar. Biol. Ecol. 241: 207-221

Belda CA, Cuff C, Yellowless D, (1993) Modification of shell formation in the giant clam *Tridacna gigas* at elevated nutrient levels in sea water. Marine Biology 117, 251-257

Berge JA, Bjerkeng B, Pettersen O, Schaanning MT, Øxnevad S, (2006) Effects of increased sea water concentrations of CO_2 on growth of the bivalve *Mytilus edulis*. Chemosphere 62, 681-687

Bibby R, Widdicombe S, Parry H, Spicer J, Pipe R, (2008) Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquatic Biology 2: 67-74

Bidwell JP, Spotte S, (1985) Artificial Seawaters: formulas and methods. Jones and Bartlett Publishing Company, Boston, 349pp.

Burnett LE, (1997) The challenges of living in hypoxic and hypercapnic aquatic environments. American Zoologist, 37: 633-640

Caldiera K, Wickett ME, (2005) Ocean model predictions of chemistry changes from carbon dioxide emission to the atmosphere and ocean. Journal of Geophysical Research, 110; C09S04, doi:10.1029/2004JC002671

Chalebrese A, Davis HC, (1996) The pH Tolerance of Embryos and Larvae of *Mercenaria mercenaria* and *Crassostrea virginica*. Bio. Bull. 131: 3, 427-436

Cowen R, (1988) The role of algal symbiosis in reefs through time. PALAIOS, V. 3, Reefs Issue, p. 221-227. Ancient Reef Ecosystems, The Society of Economic Paleontologists and Mineralogists

Dickson AG, Sabine CL, Christian JR, (Eds.) (2007) Guide to Best Practices for Ocean CO_2 Measurements. PICES Special Publication 3, 191 pp.

Dickson AG, (1981) Exact definition of total alkalinity and a procedure for the estimation of alkalinity and total inorganic carbon from titration data. Deep-Sea Res. 28A: 609-623

Dickson AG, Millero FJ, (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep-Sea Research 34: 1733-43

Doney S, (2008) Written testimony presented to the Committee on Science and Technology, Subcommittee on Energy and the Environment, United States House of Representatives, June 5, 2008, The Federal Ocean Acidification Research and Monitoring Act: HR 4174, found at http://democrats.science.house.gov/Media/File/Commdocs/hearings/2008/Energy/05june/Doney_Testimony.pdf

Ellis S, (1998) Spawning and Early Larval Rearing of Giant Clams (Bivalvia: Tridacnidae) CTSA Publication No. 130, available at

http://www.ctsa.org/upload/publication/CTSA_13063167286087309540 4.pdf.

Fabry VJ, Seibel B A, Feely RA, Orr, JC, (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES Journal of Marine Science. 65: 414-432

Fankboner PV, (1971) Intracellular digestion of symbiotic zooxanthellae by host amoebocytes in giant clams (Bivalvia:Tridacnidae), with note on the nutritional role of the hypertrophied siphonal epidermis. Bio. Bull. 141: 222-234

Fautin DG, & Buddemeier RW, (2004) Adaptive bleaching: a general phenomenon. Hydrobiologia 530/531: 459-467

Feely RA, Sabine CL, Kitak L, Berelson W, Kleypas J, Fabry VJ, Millero FJ, (2005) Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305: 5682, pp 362-366

Fisher CR, Fitt WK, Trench RK,(1985) Photosynthesis and respiration in *Tridacna gigas* as a function of irradiance and size. Biol. Bull 169: 230-245

Fitt WK, Rees TAV, Yellowlees D, (1995) Relationship between pH and the availability of dissolved inorganic nitrogen in the zooxanthella-giant clam symbiosis. Limnology and Oceanography, 40, 976-982

Fitt WK, Trench RK, (1981) Spawning, development, and acquisition of zooxanthellae by *Tridacna squamosa* (Mollusca, Bivalvia). Biol. Bull. 161:213-235

Feely, R. A., Sabine, C. L., Lee, K., Berelson, W., Keypas, J., Fabry, V. J., Millero, F. J. 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans, *Science*, 305:5682, 362-366.

Gao K, Aruga Y, Asada K, Ishihara T, Akano T, Kiyohara M, (1993) Calcification in the articulated coralline alga *Corallina pilulifera*, with special reference to the effect of elevated CO_2 concentration. Marine Biology 117: 129-132

Gattuso JP, Frankignoulle M, Bourge I, Romaine S, Buddemeier RW, (1998) Effect of calcium carbonate saturation of seawater on coral calcification. Global and Planetary Change 18, Issues 1-2, 37-46

Gazeau F, Quiblier C, Jansen JM, Gattuso J, Middelburg JJ, Heip HR, (2007) Impact of elevated CO_2 on shellfish calcification. Geophysical Research Letters 34; LO7603, doi:10.1029/2006GL028554, 2007

Goreau TF, Goreau NI, Yonge CM, (1973) On the utilization of photosynthetic products from zooxanthellae and of a dissolved amino acid in *Tridacna maxima* f. elongate (Molluca:Bivalvia). J. Zool. (London) 169: 417-454

Gloor M, Bruber N, Sarmiento J, Sabine CL, Feely RA, Rödenbeck C, (2003) A first estimate of present and preindustrial air-sea CO2 flux patterns based on ocean interior carbon measurements and models. Geophysical Research Letters, 30: 1010, doi10:1029/2002GL015594, 2003

Green MA, Jones ME, Boudreau CL, Moor RL, Westman BA, (2004) Dissolution mortality of juvenile bivalves in coastal marine deposits. Limnol. Oceanogr., 49: 3, pp 727-734

Goreau TF, (1959) The physiology of skeleton formation in corals: A method for measuring the rate of calcium deposition by corals under different conditions. Bio. Bull. 116: 1, 59-75

Haugen PM, (2004) Possible effects of ocean acidification, in Reports in Meteorology and Oceanography, Geophysical Institute, University of Bergen, Norway

Hand SC (1991) Metabolic dormancy in aquatic invertebrates. In: Weber RE, Gilles R, & Mangum CP (Eds.) 1991. Advances in Comparative and Environmental Physiology 8: pp. 1-50. Heidelberg: Springer-Verlag.

Herfort L, Thake B, Taubner I, (2008) Bicarbonate stimulation of calcification and photosynthesis in two hermatypic corals. J. Phycol. 44: pp. 91-98

Heslinga G, Fitt, WK, (1987) The domestication of reef-dwelling clams. BioScience 37: 5, Aquaculture pp.332-339

Heslinga G, Watson T, Isamu T, (1990) Giant Clam Farming. Pacific Fisheries Development Foundation (NMFS/NOAA), Honolulu, Hawaii, 179 p.

Hirose E, Iwai K, Maruyama T, (2005) Establishment of photosymbiosis in the early ontogeny of three giant clams. Marine Biology 148: 551-558

Hoegh-Guldberg H, Hoegh-Guldberg O, (2004) Biological, economic and social impacts of climate change on the Great Barrier Reef. Worldwide Fund for Nature: Brisbane, Australia

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, et al. (2007) Coral reefs under rapid climate change and ocean acidification. Science 18: 737-1742

Hoegh-Guldberg O, Smith GJ, (1989) Influence of the population density of zooxanthellae and supply of ammonium on the biomass and metabolic characteristics of the reef corals *Seriatopora hystrix* and *Stylophora pistillata* . Marine Ecology Progress Series, Oldendorf Vol. 57, No. 2, 173-186

Houghton RA, (2005) The Contemporary Carbon Cycle, pp. 473-513. In Biogeochemistry (ed. W.H. Schlesinger) Vol. 8 Treatise on Geochemistry (eds. H.D. Holland and K.K. Turekian), Elsevier-Pergamon, Oxford

Iglesias-Rodriguez M, Halloran PR, Rickaby EM, Hall IR, Colmenaro-Hidalgo E, Gittins JR, Green RH, Tyrell T, Gibbs SJ, von Dassow P, Rehm E,

Armburst EV, Boessenkook KP, (2008) Phytoplankton calcification in a high-CO_2 world. Science 20: 336-340

IPCC 2007 Climate Change 2007: The Physical Science Basis. Contribution of Working Group 1 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S.,D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tingor and H.L.Miller (eds.)]. Cambridge University Press, Cambridge United Kingdom and New York, NY, USA, 996 pp.

Kleypas J, (2008) Statement of Joan A. Kleypas, Ph.D., Scientist, Institute for the Study of Society and Environment National Center for Atmospheric Research Before the U.S. House of Representatives Committee on Science and Technology Subcommittee on Energy and Environment Chairman Nick Lampson 01 June 2008 Hearing on The Federal Ocean Acidification Research and Monitoring Act: H.R. 4174, found at http://democrats.science.house.gov/Media/File/Commdocs/hearings/2008/Energy/05june/Kleypas_Testimony.pdf

Kleypas JA, Feely RA, Fabry VJ, Langdon C, Sabine CL, Robbins LL, (2005) Impacts of ocean acidification on coral reefs and other marine calcifiers: A guide for future research, report of a workshop held 18-20 April 2005, St. Petersburg, FL, sponsored by NSF, NOAA, and the U.S. Geological Survey, 88 pp.

Knop D, (1996) Giant Clams: A Comprehensive Guide to the Identification and Care of Tridacnid Clams. Dähne Verlag GmbH, Postfach 250, D-76256 Ettlingen

Knutzen J, (1981) Effects of decreased pH on marine organisms. Marine Pollution Bulletin 12: 1, pp. 25-29

Kuffner IB, Andersson AJ, Jokiel PL, Rodgers KS, Mackenzie FT, (2007) Decreased abundance of crustose coralline algae due to ocean acidification. Nature Geoscience, Letters, Advance Online Publication, available at http://www.nature.com/ngeo/index.html

Kurihara H, Shimode S, Shirayama Y, (2005) Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins. Journal of Oceanography 60: 743-750

Langdon C, Atkinson MJ, (2005) Effect of elevated pCO_2 on photosynthesis and calcification of corals and interactions with seasonal change in temperature/irradiance and nutrient enrichment. Journal of Geophysical Research 110: C09S0, doi:10.1029/2004JC002576

Langdon C, Wallace SB, Hammond DE, Glenn E, Fitzsimmons K, Nelson SG, Peng TH, Hajdas I, Bonani G, (2003) Effect of elevated CO_2 on the community metabolism of an experimental coral reef. Global Biogeochemical Cycles 17: 1, 1011, doi:10.1029/2002G001941, 2003

Lawrence DR, Scott GI, (1982) The determination and use of condition index of oysters. Estuaries, 5: 1 pp. 23-27

Leclercq N, Gattuso JP, Jaubert J, (2002) Primary production, respiration, and calcification of a coral reef mesocosm under increased CO_2 partial pressure. Limnology and Oceanography 47: 2, 558-564

Legget W, Badger MR, Yellowlees D, (1999) Evidence for an inorganic carbon-concentrating mechanism in the symbiotic dinoflagellate *Symbiodinium* sp. Plant Physiol. 121: 1247-1255

Legget W, Rees TAV, Yellowlees D, (2000) Meeting the photosynthetic demand for inorganic carbon in an alga-invertebrate association: Preferential use

of CO_2 by symbionts in the giant clam *Tridacna gigas*. Proc. R. Soc. Lond. B. 267: 523-529

Lewis E, Wallace D, (1998) Program Developed for CO_2 Calculations. Department of Applied Science, Brookhaven national Laboratory, Upton New Your, Environmental Sciences Division Publication No. 4735, Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, Oak Ridge, TN

Libes S, (2005) An Introduction to Marine Biogeochemistry, Copely Custom Textbooks, Elsevier.

Lindinger MI, Lauren DJ, McDonald DG, (1984) Acid-base balance in the sea mussel, *Mytilus edulis*. Effects of environmental hypercapnia on intra- and extra-cellular acid-base balance. Mar. Biol. Lett. 5:371-381

Lowenstam HA, Weiner S, (1989) On Biomineralization. Oxford University Press, Inc., New York

Marine Biogeochemical Cycles, The Accumulation of Deep-Sea Sediments, (2005) The Open University, Elsevier Butterworth-Heinemann, Reed Educational and Professional Publishing, Ltd.

Marubini F, Atkinson MJ, (1999) Effects of lowered pH and elevated nitrate on coral calcification. Marine Ecological Progress Series 293:117-121

Marubini F, Davies PS, (2996) Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. Marine Biology 127:2, 319-328

McConnaughey TA, Adey WH, Small AM, (2000) Community and environmental influences on reef coral calcification. Limnology and Oceanography 45: 7, 1667-1671

Medaković D, Popović S, Gržeta B, Plazonić M, Hrs-Brenko (1977) X-ray diffraction study of calcification process in embryos and larvae of the brooding oyster *Ostrea edulis*. Marine Biology 129: 616-623

Merbach, C., Culberson, C. H., Hawley, J. E., Pytkowicz, R. M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897-907).

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO, (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovinvialis*. Marine Ecological Progressive Series 293:109-118

Mucci A, (1983) The Solubility of calcite and aragonite in seawater at various salinities, temperatures and 1 atmosphere total pressure. American Journal of Science 238: 780-799

Norton JH, Shepherd MA, Long HM, Fitt WK, (1992) The zooxanthellal tubular system in the giant clam. Bio. Bull 183: No. 2, 503-506

Ocean Chemistry and Deep-Sea Sediments, The accumulation of pelagic biogenic sediments (1994) The Open University, Elsevier Science, Ltd.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G.-K., Rodgers, K. G., Sabine, C. L., Sarmiento, J. L., Schlitzer, R.,Slater, R. D., Totterdell, I. J., Weirig, M.-F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437: doi:10.1038

Paracer S, Ahmadjian V, (2000) Symbiosis: an introduction to biological associations. Oxford University Press

Pörtner HO, Langenbuch M, Reipschläger A, (2004) Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and Earth history. Journal of Oceanography 60: pp. 705-718

The Royal Society (2005) Ocean acidification due to increasing atmospheric carbon dioxide. Policy Document 12/05. The Royal Society, London. Available at http://royalsociety.org/displaypagedoc.asp?id=13539

Rosewater J, (1965) The family *Tridacnidae* in the Indo-Pacific. Indo-Pacific Mollusca 1, 347-396

Riebesell U, Zondervan I, Rost B, Tortell PD, Zeebe RE, Morel FMM, (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 404: 21 September 200, Macmillan Magazines, Ltd.

Rebelo MF, Amaral MCR, Pfeiffer WC, 2005 Oyster condition index in Crasostrea rhizophorae (Guilding, 1828) from a heavy-metal polluted coastal lagoon. Braz. J. Biol 65: 2, Print ISSN 1519-6984

Ringwood AH, Keppler CJ, (2002) Water quality variation and clam growth: Is pH really a non-Issue in estuaries? Estuaries 25, Vol. 5, p.901-907

Seibel BA, Fabry VJ (2003) Marine Biotic Response to Elevated carbon Dioxide. Advances in Applied Biodiversity Science 4: pp. 59-67.

Taylor DL, (1969) Identity of zooxanthellae isolated from some Pacific *Tridacnidae*. J. Phycol. 5:336-340

Takeuchi K, Fujioka Y, Kawasaki Y, Shirayama Y, (1997) Impacts of high concentration of CO_2 on marine organisms: A modification of CO_2 ocean sequestration. Energy Convers. Mgmt 38: Suppl. pp. S337-S341

Trench RK, Wethey DS, & Porter JW (1981) Observations on the symbiosis with zooxanthellae among the *Tridacnidae* (Mollusca, Bivalvia). Biol Bull 161: 180-198

Wolf-Gladrow DA, Reibesell U, Burkhardt S, Bijma J, (1999) Direct effects of CO_2 concentration on growth and isotopic composition of marine plankton. Tellus B 51: 2, pp. 461-476

Woolridge SA, (2008) Mass extinctions past and present: a unifying hypothesis. Biogeosciences Discuss 52401-2423 available at http://www.biogeosciences-discuss.net/5/2401/2008/bgd-5-2401-2008.pdf

Yellowlees D, Dionisio-Sese ML, Masuda K, Maruyama, T, Abe T, Baille B, Tsuzuki M, Miyachi S, (1992) Role of carbonic anhydrase in the supply of inorganic carbon to the giant clam-zooxanthellate symbiosis. Marine Biology 115, pp. 605-611

Yonge CM, (1980) Functional morphology and evolution in the *Tridacnidae* (Mollusca:Bivalvia: *Cardiacea*. Rec. Aust. Mus. 33: 735-777

Zondervan I, Zeebe RE, Rost B, Riebesell U, (2001) Decreasing marine biogenic calcification: A negative feedback on rising atmospheric CO_2. Global Biogeochemical Cycles 15: 2, 507-516