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The Effects of Nutrient Addition and Ocean Acidification on Tropical Crustose Coralline Algae

A thesis submitted in partial fulfillment of the requirements

For the degree of Master of Science in Biology

By

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Table of Contents

List of Figures

Abstract

The Effects of Nutrient Addition and Ocean Acidification on Tropical Crustose Coralline Algae

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As the global population increases, the occurrence of multiple anthropogenic impacts on valuable coastal ecosystems, such as coral reefs, also increases. These stressors can be global and long-term, like ocean acidification (OA), or local and short-term, like nutrient runoff in some areas. The combination of these stressors can potentially have additive or interactive effects on the organisms in coral reef communities. Among the most important groups of organisms on coral reefs are crustose coralline algae (CCA), calcifying algae that cement the reef together and contribute to the global carbon cycle. This thesis studied the effects of nutrient addition and OA on *Lithophyllum kotschyanum*, a common species of CCA on the fringing reefs of Mo'orea, French Polynesia. Two mesocosm experiments tested the individual and interactive effects of OA and short-term nitrate and phosphate addition on *L. kotschyanum*. These experiments showed that nitrate and phosphate addition together increased photosynthesis, OA had interactive effects with nutrient addition, and after nutrient addition ended, calcification and photosynthetic rates changed in unpredictable ways in

different OA and nutrient treatments. Because the results of the first two experiments showed impacts of nutrients even after addition stopped, two more mesocosm experiments were conducted to study the changes in photosynthesis and calcification over hourly time scales more relevant to a single nutrient pulse event. These two experiments revealed the existence of diurnal variation in light-saturated photosynthetic rate, but not calcification rate, under ambient and elevated pCO_2. This pattern of increased maximum photosynthesis in the middle of the day can have important implications for how the time of nutrient runoff events during the day impacts CCA physiology. Finally, a field experiment was conducted to determine the effects of short- and long-term nutrient addition on *L. kotschyanum*. The results showed that a series of short-term nutrient additions did not increase photosynthesis or calcification rates above those in ambient nutrient conditions, but continual nutrient enrichment for 6 weeks increased photosynthetic rates. This increase in photosynthesis under only long-term enrichment shows the need for consideration of specific nutrient addition scenarios on coral reefs when predicting how the community will be affected.

Chapter 1

General Introduction

Ocean Acidification

Ocean acidification (OA) is one of the large-scale, long-term press disturbances that threatens marine ecosystems globally (Kroeker et al. 2010), and it will increase over the next centuries if CO_2 emissions continue (IPCC 2014). The ocean has absorbed about 30% of the anthropogenically emitted CO_2, which means that as CO_2 is added to the atmosphere, the water chemistry of the oceans changes in response (Caldeira and Wickett 2003, IPCC 2014). CO_2 reacts with water to form carbonic acid, which then dissociates into bicarbonate ions (HCO_3^-) and protons. The protons react with carbonate ions (CO_3^-) to form more bicarbonate, which leads to a reduction in carbonate in the water and a decrease in pH. Decreased carbonate ions in the water means reduced calcification rates for calcifying organisms, and at low enough carbonate concentrations, erosion is favored (Hoegh-Guldberg et al. 2007). This process of OA is projected to increase over the next century as oceanic pH is expected to decrease by 0.30-0.32 units and pCO_2 is expected to increase to 851-1370 μatm in baseline scenarios with no CO_2 mitigation efforts (IPCC 2014).

Because of the negative effects of OA on calcification, coral reef ecosystems are threatened by these water chemistry changes. The structure of coral reefs is made up of calcium carbonate deposited mainly by corals and coralline algae, and many of the organisms that live in coral reef ecosystems are calcifiers, including foraminifera, coccolithophorids, mollusks, and echinoderms (Gattuso and Buddemeier 2000, Kleypas

and Yates 2009). Therefore, OA affects both biological and geological components of the reefs (Kleypas and Yates 2009).

In addition to affecting the reef community directly, press disturbances, like OA, can affect the ability of a system to recover from other disturbances (Nyström et al. 2000). Therefore, in order to fully understand the effects of OA on marine organisms and systems, OA needs to be studied in combination with other disturbances, like shorter-term pulse disturbances. Because humans increasingly are altering natural disturbance regimes such as sediment and nutrient input on reefs and grazing by herbivorous fishes, the likelihood of multiple human-induced disturbances affecting an ecosystem is increasing as well (Nyström et al. 2000). Thus, it is especially important to understand the potentially synergistic effects of anthropogenically-induced press and pulse disturbances, like OA and periodic nutrient enrichment.

Nutrient Addition

Nutrient enrichment is an increasingly important issue in coastal waters due to anthropogenic sources (Smith and Schindler 2009, Jessen et al. 2013). Land clearing, urbanization, and fertilizer use all lead to the input of agricultural runoff, human sewage, urban waste, and industrial effluent into the ocean (Fabricius 2005, Jessen et al. 2013). Nitrogen fertilizer use globally continues to increase annually, and river-borne agricultural nitrogen and phosphorus have led to oxygen-depleted seafloor zones, in areas such as the Mediterranean Sea and the Gulf of Mexico, that are expanding in both number and size (GESAMP 2001).

Coastal ecosystems, like coral reefs, are exposed directly to the nutrients, sediments, and pollutants that are washed off the land (Fabricius 2005). Increases in nutrients potentially can have serious impacts on coral reefs (Koop et al. 2001). In many places, nutrient addition has led to decreases in coral cover, and in some areas, the dominant organisms in the reef ecosystems shift with increasing nutrients (Fabricius 2005). Nutrient enrichment can affect the growth rates and reproductive abilities of corals negatively (Koop et al. 2001), and it can increase the likelihood of corals becoming diseased or bleached (Vega-Thurber 2014). It can change the percent cover of different functional groups on the reef over time, therefore changing the community composition and affecting interactions between different groups of reef organisms (Jessen et al. 2013). Additionally, it can lead to changes in the growth of important reef builders, like crustose coralline algae (Bjork et al. 1995).

However, most previous studies on the effects of nutrients on coral reefs have looked at the effects of long-term nutrient enrichment. Far less attention has been paid to episodic nutrient pulses entering the water over coral reefs. These pulses last only a few hours, and they can fluctuate seasonally or with rain events and result in high concentrations of nutrients (Nyström et al. 2000, den Haan et al. 2016). When combined with other press stressors, like OA, the effects of periodic nutrient input on reef ecosystems may be exacerbated. When multiple disturbances impact a reef simultaneously, the ability of reef organisms to respond to even one of them is degraded, and the negative effects can be amplified (Nyström et al. 2000).

Coralline Algae

One of the most common groups of calcifying algae is coralline red algae in the Phylum Rhodophyta (Family Corallinaceae). These algae can be of either branching or crustose morphology, and they deposit high-magnesium (Mg) calcite, which is the most soluble form of marine carbonate minerals. These algae are found throughout the oceans across all latitudes and to the greatest depths for benthic photosynthetic species (Kleypas and Yates 2009).

Crustose coralline algae (CCA) have important ecological roles on coral reefs. They reinforce the skeletal structure of corals, bind the reef together, fill in cracks, bind together sand, dead coral, and debris to create solid substrata, and produce carbonate sediments (Kuffner et al. 2008, Nelson 2009, Diaz-Pulido et al. 2012). CCA also induce the larval settlement of reef invertebrates, including corals, mollusks, and urchins (Nelson 2009, Diaz-Pulido et al. 2012). This settlement is governed by chemosensory recognition and can either be species-specific or more general (Nelson 2009). Finally, CCA are major contributors to the global carbon cycle due to their photosynthesis, respiration, and calcification (Nelson 2009).

The relative dominance of CCA in reef communities has been summarized in multiple conceptual models that take into account nutrient enrichment, herbivory, and life-history characteristics (Szmant 2002). One such model suggested that under high concentrations of nutrients and intense herbivory, CCA would be the dominant functional group (Littler and Littler 1984). Another model proposed that CCA coexist with coral under low nutrient conditions, but as nutrient concentrations increase, fleshy algae outcompete both groups (Birkeland 1987). A third suggested that fleshy algae could

outcompete CCA under decreased herbivory even without increased nutrients (Steneck and Dethier 1994). Overall, these conflicting ideas lead to the conclusion that the relative dominance of CCA on coral reefs is likely to be affected by changes in water chemistry due to OA and nutrient input. Therefore, the effects of these disturbances on CCA should be studied together.

Carbon, Nitrogen, and Phosphorus Metabolism of CCA

Calcification, photosynthesis, respiration, nitrogen metabolism, and phosphorus metabolism in crustose coralline algae all are interconnected (Turpin 1991, Lobban and Harrison 1997, Comeau et al. 2013a). Calcification in CCA occurs in the cell wall, and the process was described by Comeau et al. (2013a). When carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) enter the cell wall, the carbonate remains while the bicarbonate is removed with hydrogen ions (H^+) via a symport. The removal of H^+, along with the influx of OH^- into the cell wall from photosynthesis, increases the pH in the cell wall facilitating the precipitation of calcium carbonate ($CaCO_3$). Inside the cell, carbonic anhydrase facilitates the reaction of bicarbonate with H^+ to create water and CO_2. This CO_2 then is used in photosynthesis.

Photosynthesis uses both the CO_2 from bicarbonate and the CO_2 released in respiration to turn the Calvin Cycle (Turpin 1991, Comeau et al. 2013a). One of the carbon compounds from the Calvin Cycle then is used in respiration (Turpin 1991). Photosynthesis and respiration counter each other in their effects on pH, and therefore, they also have opposite effects on calcification during the day. Photosynthesis increases pH, favoring calcification, and respiration decreases pH, hindering calcification (Martin

et al. 2013). However, ATP is also produced through photosynthesis, which may aid calcification indirectly by fueling the HCO_3^-/H^+ symport (Turpin 1991, Comeau et al. 2013a).

Nitrogen is used in the creation of amino acids, protein complexes, and phycobilin photosynthetic pigment molecules (Ryder et al. 1999). The cells can take up any form of nitrogen, but it can only assimilate ammonium (NH_4^+) and therefore must convert other forms to ammonium within the cell (Lobban and Harrison 1997). When nitrate (NO_3^-) is taken up, it must first be converted to nitrite (NO_2^-) and then to ammonium (Turpin 1991). In order to make compounds with nitrogen, ammonium then is combined with carbon-based molecules, whose synthesis is fueled by respiration. Therefore, because of the connection between photosynthesis and respiration and the requirement of N-containing enzymes in all pathways (e.g., RUBISCO, nitrate reductase), nitrogen metabolism is connected integrally to both (Turpin 1991).

In addition, because chlorophyll and phycobilin pigments contain nitrogen (Turpin 1991, Ryder et al. 1999), changes in nitrogen availability can lead to changes in pigment concentrations. Pigment increases can be manifest as an increase in the number of photosynthetic units (functional clusters of pigments) or an increase in the number of antennae pigments within photosynthetic units (Lobban and Wynne 1981). Both of these changes result in an overall increase in pigment concentration, and because energy is passed from photosynthetic units to the reaction centers where photochemistry occurs, changes in pigment concentration can affect rates of photosynthesis through both light capture and transfer, as well as altered rates of photochemistry (Lobban and Wynne 1981).

Phosphorus is used to produce DNA, RNA, ATP, and other molecules used in carbon metabolism (Turpin 1991, Lobban and Harrison 1997). Algae acquire phosphorus primarily as phosphate (PO_4^{3-}), and because of the use of ATP in all metabolic processes, phosphorus availability is important to overall cell functioning (Lobban and Harrison 1997). In addition, there is some evidence that at very high concentrations (30-150 μM), phosphate can inhibit calcification in calcifying algae (Lobban and Harrison 1997).

OA and Nutrient Effects on Calcification of CCA

The oceanic carbonate chemistry changes caused by OA directly affect the calcification of CCA due to the change in pH and carbonate concentration in seawater (Nelson 2009). Because the high-Mg calcite that CCA deposit is more soluble than aragonite, the carbonate phase deposited by corals, CCA could initially be more susceptible to OA (Kuffner et al. 2008). They have been shown to be more sensitive than both branching and massive coral species under projected pCO_2 concentrations (Nelson 2009). However, the projected pCO_2 increases in the business-as-usual scenarios for the rest of the century are large enough to affect calcification in both CCA and corals (Kuffner et al. 2008). Past experiments have shown that OA can affect the recruitment and early growth of CCA and can lead to dissolution in mature thalli (Jokiel et al. 2008). Increased pCO_2 also has been shown to inhibit or significantly decrease calcification (Nelson 2009, Johnson et al. 2014b).

The effects of nutrients on CCA calcification have not been studied widely. One study on CCA determined that nitrogen enrichment with ammonium or nitrate had no significant effect on calcification (Björk et al. 1995). The same study also determined that

calcification decreased at high levels of phosphate addition, potentially due to its ability to inhibit calcite crystal growth by settling on the crystal lattice. The study did find that at moderate levels of phosphate enrichment (0.2-0.4 μM) the magnitude of the effects on calcification was low. Another study found no significant effect of ammonium or phosphate (2.4-5.14 μM) addition on the calcification of coralline algae (Koop et al. 2001).

OA and Nutrient Effects on Photosynthesis and Respiration of CCA

In coralline algae, increased pCO_2 does not strongly affect photosynthetic rates (Hofmann et al. 2012). Although increased pCO_2 means that there is more CO_2 available for photosynthesis, there is evidence that coralline species in the genus *Corallina* either show decreased rates of photosynthesis or no change in rates (Hofmann et al. 2012, 2013). CCA also use respiratory CO_2 and CO_2 from HCO_3^- (via carbonic anhydrase use) to fuel photosynthesis. The use of multiple sources of CO_2 suggests that under OA conditions, photosynthesis should increase if the algae are carbon limited. However, the response of photosynthesis to bicarbonate also is controversial due to varying responses of CCA photosynthesis to elevated pCO_2 (Comeau et al. 2013a), and CCA have shown either decreased rates of photosynthesis (Martin et al. 2013) or no change in rates under increased pCO_2 (Johnson et al. 2014b). Therefore, it is unclear how CCA photosynthesis will respond to elevated levels of pCO_2, and the responses may be species-specific.

Changes in pCO_2 have not been shown to affect respiration rates of CCA (Hofmann et al. 2012, Martin et al. 2013).

There have been very few studies looking at the effects of nutrient enrichment on CCA photosynthesis and respiration. One study found that the addition of nutrients (fertilizer) did not affect CCA photosynthesis (Russell et al. 2009). In another study on a non-calcifying species of red algae, nitrate enrichment resulted in increased photosynthetic rates (Zheng and Gao 2009). A second study on a different non-calcifying red alga found that photosynthesis increased and respiration increased then decreased with increasing levels of nitrate addition (Martins et al. 2011). They also found that with the addition of nitrate and phosphate, photosynthesis rates decreased and respiration rates increased compared to the nitrate-enriched treatments. The effects of nutrient addition on CCA photosynthesis and respiration may be different from non-calcifying red algae due to the coupling between calcification, photosynthesis, and respiration.

OA and Nutrient Effects on Photosynthetic Pigment Concentrations of CCA

CCA contain chlorophyll *a* as their major pigment and contain three phycobilins (phycocyanin, allophycocyanin, and phycoerythrin) as their main accessory pigments (Glazer 1977). One study found that chlorophyll *a* concentrations were not affected by elevated pCO_2 (Martin et al. 2013), while another found that they were affected negatively (Gao and Zheng 2010). Gao and Zheng (2010) also found that phycoerythrin concentrations decreased with increased CO_2. They suggested that increased CO_2 could lower the energy demand for bicarbonate utilization, leading to lower pigmentation.

There are few data on the effects of nitrate and phosphate enrichment on CCA pigment composition. One study that investigated the effects of nitrate and phosphate on a non-calcifying species of red algae found that the addition of nitrate resulted in an increase in the three phycobilin pigments, but did not produce a significant change in chlorophyll *a* concentrations (Martins et al. 2011). Another study on a non-calcifying red alga found increases in chlorophyll *a* and phycobilin concentrations with an increase in nitrogen (Chopin et al. 1995). Martins et al. (2011) also found that pigment concentrations increased with increased phosphate addition. However, Chopin et al. (1995) found no significant effect of phosphorus on pigment concentrations. Therefore, it is unknown what effects nitrate and phosphate addition have on tropical CCA.

Thesis Objectives

As ocean acidification becomes an increasingly important problem in oceans globally (IPCC 2014), and coastal nutrient addition increases due to coastal development and land use change, these two factors will increasingly impact vital coastal ecosystems, such as coral reefs. Understanding how these anthropogenic stressors affect important members of coral reef communities, such as crustose coralline algae, is necessary so that we can predict how these systems may change through the rest of this century. The objectives of this thesis were to understand the physiological effects of ocean acidification and nutrient addition, individually and synergistically, on tropical crustose coralline algae found on the fringing reefs of Mo'orea, French Polynesia. This involved conducting several different experiments and measuring the photosynthetic response of *Lithophyllum kotschyanum*. Additionally, calcification rates, respiration rates, and

photosynthetic pigment concentrations were measured to fully understand the complexities of the photosynthetic metabolic response of this species of CCA.

In Chapter 2, two experiments were conducted to test the effects of elevated pCO_2 crossed with nitrate, phosphate, and nitrate plus phosphate addition. The algae were subjected to 3.5 weeks of either ambient or elevated pCO_2, and in the middle of the 3.5 weeks experienced 9 days of one of three nutrient treatments. Photosynthesis, respiration, calcification, and pigment concentrations were measured in half of the samples at the end of the nutrient pulse and in the other half at the end of the experiment (10 days later). It was predicted that nutrient addition would mitigate some of the effects of OA on these algae.

In Chapter 3, a short-term experiment was conducted to determine the effects of a single 8-hour nutrient pulse under ambient and elevated pCO_2 on the photosynthetic and short-term calcification rates of *L. kotschyanum*. This experiment showed that there may be a strong diurnal pattern of light-saturated photosynthesis in this species of algae, so a second experiment was conducted to describe this pattern under ambient and elevated pCO_2 conditions and determine if a diurnal pattern existed for short-term calcification.

In Chapter 4, a field experiment was conducted to determine the effects of press vs. pulse nutrient enrichment via fertilizer addition. Six weeks of nutrient addition was compared to four 3-day nutrient pulses over six weeks and an ambient nutrient treatment. It was predicted that at the end of the 6-week experiment, the pulse treatment would result in intermediate responses of photosynthesis, long-term calcification, and photosynthetic pigment concentrations compared to the ambient and press treatments but would have a short-term calcification rate similar to the ambient treatment.

Chapter 2

Interactive effects of ocean acidification and nutrient addition on the crustose coralline alga *Lithophyllum kotschyanum*

Introduction

Coral reefs increasingly are threatened globally by anthropogenic stressors, such as climate-induced changes, coastal development, and chemical and sewage discharge (Ateweberhan et al. 2013). Additionally, these stressors rarely are experienced individually, and therefore, the impacts on coral reefs may be greater than or different from the impacts expected by a single stressor (Nyström et al. 2000, Ateweberhan et al. 2013). One of the greatest long-term global stressors to coral reefs is ocean acidification (OA) (Hoegh-Guldberg et al. 2007, Kroeker et al. 2010), and one of the most detrimental local stressors is nutrient addition (Koop et al. 2001, Costa et al. 2008, Smith and Schindler 2009). Together these anthropogenic impacts can have interactive effects on coral reef communities due to the reduced ability of organisms to respond to a disturbance event, such as a short-term nutrient influx, while under constant stress from another stressor, such as ocean acidification (Nyström et al. 2000).

Among the most important groups of organisms on coral reefs are crustose coralline algae (CCA). These algae reinforce reef structure, provide habitat for other organisms, and contribute to the global carbon cycle (Nelson 2009). Additionally, they can cover 20% or more of the reef surface (Fabricius and De'ath 2001). However, due to their high-Mg calcite skeleton, they are susceptible to OA and have been shown to have decreased rates of calcification under elevated pCO_2 conditions (Johnson et al. 2014b).

Elevated pCO_2 also can decrease photosynthetic pigment concentrations, as has been shown in the coralline alga *Corallina sessilis* (Gao and Zheng 2010). Additionally, nutrient enrichment by phosphate at high concentrations has been shown to inhibit calcification in CCA by binding to the crystal lattice and preventing calcite crystal growth (Björk et al. 1995). In contrast, nutrient enrichment by nitrate has been shown to increase photosynthesis in red algae (Zheng and Gao 2009, Martins et al. 2011), and OA has not been shown to affect CCA photosynthetic rates (Comeau et al. 2016a).

The interconnectedness of photosynthesis, calcification, and nutrient metabolism in CCA means that OA and nutrient addition could have unanticipated interactive effects on several aspects of algal physiology (Turpin 1991, Lobban and Harrison 1997, Comeau et al. 2013a). Calcification and photosynthesis are linked through the exchange of OH^- and CO_2 molecules (Comeau et al. 2013a). Photosynthesis and respiration are linked through the exchange of CO_2 and carbon compounds (Turpin 1991), and these processes change the pH within cells in opposing directions, which impacts calcification (Martin et al. 2013). Photosynthetic pigments, the carbon fixing enzyme ribulose bisphosphate carboxylase oxygenase (RUBISCO), and ribulose bisphosphate (RuBP) are vital for photosynthesis, and these molecules all require nitrogen and phosphorus, common water column nutrients (Turpin 1991, Ryder et al. 1999). Additionally, nitrogen and phosphorus are necessary for the synthesis of other enzymes and ATP, which are required for all cellular processes (Turpin 1991, Lobban and Harrison 1997, Ryder et al. 1999).

With all of these metabolic links to take into account, predicting the effects of OA and nutrient addition on CCA can be difficult. Additionally, very few studies have investigated the combined effects of these two factors on calcifying algae, despite their

likely occurrence together on coral reefs. The goal of the present study was to quantify the effects of nitrate, phosphate, and nitrate + phosphate addition in combination with ocean acidification on the rates of metabolic processes of a common tropical CCA species. I hypothesized that elevated pCO_2 would affect calcification and pigment concentrations negatively (Gao and Zheng 2010, Johnson et al. 2014b), nutrient addition would affect calcification, photosynthesis, and pigment concentrations positively (Turpin 1991, Martins et al. 2011, Comeau et al. 2013a), and nutrient addition would mitigate the negative effects of OA. The results indicate that these two stressors have variable effects on CCA metabolic processes and provide insight into the complexities of the effects of multiple anthropogenic stressors on CCA.

Methods

To test the hypothesis that nutrient addition has an interactive effect with ocean acidification on CCA physiology, two manipulative experiments were conducted. The first tested the effects of nitrate and nitrate + phosphate addition crossed with OA, and the second tested the effects of phosphate and nitrate + phosphate addition crossed with OA. These experiments were performed separately due to logistical constraints on the number of mesocosm tanks available. Together these experiments show the effects of two of the most commonly added nutrients in coastal waters in combination with elevated pCO_2 on *Lithophyllum kotschyanum*.

Experiment 1

Sample collection

One hundred sixty-eight individuals of *Lithophyllum kotschyanum* (Rhodophyta) were collected from the fringing reef on the north shore of Mo'orea, French Polynesia using a hammer and chisel, at ~1-2-m depth. The samples were transported to the Richard B. Gump Research Station where they were cleaned of epibionts, acclimated in a flowing seawater table for 6 days, and glued to a plastic base using Coral Glue (EcoTech Marine). The samples then were acclimated for 1 d under ambient conditions (27 °C, 400 µatm pCO_2, and ~600 µmol quanta m^{-2} s^{-1} photosynthetically active radiation [PAR]). Fourteen samples were assigned randomly to each of 12 treatment tanks. This sample size was determined by power analyses using variances from a short preliminary nutrient enrichment experiment, a 0.5 effect size, and 0.8 power. Once in the treatment

conditions, the samples were cleaned every 2-4 days to remove epiphytic algae and were repositioned within the tanks daily to prevent any growth differences due to location.

Treatment maintenance

The 150-L experimental tanks were maintained at one of two target pCO_2 concentrations (ambient = 400 μatm; elevated = 1000 μatm) and one of three nutrient treatments (ambient = no nutrient addition; nitrate = peak of 5.5 μM NO_3^-; nitrate + phosphate = peak of 5.5 μM NO_3^- and 1.7 μM PO_4^{3-}). The elevated pCO_2 is consistent with a pessimistic projection for the end of the current century (IPCC 2014). As the ambient levels of nitrate + nitrite and phosphate in the lagoon waters of Mo'orea are 0.55 μM and 0.17 μM respectively (Alldredge 2013), elevated nutrient treatments represented a roughly 10-fold increase in nitrate, which is within the concentration range seen during runoff pulses on other tropical reefs (Devlin and Brodie 2005, den Haan et al. 2016). The 10-fold increase in phosphate kept the ratio of nutrients the same as the ratio in the ambient treatment. Each treatment combination was replicated for a total of 12 tanks.

The 25-d treatment length consisted of 3 phases: 6 days of pCO_2 treatment acclimation, 10 days of nutrient addition for the elevated nutrient treatments, and 9 days of post-nutrient pCO_2 treatment. Treatment effects were measured on half of the samples in each tank at the end of the 10-d enrichment period (time point 1) and on the remaining half at the end of the 25-d experiment (time point 2). All samples were analyzed for the same response variables.

pCO_2 treatments were achieved by bubbling CO_2 into 6 of the tanks (elevated) and air into the other 6 tanks (ambient). CO_2 addition was controlled by a pH-stat aquacontroller (Neptune Systems, Morgan Hill, CA), and pH was calculated daily by measuring the mV (Thermo Scientific Orion 3-star portable meter with Orion pH probe) and temperature (Fisher Scientific Digital Thermometer, resolution: 0.001°C) in the tanks. Salinity was measured using a conductivity meter (Orion Star A212 Conductivity Benchtop Meter) every other day. The total alkalinity of the tanks was calculated every other day from measurements using open-cell potentiometric titrations (Mettler Toledo T50 with Mettler Toledo DGi115-SC probe) of 50-mL seawater samples and was compared against certified reference material provided by A. G. Dickson (Dickson et al. 2007). pH, temperature, salinity, and total alkalinity were used to calculate the carbonate chemistry with the R package seacarb (Lavigne and Gattuso 2013).

The tanks were maintained at 27 °C to mimic austral winter temperatures on the fringing reef (Edmunds et al. 2010) using a chiller system and aquarium heaters in each tank. Light was supplied individually to each tank by 75 W LED (Light Emitting Diode) modules (Aquaillumination) at ~600 μmol quanta m^{-2} s^{-1} (measured with LI-COR LI-1400 data logger, 2Π underwater sensor model LI-192 UWQ 7060) on a 12:12 h light:dark cycle with 4-h ramp times to mimic ambient irradiances on the reef (Johnson et al. 2014a).

The elevated nitrate levels were achieved by adding 1.5 mL of 0.5M KNO_3 (Sigma-Aldrich, ACS reagent ≥99%) via pipette to the "nitrate" and "nitrate + phosphate" tanks twice day^{-1} at 12 h intervals. The elevated phosphate levels were achieved by adding 0.459 mL of 0.5M KH_2PO_4 (Sigma-Aldrich, ACS reagent ≥99%) via

pipette to the "nitrate + phosphate" tanks twice day^{-1} at 12 h intervals. The water turnover time in the tanks was 8 hours. This method mimicked pulsed additions of nutrients as it occurs from storm runoff events on fringing reefs. It did not maintain concentrations at the pulse levels (5.5 μM NO_3^- and 1.7 μM PO_4^{3-}), but resulted in an average increase in nutrient concentration slightly below the mean between the ambient and peak pulse levels in each mesocosm. This method of nutrient addition more closely reflects increased nutrient flux over a period of time instead of increased concentration, which mimics increased runoff events on the fringing reef (IPCC 2014, den Haan et al. 2016) instead of a long-term enrichment scenario.

The nitrate and phosphate levels in the elevated nutrient tanks were measured 15 minutes after each nutrient addition on 4 days during the nutrient pulse to quantify the peak values of those nutrients. In addition, on 2 of those days, nitrate and phosphate were measured 10 times over a 24-h period to determine daily fluctuations in concentration. The levels in the ambient tanks were measured once per day on those 4 days. During the experiment, water samples containing high nitrate levels were analyzed using an enzyme-based test kit (NECi Superior Enzymes; limits/range of detection: 3.6 – 71.4 μM nitrate), and the phosphate levels in all samples were analyzed via a modified ascorbic-acid method using the Hanna Phosphate Low Range handheld colorimeter (Hanna Instruments; limits/range of detection: 0.1 – 26.3 μM). Following the experiment, selected water samples were sent to the MSI Analytical Lab (UCSB) for nitrate and phosphate determination by flow injection analysis with a QuikChem 8500 flow injection analyzer (Lachat Instruments Div., Zellweger Analytics, Inc., Loveland, CO USA,

limits/range of detection: 0.2-300 μM nitrate + nitrite, 0.1-200 μM phosphate) to confirm the nutrient levels in the mesocosms determined by methods during the experiment.

Response variables

Photosynthetic rates of all samples were measured to determine the effects of treatment conditions on *L. kotschyanum*. Calcification, respiration, and photosynthetic pigment concentrations also were measured to better explain the photosynthetic responses due to the interconnectedness of these aspects of CCA metabolism. Half of the *L. kotschyanum* samples were removed from the experimental conditions at the end of the nutrient pulse (day 16), and the other half were removed at the end of the experiment (day 25). All variables were standardized to the final surface area of the samples. Calcification was calculated by buoyant weighing (Mettler Toledo PB303-S balance, resolution: 0.001 g) the samples at the beginning of the experiment and when the samples were removed, using the method of Spencer Davies (1989).

Photosynthesis and respiration were measured for each sample for each time period by incubating the samples for 1 h in the light and 1 h in the dark, respectively. Each sample was placed in an acrylic incubation chamber (250-mL volume) with water from the appropriate treatment tank. The chamber was surrounded by an outer water jacket that was supplied with constant water flow from a circulating bath to maintain a temperature of 27 °C in the chambers during the incubations to match the treatment temperature in the mesocosms. Within the chambers, a stir bar provided constant water mixing, and a piece of mesh was placed over the stir bar to keep the algal pieces above it. A PreSens (Precision Sensing GmbH, Germany) oxygen optode (calibration: 0% O_2 –

sodium dithionite, 100% O_2 – water-saturated air) and temperature probe made measurements in the chambers every second throughout the incubation. Two fiber optic halogen lights (Ace 1, Schott North America, Inc.) were used to supply saturating light (~600 μmol quanta m^{-2} s^{-1}, measured with 2Π sensor, Walz Diving PAM, Germany). Light levels matched maximum light levels in the mesocosms, and PI curves were calculated using a Walz Diving PAM (Germany) to determine saturating light (~200 μmol quanta m^{-2} s^{-1}). After 1 h of incubation under the light, the lights were turned off and a black tarp was placed over each chamber to prevent light from reaching the algae. The samples then were incubated for another hour in the same water in the dark. Samples were incubated for an hour in light and dark conditions to allow for a 5-10% change in oxygen concentration, which allowed for accurate determination of oxygen evolution rate by the sample in the chamber over the course of the incubation. Oxygen concentrations ranged between 76% and 133% saturation over the course of the incubations. Control incubations without samples in the chamber were run with water from each treatment, and control values were subtracted from algal photosynthetic and respiration rates. Metabolic rates were calculated from the linear slope over time of the dissolved oxygen concentrations in the chambers. The metabolic rates were standardized to the surface area of each sample.

The algal samples were frozen after incubations to preserve the photosynthetic pigments (Holm-Hansen and Riemann 1978) and transported to California State University, Northridge, for pigment processing. Two subsamples (~1 cm^2 each) were taken from each individual, and one was used for chlorphyll *a* extraction and the other was used for phycobilin pigment extraction. Chlorophyll *a* was extracted in 90% acetone,

and the phycobilin pigments were extracted in 1M potassium phosphate buffer, both using mortar and pestle (similar to Sudhakar et al. 2014, Parsons et al. 1984, and Carpentier 2004, using the equations of Bennett and Bogorad 1973 [phycobilins] and Jeffrey and Humphrey 1975 [chl *a*]). The solutions of extracted pigments were placed in the refrigerator (4 °C) for two days, and then warmed to room temperature and centrifuged at ~2500 RPM for 5 minutes. The absorbance of the samples was measured in a Beckman DU-65 spectrophotometer. The acetone slurry samples were read at 750 nm and 664 nm, and the phosphate buffer slurry samples were read at 750 nm, 652 nm, 615 nm, and 562 nm. These absorbances then were used to calculate the chlorophyll *a* and phycobilin (summed phycocyanin, allophycocyanin, and phycoerythrin) concentrations. Pigment concentrations were standardized to the surface area of the sample piece used for pigment extraction.

Surface area was calculated for each algal sample via the dye-dipping method (Hoegh-Guldberg 1988). Samples were dipped in a solution (400 ml) of methylene blue dye (~0.4 g), detergent (~0.10% Triton X-100), and DI water and then rinsed in a known volume of seawater. The absorbance of the seawater solution then was measured on a spectrophotometer at 620 nm and compared to a standard curve created by dipping pieces of foil with known surface areas into the dye and seawater to estimate the algal surface area. The dye then was removed from the samples by scrubbing in seawater before freezing for pigment analysis.

Statistical Analysis

The photosynthesis, calcification, respiration, Chl *a*, and phycobilin data were analyzed using partially-nested ANOVAs (tank nested within pCO_2 x nutrient treatment and crossed with duration) with tank as a random factor, and pCO_2, nitrate, and duration as fixed factors. If tank and tank x duration factors were non-significant (≥0.25), they were dropped from the model (Quinn and Keough 2002). Even when tank factors were significant in the analysis of response variables, physical and chemical tank parameters were not different between tanks. Data were log transformed when necessary to meet ANOVA test assumptions. Analyses were run in SYSTAT (v 12.0).

Experiment 2

Sample collection, response variables, and statistical analysis were the same as in Experiment 1. Treatment maintenance was the same as Experiment 1 except for the nutrient treatments. In this experiment, the three nutrient treatments were: ambient (no nutrient addition), phosphate (peak of 1.7 μM PO_4^{3-}), and nitrate + phosphate (peak of 5.5 μM NO_3^- and 1.7 μM PO_4^{3-}).

Results

Nutrients

The mean (± SE) peak nitrate concentration after nitrate addition was 6.54 ± 0.08 μM (n=16) in the first experiment and 6.56 ± 0.06 μM (n=8) in the second experiment. The mean (± SE) ambient nitrate concentration was 0.16 ± 0.03 μM (n=4) in the first experiment and 0.18 ± μM (n=8) in the second. The mean (± SE) peak phosphate concentration after phosphate addition was 2.45 ± 0.03 μM (n=8) in the first experiment and 2.39 ± 0.04 μM (n=12) in the second experiment. The mean (± SE) ambient phosphate concentration was 0.18 ± 0.02 μM (n=8) in the first experiment and 0.22 ± 0.03 μM (n=4) in the second. Concentrations of both nutrients returned to ambient levels 1-2 h before each new pulse.

Experiment 1

Photosynthetic rates showed a three-way interaction between nutrients, pCO_2, and duration ($F_{2,6}$=4.92, p=0.05) (Fig. 1a). At the end of the 10-d nutrient addition, photosynthetic rates (mean ± SE) under elevated pCO_2 conditions in ambient and elevated nitrate (N) nutrient treatments were both 0.13 ± 0.01 μmol O_2 cm^{-2} h^{-1}, which were higher than the rates under ambient pCO_2 in those nutrient treatments by 35% and 30%, respectively. In the nitrate and phosphate (N+P) treatment, there was no pCO_2 effect, but photosynthetic rates (ambient pCO_2: 0.20 ± 0.01 μmol O_2 cm^{-2} h^{-1}, elevated pCO_2: 0.20 ± 0.02 μmol O_2 cm^{-2} h^{-1}) were higher than in the other two nutrient treatments. Nine days after the end of the nutrient pulse, photosynthetic rates had dropped in the N+P treatment by 27% under ambient pCO_2 and 41% under elevated pCO_2. Also,

in both the N and N+P treatments, photosynthetic rates were lower under elevated pCO_2 than ambient pCO_2, and lower than in the ambient nutrient treatment.

There was also a significant three-way interaction of nutrients, pCO_2, and duration for the respiration response ($F_{2,155}$=4.41, p=0.01) (Fig. 1b). This response was driven by a lower rate of respiration (-0.011 ± 0.002 μmol O_2 cm^{-2} h^{-1}) in the N+P treatment under elevated pCO_2 and a higher rate (-0.027 ± 0.002 μmol O_2 cm^{-2} h^{-1}) in the N+P treatment under ambient pCO_2 at the end of the nutrient pulse only. These rates were 31% lower (elevated pCO_2) and 50% higher (ambient pCO_2) than the rates under ambient nutrients at that time point. Besides this treatment, the respiration rates showed no pCO_2 effect, and there was a slight trend of elevated respiration rates in the N and N+P treatments at the end of the nutrient pulse. All of these differences disappeared nine days later at the end of the experiment.

Calcification rates also showed a three-way interaction ($F_{2,155}$=3.00, p=0.05) (Fig. 2). At the end of the nutrient pulse, there was no pCO_2 effect in any of the nutrient treatments, but in the N+P treatment, rates were higher than in the other nutrient treatments. Nine days later, there were lower calcification rates in the elevated pCO_2 treatments than in the ambient treatments, but only when crossed with the N and N+P nutrient treatments. Mean (± SE) calcification rate in the N treatment was 0.25 ± 0.02 mg cm^{-2} d^{-1} under ambient pCO_2 and 0.16 ± 0.01 mg cm^{-2} d^{-1} under elevated pCO_2. In the N+P treatment, calcification was 0.25 ± 0.02 mg cm^{-2} d^{-1} under ambient pCO_2 and 0.17 ± 0.01 mg cm^{-2} d^{-1} under elevated pCO_2.

There were significant effects of nutrients and of duration on Chl *a* concentrations ($F_{2,155}$=36.67, p<0.01; $F_{1,155}$=12.96, p<0.01) (Fig. 3a). There also was a significant effect

of nutrients on phycobilin concentrations ($F_{2,155}$=4.65, p=0.01) (Fig. 3b). At the end of the nutrient pulse, there was higher pigment content in the N and N+P treatments than the ambient treatments. At the end of the experiment, pigment contents had dropped in the nutrient addition treatments to levels similar to or closer to pigment contents in the ambient nutrient treatment.

Experiment 2

Photosynthetic rates were affected significantly by the interaction between experimental duration and pCO_2 treatment ($F_{1,6}$=7.92, p=0.03) and by the interaction between duration and nutrient treatment ($F_{2,6}$=14.95, p<0.01) (Fig. 4a). Tank was significant and could not be dropped from the model ($F_{6,144}$=3.34, p<0.01), but it did not hinder the interpretation of the within tank factors (experimental duration and its interactions). At the end of the nutrient pulse, there were higher rates of photosynthesis in the N+P treatment than the ambient and elevated phosphate (P) treatments. Additionally, there were lower rates of photosynthesis under elevated pCO_2 in the ambient and P nutrient treatments. Nine days after the nutrient pulse ended, photosynthetic rates had dropped in the N+P treatment. There were still lower rates of photosynthesis under elevated pCO_2 in the P treatment, but rates were not different between the pCO_2 treatments under ambient nutrients.

There was a significant effect of nutrients on respiration ($F_{2,6}$=11.51, p=0.01) (Fig. 4b). Under both ambient and elevated pCO_2, the P treatment had lower respiration rates than almost all of the other treatments at both times.

Calcification rates were not significantly different between any of the treatments at either time point (Fig. 5). There was a slightly lower calcification rate (0.17 ± 0.18 mg cm^{-2} d^{-1}) under elevated pCO_2 but only in the ambient nutrient treatment at the end of the 10-d nutrient pulse.

There was a significant interaction of nutrients, pCO_2, and experiment duration on Chl *a* concentrations ($F_{2,156}$=4.38, p=0.01) (Fig. 6a). There was a positive effect of elevated pCO_2 on Chl *a* content in the N+P treatment at the end of the nutrient pulse (0.40 ± 0.04 μg cm^2, compared to 0.23 ± 0.02 μg cm^2 under ambient pCO_2), but there were no pCO_2 effects in other treatments or at the other time point. There was a trend of increasing Chl *a* with nutrient addition at the end of the experiment only. For phycobilins, there were two-way interactions between nutrients and pCO_2 ($F_{2,156}$=3.27, p=0.04), pCO_2 and duration ($F_{1,156}$=9.80, p<0.01), and nutrients and duration ($F_{2,156}$=4.87, p=0.01) (Fig. 6b). Under elevated pCO_2, phycobilin concentrations were higher in the P and N+P treatments at the second time point than the first. The only nutrient effect at the end of the nutrient pulse was the low phycobilin content in the P treatment under elevated pCO_2 (0.001 ± 0.0002 mg cm^2, compared to 0.002 ± 0.0006 mg cm^2 under ambient pCO_2).

Discussion

Coral reefs increasingly are impacted by multiple global stressors, such as ocean acidification and climate change, and local stressors, such as nutrient addition. Together these can have complicated and interactive effects on coral reef organisms. The present study examined the effects of these stressors on metabolic responses of one species of tropical crustose coralline algae and found different responses to elevated pCO_2 under different nutrient treatments and delayed effects of nutrient addition that were observed more than one week after nutrient addition ceased.

It has been shown for other macroalgae, that nutrient addition can lead to increases in photosynthesis and growth (Schaffelke and Klumpp 1998, Zheng and Gao 2009, Hofmann et al. 2015). The results of the present study demonstrated that the addition of nitrogen and phosphorus led to elevated photosynthetic rates. However, nitrogen and phosphorus individually did not increase photosynthesis. Only N+P resulted in elevated rates, and this pattern was observed under both pCO_2 treatments. Despite the fact that tropical coastal waters are typically P limited (Smith 1984), specific nutrient limitation can vary by species (Lapointe et al. 1987). Some species also have been shown to be limited by both N and P (Shaffelke and Klumpp 1998). This dual limitation may exist in *L. kotschyanum* in the present study and could explain the effects of nutrient enrichment on photosynthesis. Nine days after the nutrient pulse ended in this study, the patterns of photosynthetic responses were very different than at the end of the pulse. In the N+P treatments, photosynthetic rates dropped closer to ambient levels, but there were different responses under different pCO_2 treatments. Additionally, the responses were not the same in the first and second experiments. This difference in response suggests that

there is some interactive effect of nutrients and pCO_2 that is potentially variable. The stored nutrients could be being used at different rates within the algal cells and, therefore, cause the photosynthetic rates to respond to external nutrient changes at different speeds. Alternatively, the increases in nitrate uptake and nitrate reduction within the cell that occur during nitrate addition in the surrounding seawater (Eppley et al. 1969, D'Elia and DeBoer 1978) may result in excess nitrate reductase in the cell after nutrient addition stops. Then, the already variable response of photosynthesis to elevated pCO_2 could interact with the reduction in the cellular content of nitrate reductase to produce unpredictable changes in photosynthetic rates.

The effects of moderate phosphate enrichment on *L. kotschyanum* are still unclear. Previously it has been shown in fungi that nitrate reductase activity depends on phosphate availability (Nicholas and Scawin 1956), and therefore, under elevated pCO_2, phosphorus limitation could lead to decreases in nitrate reduction in calcifying algae (Hofmann et al. 2014). However, in the present study, photosynthesis increased after phosphate addition ceased, and respiration rates were low in the phosphate treatment at both time points. An alternative possibility that was suggested by Hofmann et al. (2014) in a similar experiment with *Halimeda opuntia* where the algae were subjected to different pCO_2 and nutrient conditions, is that the excess phosphate in combination with elevated CO_2 gave epiphytic diatoms a competitive advantage over the macroalgae on which they were growing. Although the samples in the present experiment were cleaned regularly to minimize epiphytic algal growth, it is still possible that diatoms in the water settled on the CCA samples after cleaning and negatively affected CCA photosynthetic rates.

Photosynthetic pigment concentrations have been shown to increase under elevated nutrients in many algae due to the storage of nitrogen in pigment molecules (Chopin et al. 1995, Naldi and Wheeler 1999, Martins et al. 2011). The most common cause of variation in pigment content is varying light levels (Lobban and Harrison 1997), but because PAR (photosynthetically active radiation) was not varied in the present experiment, the increased pigments observed can be attributed to nutrient addition. The variable responses of pigment concentrations between the end of the nutrient pulse and the end of the experiment may be related to the varying rates of use of stored nutrients. The increases in pigments may also be a reflection of nutrient replete conditions in the algae, which correspond with increases in nitrogen storage as amino acids and proteins (Naldi and Wheeler 1999). Pigment concentrations could be variable depending on how much nitrogen is allocated to each of several storage pools. It is therefore possible that nitrogen could be stored in different pools depending on where it is most needed and pigment concentrations are not the best reflection of nutrient replete conditions.

The calcification responses at the end of the first experiment showed reduced calcification under elevated pCO_2 in the N and N+P treatments. The calcification rates in the ambient nutrient treatment at the first time point in the second experiment also showed this trend. These results are consistent with previous work showing reduced calcification in CCA under elevated pCO_2 (Johnson and Carpenter 2012, Comeau et al. 2013b), and the rates are similar to those seen in previous work (Comeau et al. 2016b). However, the remaining treatments showed no effect of pCO_2 on calcification. This lack of response may be due to the length of the experiment, the variability in calcification rates between samples, or some other effect of nutrients. It has been shown previously

that phosphate addition can reduce calcification at high concentrations by inhibiting calcite crystal growth, but that moderate levels of phosphate enrichment did not have a negative effect (Björk et al. 1995). These previous findings are consistent with the results of the present study, which showed no effect of phosphate on calcification. Also, photosynthesis and calcification are linked metabolically in CCA (Comeau et al. 2013a). There may be a link between the changes in photosynthetic rate between treatments and the calcification response. Because the last calcification measurement was an integration over the entire 25-d experiment, any changes in calcification rate that may have occurred in the middle due to the nutrient pulse or changes in photosynthetic metabolism could have averaged out so that no effect was observed over the whole experiment. In order to investigate short-term effects more fully, further experiments should be done with multiple measurements of buoyant weight taken on the same samples over a longer experiment that incorporates nutrient addition. Future study should quantify the nitrogen and phosphorus content of the algae as well as the importance of seawater N:P ratios.

The results of the present study demonstrate complex effects resulting from the interaction of multiple anthropogenic stressors on CCA metabolism. The combination of ocean acidification, a long-term stressor, and nutrient addition, a short-term stressor on many coral reefs, can have unanticipated and variable results. A short nutrient pulse can lead to delayed or lasting effects on CCA that are different under different pCO_2 conditions. This finding emphasizes the importance of continued study of these interactive stressors as well as managing these anthropogenic impacts. The present study suggests that protecting coral reefs from nutrient enrichment may be even more important in the future when pCO_2 in the ocean will be higher, and further work should be done on

the combined effects of OA and local anthropogenic stressors to better predict the impacts of these combined effects.

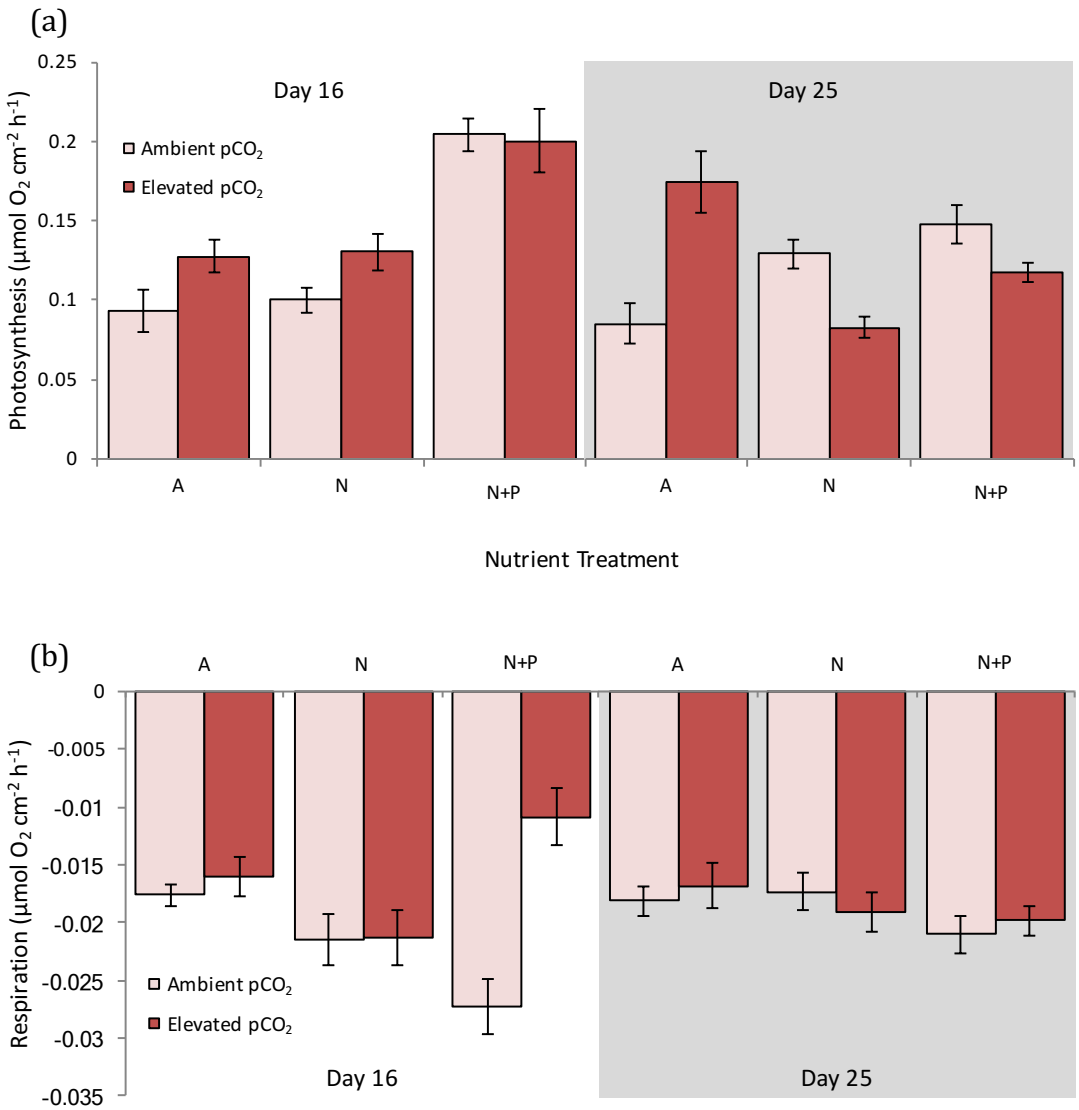

Figure 1. Photosynthesis (a) and respiration (b) rates for Experiment 1. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7, except Day 25 ambient pCO_2 ambient nutrients n=6). There is a significant three-way interaction between nutrients, pCO_2, and duration in both graphs. Levels of significance are in the text.

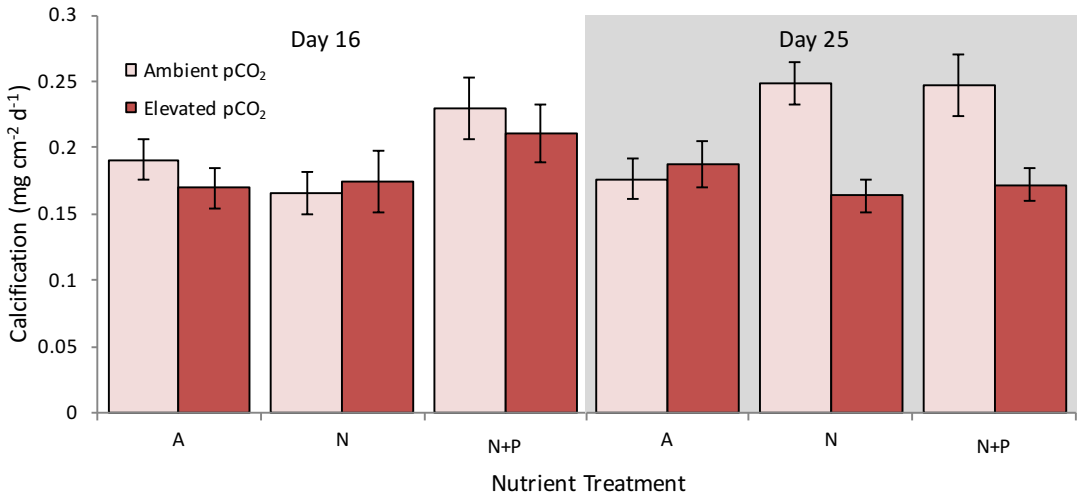

Figure 2. Calcification rates for Experiment 1. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7, except Day 25 ambient pCO_2 ambient nutrients n=6). There is a significant three-way interaction between nutrients, pCO_2, and duration in both graphs. Levels of significance are in the text.

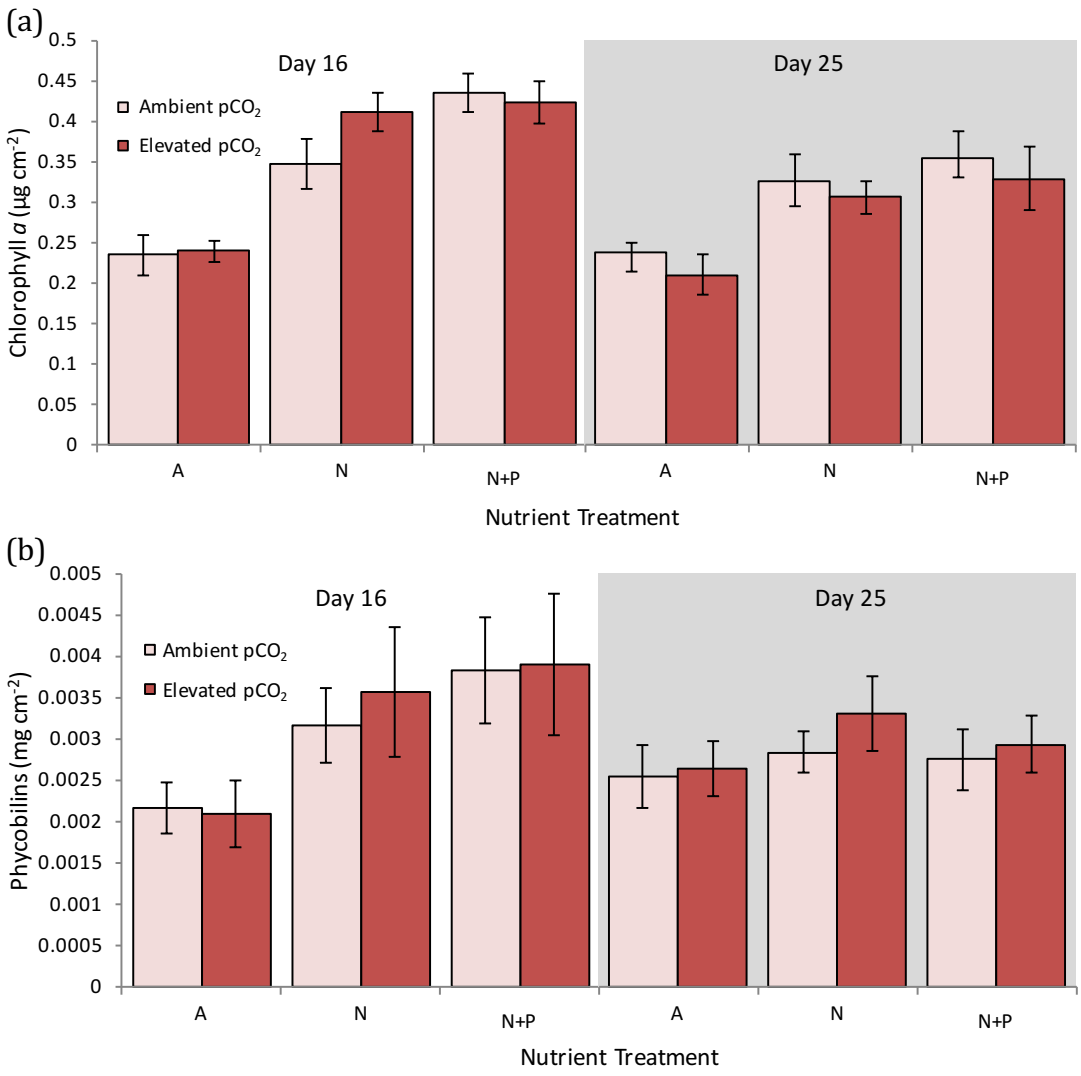

Figure 3. Chlorophyll *a* (a) and total phycobilin accessory pigment (b) concentrations for Experiment 1. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7, except Day 25 ambient pCO_2 ambient nutrients n=6). There are significant effects of nutrients and of duration in the chlorophyll *a* graph, and there is a significant effect of nutrients in the phycobilins graph. Levels of significance are in the text.

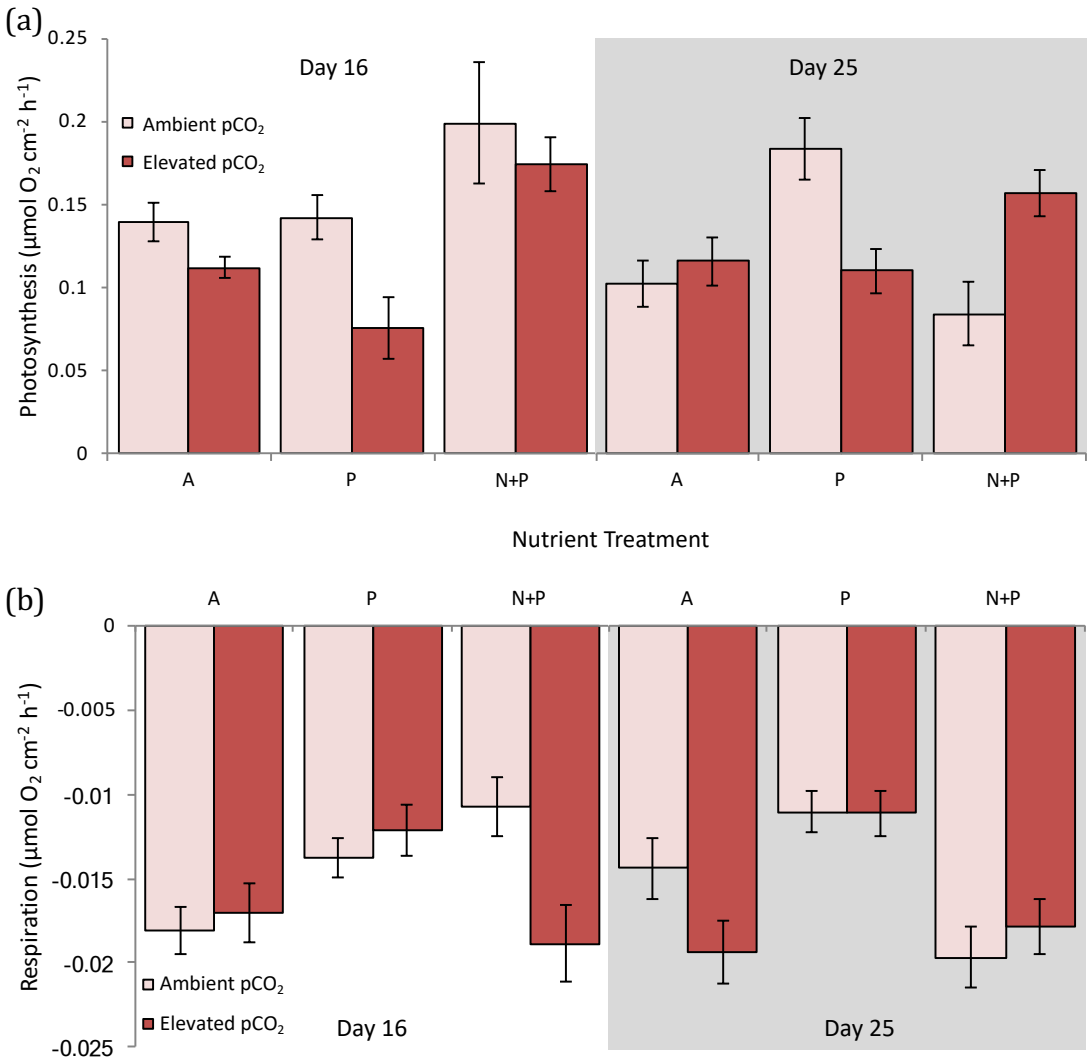

Figure 4. Photosynthesis (a) and respiration (b) rates for Experiment 2. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7). There are significant two-way interactions between duration and pCO_2 and between duration and nutrients in the photosynthesis graph. There is a significant effect of nutrients in the respiration graph. Levels of significance are in the text.

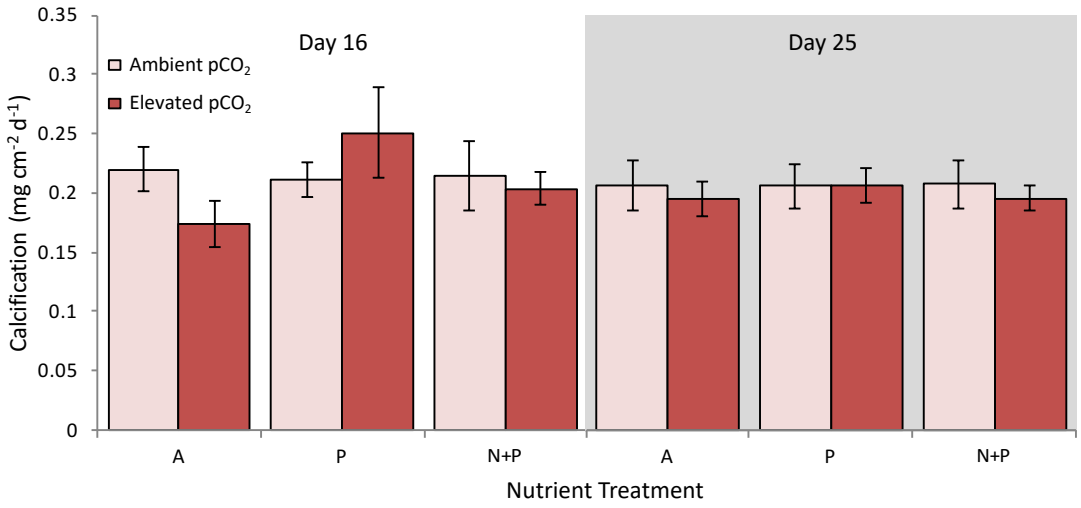

Figure 5. Calcification rates for Experiment 2. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7). The responses are not significantly different between any treatment.

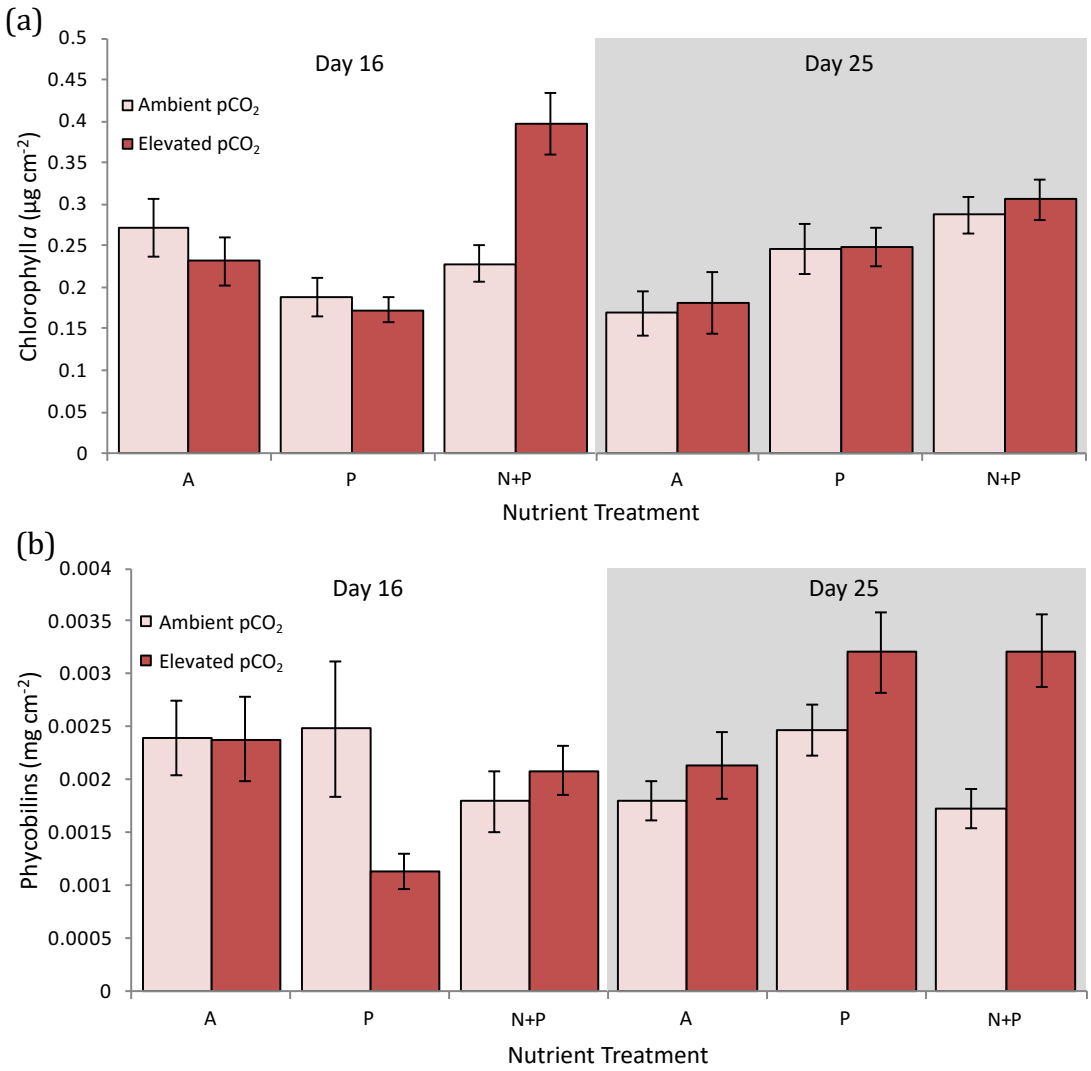

Figure 6. Chlorophyll *a* and total phycobilin accessory pigment concentrations for Experiment 2. Nutrient treatments are ambient (A), nitrate added (N), and nitrate and phosphate added (N+P). Graphs show means ± SE (n=7, except Day 16 ambient pCO_2 ambient nutrients n=6). There is a significant 3-way interaction between nutrients, pCO_2, and duration in the chlorophyll *a* graph. There are significant 2-way interactions between nutrients and pCO_2, pCO_2 and duration, and nutrients and duration in the phycobilins graph. Levels of significance are in the text.

Chapter 3

Effects of ocean acidification, short-term nutrient pulses, and diurnal photosynthetic patterns on tropical CCA

Introduction

Increasingly, coral reefs around the globe are threatened by climate change and other anthropogenic stressors, including ocean acidification (OA) and coastal eutrophication (Smith and Schindler 2009, Kroeker et al. 2010). Additionally, multiple stressors are impacting reefs simultaneously, potentially leading to interactive effects or changes in the ability of reef organisms to recover from a single stressor (Nyström et al. 2000). This may be true especially when the disturbances occur on different time scales. Ocean acidification, the reduction in pH of seawater due to increased CO_2, already is occurring and will continue past the end of the century in all oceans (Hoegh-Guldberg et al. 2007, IPCC 2014). In contrast to this long-term stressor, nutrient addition on coral reefs often occurs in short pulses due to runoff plumes entering the water for a few hours after rain events (den Haan et al. 2016).

Among the most important groups of organisms on coral reefs are crustose coralline algae (CCA). These algae reinforce reef structure, provide habitat for other organisms, and contribute to global carbon cycles (Nelson 2009). However, because they build high-Mg calcite skeletons, they are susceptible to OA and have been shown to have decreased rates of calcification under elevated pCO_2 conditions (Johnson et al. 2014b). There have not been any demonstrated effects of OA on photosynthesis in CCA (Comeau et al. 2016a). OA is not the only stressor affecting these algae though, and nutrient

enrichment by nitrate has been shown to increase photosynthetic rates in red algae (Zheng and Gao 2009, Martins et al. 2011). There have been no demonstrated effects of elevated nitrate concentrations on calcification for CCA (Björk et al. 1995). Additionally, these previous nutrient enrichment experiments have been performed over long time periods and have not taken into account the short duration that is characteristic of many nutrient pulses. Therefore, it is important to determine the effects of OA in combination with short nutrient pulses on an hourly time scale to fully understand the interactive effects of these anthropogenic stressors.

Over hourly time scales, diurnal metabolic patterns in algae also can influence algal photosynthetic rates (Henley et al. 1991, Roenneberg and Mittag 1996). These diurnal photosynthetic patterns include circadian photosynthetic patterns, endogenously controlled changes in light-saturated photosynthesis that follow the light-dark cycle over a diel period (Roenneberg and Mittag 1996). Light-saturated photosynthesis is the maximum photosynthetic rate of an alga when light is not limiting (Lobban and Harrison 1997). The circadian photosynthetic pattern is different from the change in photosynthesis that occurs over the course of the day due to changing light levels because the circadian pattern is still observed when light levels are held constant (Harding et al. 1981, Lobban and Harrison 1997). Often, natural variation in algal photosynthetic rate over the course of the day is not taken into account in lab experiments when the photosynthetic response of an alga is measured. However, diurnal changes in photosynthetic rate, such as those caused by circadian patterns, could confound measured rates of photosynthesis at certain times of day. These patterns may not be taken into account in many measurements of photosynthesis because the times at which the rates are

measured are randomized with respect to time of day or because a specific manipulated factor in an experiment produces a greater change in photosynthesis than the natural diurnal fluctuations. This would lead to a noticeable effect of the manipulated factor despite the inherent diurnal photosynthetic changes in the algae. Additionally, not all algae have diurnal or circadian photosynthetic patterns, so hourly photosynthetic changes may not be a concern in all experiments (Harding et al. 1981). However, these hourly changes in light-saturated photosynthesis may be important when short-term responses are being measured on the scale of hours.

The first goal of the present study was to determine the effect of short-term nutrient pulses on the light-saturated photosynthetic rate and short-term calcification rate (calcification over the course of one hour) of *Lithophyllum kotschyanum*, a species of CCA, under two pCO_2 treatments. Subsequently, based on the results of the first experiment, the second goal was to quantify the diurnal pattern of light-saturated photosynthesis and short-term calcification in this species of CCA. I hypothesized that elevated pCO_2 would decrease calcification rates, and that short-term photosynthetic rates would increase over the course of an 8-h nutrient pulse. I did not expect a diurnal pattern of light-saturated photosynthesis to impact the effects of the nutrient pulse on photosynthetic rate, and I hypothesized that there would not be a diurnal pattern in net calcification. The results of the first experiment suggest the existence of a diurnal pattern in light-saturated photosynthesis in this species, and the second experiment confirms and describes this pattern. Overall, the present study emphasizes the complexities of measuring short-term metabolic responses of CCA to nutrient pulse events, and therefore,

the difficulties in predicting how increased runoff will affect these algae in the context of the whole coral reef community.

Methods

Experiment 1

To test the hypothesis that photosynthetic and calcification rates of CCA would change in the 8 hours following a nutrient pulse, and that there would be different responses under different pCO_2 concentrations, a manipulative experiment was conducted with replicate mesocosm tanks at two levels of pCO_2. Photosynthesis and calcification both were measured over 1-h incubation periods to quantify the rates of these processes on short timescales, and therefore how they changed over short timescales.

Sample collection

Sixty individuals of a common species of CCA, *Lithophyllum kotschyanum*, were collected from the fringing reef on the north shore of Mo'orea, French Polynesia using a hammer and chisel, at ~1-2-m depth. The samples were transported to the Richard B. Gump Research Station where they were cleaned of epibionts, acclimated in a flowing seawater table for 4 days, and glued to a plastic base using Coral Glue (EcoTech Marine). Then, the samples were acclimated for 1 d under ambient conditions (27 °C, 400 µatm CO_2, and ~600 µmol quanta m^{-2} s^{-1} photosynthetically active radiation [PAR]). Fifteen samples were assigned randomly to each of 4 treatment tanks. Once in the treatment conditions, samples were cleaned as needed to remove epiphytic algae and were repositioned within the tanks daily to prevent any growth differences due to location.

Treatment maintenance

The 150-L experimental tanks were maintained at two target pCO_2 concentrations: ambient (400 µatm) or elevated (1000 µatm). The elevated concentration is a pessimistic projection for the end of the current century (IPCC 2014). Each pCO_2 treatment was replicated in 2 tanks. CCA samples were acclimated in one of the two pCO_2 treatments for 10 days. On the tenth day, a nitrate pulse (peak of 5.5 µmol/L NO_3^-; achieved by adding 1.5 mL of 0.5M KNO_3 [Sigma-Aldrich, ACS reagent ≥99%]) was added to each tank. As the ambient levels of nitrate + nitrite in the lagoon waters surrounding Mo'orea are 0.55 µM (Alldredge 2013), this nutrient addition reflects a roughly 10-fold increase in nitrate, which is within the concentration range seen during pulses of nutrients on other tropical reefs (Devlin and Brodie 2005, den Haan et al. 2016). The water turnover rate in the tanks was ~8 hours, so nutrient concentrations peaked when the pulse was added and slowly decreased over the following 8 hours to ambient levels.

pCO_2 treatments were achieved by bubbling CO_2 into 2 of the tanks (elevated) and air into the other 2 tanks (ambient). CO_2 addition was controlled by a pH-stat aquacontroller (Neptune Systems, Morgan Hill, CA), and pH was calculated daily by measuring the mV (Thermo Scientific Orion 3-star portable meter with Orion pH probe) and temperature (Fisher Scientific Digital Thermometer, resolution: 0.001°C) in the tanks. Salinity was measured using a conductivity meter (Orion Star A212 Conductivity Benchtop Meter) every other day. The total alkalinity of the tanks was calculated every other day from measurements using open-cell potentiometric titrations (Mettler Toledo T50 with Mettler Toledo DGi115-SC probe) of 50-mL seawater samples and was

compared against certified reference material provided by A. G. Dickson (Dickson et al. 2007). pH, temperature, salinity, and total alkalinity were used to calculate the carbonate chemistry with the R package seacarb (Lavigne and Gattuso 2013).

The tanks were maintained at 27 °C to mimic austral winter temperatures on the fringing reef (Edmunds et al. 2010) using a chiller system and aquarium heaters in each tank. Light was supplied individually to each tank by 75 W LED (Light Emitting Diode) modules (Aquaillumination) at ~600 μmol quanta m^{-2} s^{-1} (measured with LI-COR LI-1400 data logger, 2Π underwater sensor model LI-192 UWQ 7060) on a 12:12 h light:dark cycle with 4-h ramp times to mimic ambient irradiances on the reef (Johnson et al. 2014a).

Water samples for nitrate concentrations were taken from each tank immediately before nitrate was added, and 5 minutes, 1, 2, 4, and 8 h after nitrate was added to determine nitrate concentrations over the course of the pulse. The water samples were sent to the MSI Analytical Lab (UCSB) for nitrate determination by flow injection analysis with a QuikChem 8500 flow injection analyzer (Lachat Instruments Div., Zellweger Analytics, Inc., Loveland, CO USA, limits/range of detection: 0.2-300 μM nitrate + nitrite).

Response variables

Rates of photosynthesis and short-term calcification of the samples were measured following the nutrient pulse. Three samples from each tank were removed at each of five time points: immediately before nitrate was added and 1, 2, 4, and 8 hours after nitrate was added. Light-saturated photosynthesis was measured at all 5 time points,

and short-term calcification was measured before the nitrate pulse and 4 hours after the pulse. Both photosynthesis and short-term calcification rates were standardized to the final surface area of the samples.

Photosynthesis was measured by incubating samples for 1 h in an acrylic incubation chamber (250-mL volume) using water from the appropriate treatment tanks. The chamber was surrounded by an outer water jacket that was supplied with constant water flow from a circulating bath to maintain a temperature of 27 °C in the chambers during the incubations to match the treatment temperature in the mesocosms. Within the chambers, a stir bar provided constant water mixing, and a piece of mesh was placed over the stir bar to keep the algal pieces above it. A PreSens (Precision Sensing GmbH, Germany) oxygen optode (calibration: 0% O_2 – sodium dithionite, 100% O_2 – water-saturated air) and temperature probe made measurements in the chambers every second throughout the incubation. Two fiber optic halogen lights (Ace 1, Schott North America, Inc.) were used to supply saturating light (~600 μmol quanta m^{-2} s^{-1}, measured with 2Π sensor, Walz Diving PAM, Germany). Light levels matched maximum light levels in the mesocosms, and PI curves were calculated using a Walz Diving PAM (Germany) to determine saturating light at midday (~200 μmol quanta m^{-2} s^{-1}). Samples were incubated for one hour to allow for a 5-10% change in oxygen concentration, which allowed for accurate determination of oxygen evolution rate by the sample in the chamber over the course of the incubation. Control incubations without samples in the chamber were run with water from each treatment, and control values were subtracted from algal photosynthetic rates. Photosynthetic rate was calculated from the linear slope over time of the dissolved oxygen concentrations in the chambers.

Short-term calcification was measured using the total alkalinity anomaly method (Smith and Kinsey 1978). Total alkalinity of the water was measured at the beginning and end of the hour-long photosynthesis incubation for each sample. The change in alkalinity was used to calculate calcification under the model that alkalinity is lowered two units for every one mole of calcium carbonate precipitated (Smith and Kinsey 1978). Calcification in the control incubations was also measured and was subtracted from sample calcification values.

Surface area was quantified for each algal sample via the dye-dipping method (Hoegh-Guldberg 1988). Samples were dipped in a solution (400 ml) of methylene blue dye (~0.4 g), detergent (~0.10% Triton X-100), and DI water and then rinsed in a known volume of seawater. The absorbance of the seawater solution then was measured on a spectrophotometer at 620 nm and compared to a standard curve created by dipping pieces of foil with known surface areas into the dye and seawater to estimate the algal surface area.

Experiment 2

To test the hypotheses that there was a diurnal pattern of light-saturated photosynthesis and that there was not a diurnal pattern of short-term calcification, under both ambient and elevated pCO_2, an experiment was designed with replicate mesocosm tanks at each pCO_2 level.

Sample collection and response variables were the same as in Experiment 1. Maintenance of the tanks and pCO_2 treatments were similar. The light in the tanks was still controlled on a 12:12 h light:dark cycle with 4-h ramp times. However, no nutrient

pulses were added at the end of the 10 days. Instead, on the last day, three samples were removed (without replacement) from each of the four tanks at five time points over the course of the 12 hours of daylight (0600, 0900, 1200, 1500, and 1800 h local time) in order to determine the diurnal pattern of light-saturated photosynthesis and short-term calcification.

Statistical Analyses

Each response variable in Experiment 1 was analyzed using a partially-nested ANOVA with Tank nested within pCO_2 treatment and Time Since Nutrient Addition within Tank (SYSTAT v12.0). The photosynthesis and calcification responses then were plotted against time of day in order to compare the results to those from the second experiment.

In the second experiment, partially-nested ANOVAs were run on the photosynthetic rates and calcification rates to test for significant differences between tanks of the same treatment. pCO_2 level was the between Tank factor and Time of Day was the within Tank factor. 1^{st}-4^{th} order polynomial curves were fit to the responses in each tank and the curve with the best fit was used to analyze differences between pCO_2 treatments. Two tailed *t*-tests were used to statistically compare the fitted curve maxima (y max), the time of day of the fitted curve maximum, and the photosynthetic rates at 1200 h between ambient and elevated pCO_2 treatments.

Results

The ambient nitrate + nitrite concentration in the tanks was ~0.5 μM. With the nitrate pulse, concentrations increased to ~7.3 μM. Concentrations dropped gradually over the 8 hours following the pulse to ~2 μM (Values from one tank shown in Fig. 1).

The results of the nutrient pulse experiment showed that there were significant differences between tanks of the same pCO_2 concentration for both photosynthetic rates (μmol O_2 cm^{-2} h^{-1}) and calcification rates (mg cm^{-2} d^{-1}) (ANOVA: photosynthesis Tank $F_{2,40}$=7.85 $p<0.01$; calcification Tank $F_{2,14}$=7.52 p=0.01). When plotted against time of day, photosynthetic rates under saturating light showed a pattern of elevated rates in the middle of the day (up to 0.14 ± 0.03 μmol O_2 cm^{-2} h^{-1} in elevated pCO_2 tank 1) when there was maximum light in the mesocosms (~600 μmol quanta m^{-2} s^{-1}) and lower rates at dawn and dusk (down to 0.06 ± 0.02 μmol O_2 cm^{-2} h^{-1} in ambient pCO_2 tank 1) when the mesocosm lights were just turning on or off (0 μmol quanta m^{-2} s^{-1}) (Fig. 2). There were higher rates of photosynthesis in the elevated pCO_2 treatment before 1300 h and higher rates in the ambient treatment after 1300 h. Calcification was higher in the ambient pCO_2 treatment later in the day, but there was not a clear pattern over the course of the day (Fig. 3).

In the second experiment, a 4^{th} order polynomial curve fit both ambient and elevated pCO_2 responses best ($r^2>0.999$) (Fig. 4). The maximum photosynthetic rates for the fitted curves were not significantly different between ambient and elevated pCO_2 (ambient mean ± SE: 0.18 ± 0.01 μmol O_2 cm^{-2} h^{-1}, elevated mean ± SE: 0.16 ± 0.03 μmol O_2 cm^{-2} h^{-1}) (*t*-test: t_2=0.54, p=0.64), and the photosynthetic rates at 1200 h also were not significantly different between treatments (ambient mean ± SE: 0.18 ±

0.008 μmol O_2 cm^{-2} h^{-1}, n=2; elevated mean ± SE: 0.15 ± 0.038 μmol O_2 cm^{-2} h^{-1}, n=2) (*t*-test: t_2=0.67, p=0.62). The elevated pCO_2 peak photosynthetic rate occurred at 1225 h and the ambient peak occurred at 1113 h, but the times were not significantly different (*t*-test: t_2=-1.43, p=0.38).

Calcification rates were different between tanks of the same pCO_2 treatment (ANOVA: $F_{2,40}$=4.76, p=0.01). Additionally, they did not follow a curve like the photosynthetic rates (Fig. 5). Rates increased in the elevated pCO_2 treatment from 0.35 ± 0.14 mg cm^{-2} d^{-1} at 06:00 to 0.60 ± 0.07 mg cm^{-2} d^{-1} at 18:00, but there was not a clear pattern in calcification rates over the course of the day.

Discussion

Light-saturated photosynthesis in *L. kotschyanum* follows a diurnal pattern that directly influences the physiological responses of the alga to inputs such as nutrient addition. This daily oscillation of photosynthetic rate is suggested in the first experiment and clearly shown in the second experiment. This pattern can be explained by several hypotheses. The diurnal pattern of light-saturated photosynthesis may be a result of diel ambient nutrient fluctuations, photoinhibition in the afternoon, or feedback inhibition of accumulated photosynthetic products later in the day. However, each of these was shown not to be the cause of similar diurnal patterns of photosynthesis in *Ulva rotundata* by Henley et al. (1991). It is also possible that diurnal patterns of RUBISCO (ribulose bisphosphate carboxylase oxygenase) concentration control photosynthetic patterns due to the importance of RUBISCO in carbon fixation in photosynthesis (Turpin 1991). A diurnal pattern in the production of the RUBISCO large subunit transcript has been shown in some red and green algae (Jacobsen et al. 2003, Recuenco-Muñez et al. 2015). However, Recuenco-Muñez et al. (2015) also found that the diurnal pattern did not persist through all steps of RUBISCO assembly, so there may not be diurnal changes in RUBISCO abundance in the cell in some algae.

Alternatively, the pattern shown in the present study is consistent with circadian patterns of light-saturated photosynthesis that have been shown in many unicellular algae and some multicellular algae (Harding et al. 1981, Samuelsson et al. 1983, Roenneberg and Mittag 1996, Sorek et al. 2013). Circadian patterns are endogenous daily rhythms that are controlled by internal oscillators and synchronized to the 24-h solar day by zeitgebers (Roenneberg and Mittag 1996) These circadian patterns almost always are

synchronized by the solar light-dark cycle, but in some unicellular species, they also can be synchronized by nitrate availability (Roenneberg and Mittag 1996). In species that have this intrinsic circadian pattern of photosynthesis, diurnal changes in photosynthetic rates are maintained even when individuals are moved into a constant light environment, and they are not correlated with changes in respiration or pigment content (Harding et al. 1981). This is due to circadian oscillators, components of a cell that regulate different aspects of the photosynthetic process, as well as other functions (Roenneberg and Mittag 1996). In some dinoflagellates, it has been shown that photosystem (PS) II accounts for the circadian oscillations in photosynthesis, and in *Euglena*, photosystem I and II both show rhythmicity (Samuelsson et al. 1983). In other algae and plants, the circadian system regulates chloroplast components, number, structure, shape, and migration, which would affect photosynthesis (Roenneberg and Mittag 1996). In *Symbiodinium*, the expression of a gene involved in the oxygen-evolution complex of PS II was shown to have daily oscillations, thereby controlling patterns of photosynthetically evolved oxygen (Sorek et al. 2013). Whether or not the diurnal pattern of photosynthetic rate quantified in the present study is controlled by a circadian oscillator can be determined by measuring photosynthetic rates of *L. kotschyanum* over multiple days under both changing light conditions and constant light conditions. The further study of the photosynthetic processes of this alga, and CCA in general, has important implications for how these algae will respond to varying anthropogenic stressors, especially on short time scales of hours to days.

Because of the pattern of changing light-saturated photosynthetic rates over the course of the day that was described in experiment 2 of the present study, it is not

possible to quantify the effects of nutrient addition in the first experiment. It is possible that there was some increase in photosynthesis due to the nutrient addition because the maximum rates of photosynthesis in the first experiment were higher than those in the second experiment without nutrients. However, any effects of the nutrient pulses were not strong enough to be observed due to the underlying diurnal photosynthetic pattern. This clear diurnal pattern also means that the time of nutrient addition in the field may impact how much it affects the photosynthetic physiology of this alga. In addition, diurnal patterns of nitrate reductase and nutrient uptake have been shown in seagrass and some algae (D'Elia and DeBoer 1978, Turpin 1991, Touchette and Burkholder 2000). These two patterns combined could interact to determine how much of an effect a short-term nutrient pulse from a runoff event has on the physiology of CCA. If nutrients are added to the water at night, they may not be taken up by the algae due to the absence of photosynthesis and the downregulation of nutrient uptake. However, if a nutrient pulse occurs during the day during maximum rates of photosynthesis, it may have a larger impact on algal metabolism. Similarly, a pulse that lasts for 8 hours during daylight will have more of an impact on an alga than a pulse for the same length that only occurs during 2 hours of daylight. These impacts can also last for shorter or longer time periods after the pulse ends depending on whether there are higher rates of photosynthesis or increased levels of nitrogen being stored. Due to the many factors that need to be considered in order to predict the effects of nutrient addition on CCA, the physiology of these algae needs to be better understood.

The description of this diurnal photosynthetic pattern also has important implications for laboratory studies of CCA physiology. When measuring photosynthetic

responses to experimental conditions, time of day needs to be considered in order to avoid confounding treatment effects. This means considering both the length of measurement or incubation periods as well as the time of day that measurements are made. This is especially important if the diurnal photosynthetic pattern described in the present study is controlled by a circadian oscillator because circadian photosynthetic patterns become evident when measuring photosynthesis under constant light levels, which has important implications for lab measurements.

The results of the present study also suggest that there is a disconnect between the photosynthetic response and the short-term calcification response in CCA because short-term calcification did not show a diurnal pattern similar to photosynthesis in either experiment. It is possible that although the processes are metabolically connected via the transfer of OH^- ions from photosynthesis into the cell wall and the conversion of bicarbonate to CO_2 (Comeau et al. 2013a), they do not affect each other on a short enough time scale to follow the same diurnal pattern. This may be because the pH in the cell wall is also buffered by the amount of carbonate and bicarbonate in the cell wall, so the pH in the cell wall may not change as quickly as the photosynthetic rate changes. Alternatively, photosynthesis and calcification responses may not be tightly correlated at all. Photosynthesis uses CO_2 directly from the surrounding seawater and from respiration, not just the CO_2 that is a byproduct of calcification, and the pH of the cell wall is increased for calcification as a side effect of moving bicarbonate into the cell, not just by the input of OH^- from photosynthesis (Lobban and Harrison 1997, Comeau et al. 2013a). Therefore, each process is not dependent solely on the other. Additionally, it has been shown in coccolithophores that calcification can be inhibited while photosynthesis is

unaffected (Bach et al. 2013). Therefore, although these processes are connected, they are not completely dependent on one another, and their responses may not be correlated on the short time scale of the present study.

The experiments in the present study also demonstrated that there was not a clear effect of elevated pCO_2 on either photosynthesis or calcification. The lack of a photosynthetic response under OA is consistent with previous experiments (Comeau et al. 2016a). However, it has been shown previously that elevated pCO_2 decreases calcification in CCA (Johnson et al. 2014b). The lack of effect in these experiments may be due to the short length of the acclimation time and the slow carbon fixation rates in CCA (Goreau 1963, Comeau et al. 2016b). In the experiments of the present study, samples were acclimated for 10 days, which is shorter than other experiments with 12-14 d (Johnson et al. 2014b) and 4 wk exposure times (Comeau et al. 2016b) where there was a reduction in calcification with increased pCO_2. This means that the experiment may need to be longer to detect a reduction in calcification under ocean acidification. Additionally, there were only two replicate tanks per pCO_2 treatment in the present experiment due to logistical constraints, and there was a significant effect of tank in the analysis. Adding more tanks and extending the acclimation period of the experiment may clarify the effect of different levels of pCO_2 on calcification.

Overall, the present study showed that the impacts of short-term nutrient pulses can be complicated by the photosynthetic physiology in CCA. It also demonstrated a clear diurnal pattern in light-saturated photosynthesis in *L. kotschyaum*. Together these results suggest that short-term nutrient pulses can have different effects on CCA photosynthesis depending on how long they last and when they occur during the day. The

ability of the algae to take up, store, or use the nutrients that are added during a short pulse event will depend on where the energy balance and allocation is at that time of day. It is plausible that nutrient pulses during the day would be assimilated more than those at night because of the increased rates of photosynthesis and nutrient uptake. This possibility emphasizes the importance of distinguishing effects between long-term and short-term nutrient additions. Pulse events that last only a few hours may have different effects than longer ones because of both their length and their time of occurrence. Nutrient addition that lasts many days or weeks could overcome the temporal variation in CCA metabolism and therefore be more likely to strongly impact photosynthetic or calcification rates. Therefore, further study of CCA responses to hourly or daily nutrient addition is required to better understand the impacts of short-term nutrient runoff that is common on fringing coral reefs in order to determine how coastal development will impact these reefs into the future.

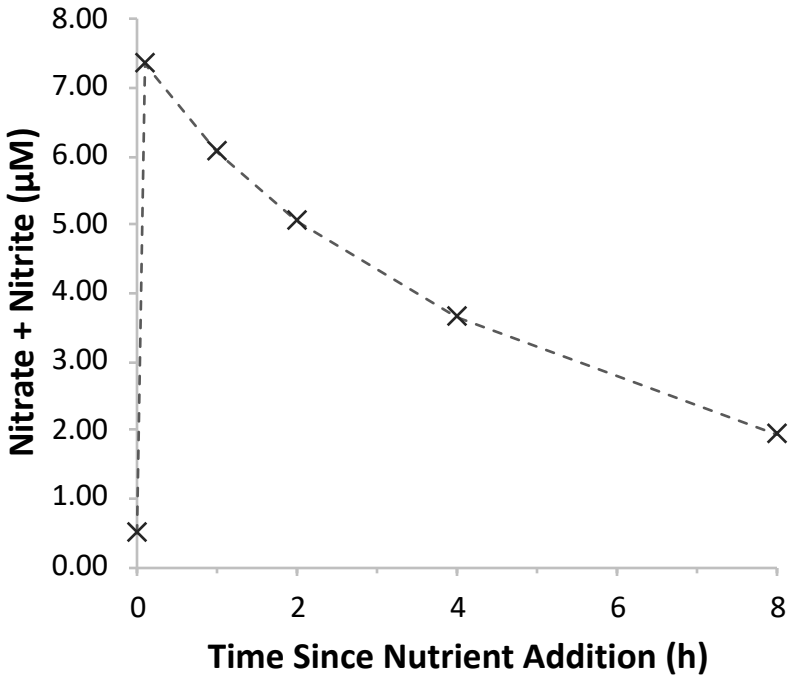

Figure 1. Nitrate + nitrite concentrations in one tank during nutrient pulse. Time is relative to nutrient addition with addition occurring at 0 h.

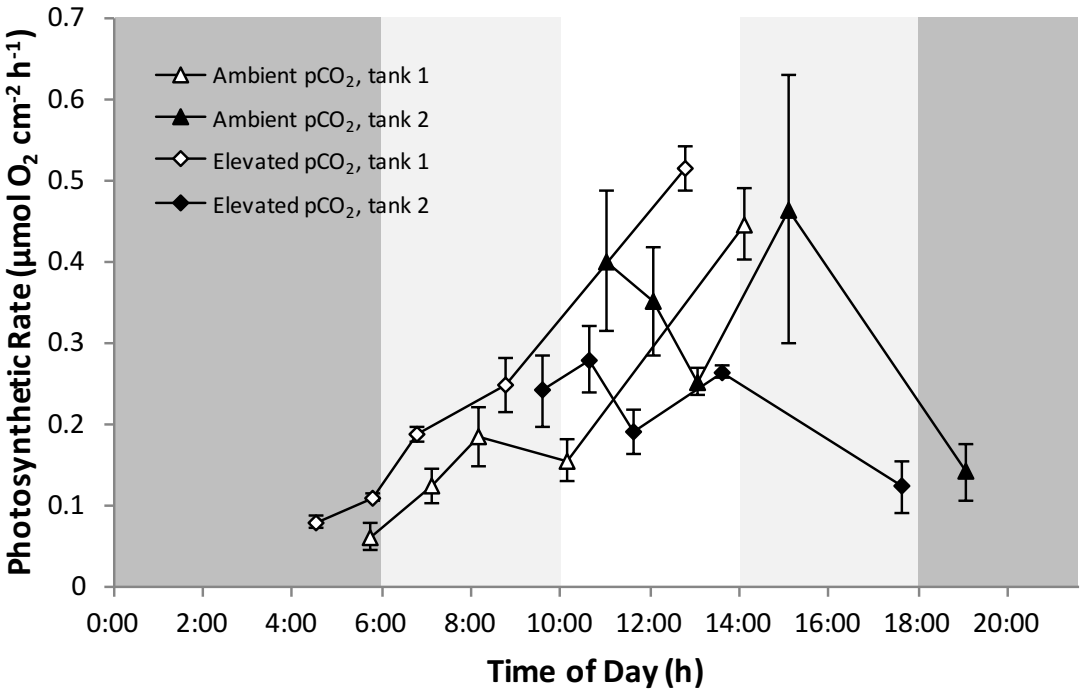

Figure 2. Mean photosynthetic rates ± SE at ~600 µmol quanta m^{-2} s^{-1} (n=3) in experiment 1. Dark gray areas are periods when lights in mesocosms are off, light gray areas are ramp periods, and white area is when lights in mesocosms are fully on.

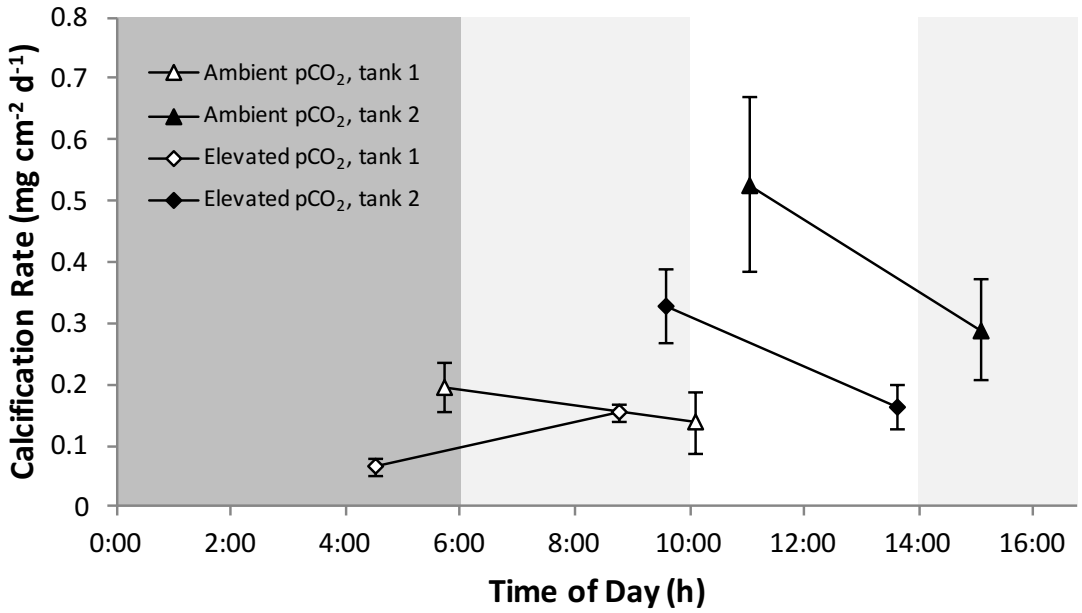

Figure 3. Mean calcification rates ± SE at ~600 µmol quanta m^{-2} s^{-1} (n=3) in experiment 1. Dark gray areas are periods when lights in mesocosms are off, light gray areas are ramp periods, and white area is when lights in mesocosms are fully on.

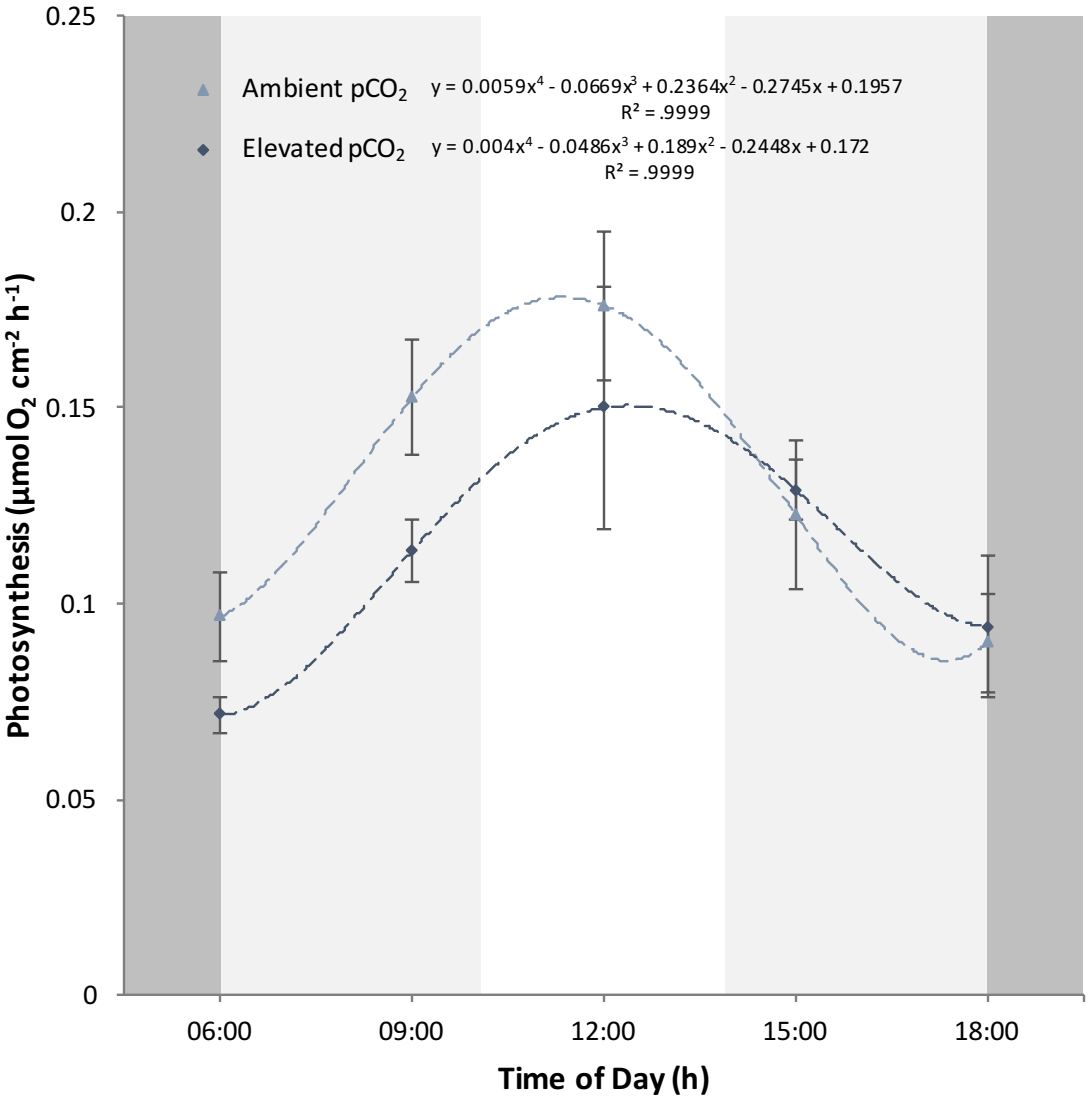

Figure 4. Mean photosynthetic rates ± SE at ~600 µmol quanta m^{-2} s^{-1} (n=3) in experiment 2. Dashed lines are 4th order polynomial fit curves, with equations and R^2 next to corresponding line in legend. Dark gray areas are periods when lights in mesocosms are off, light gray areas are ramp periods, and white area is when lights in mesocosms are fully on.

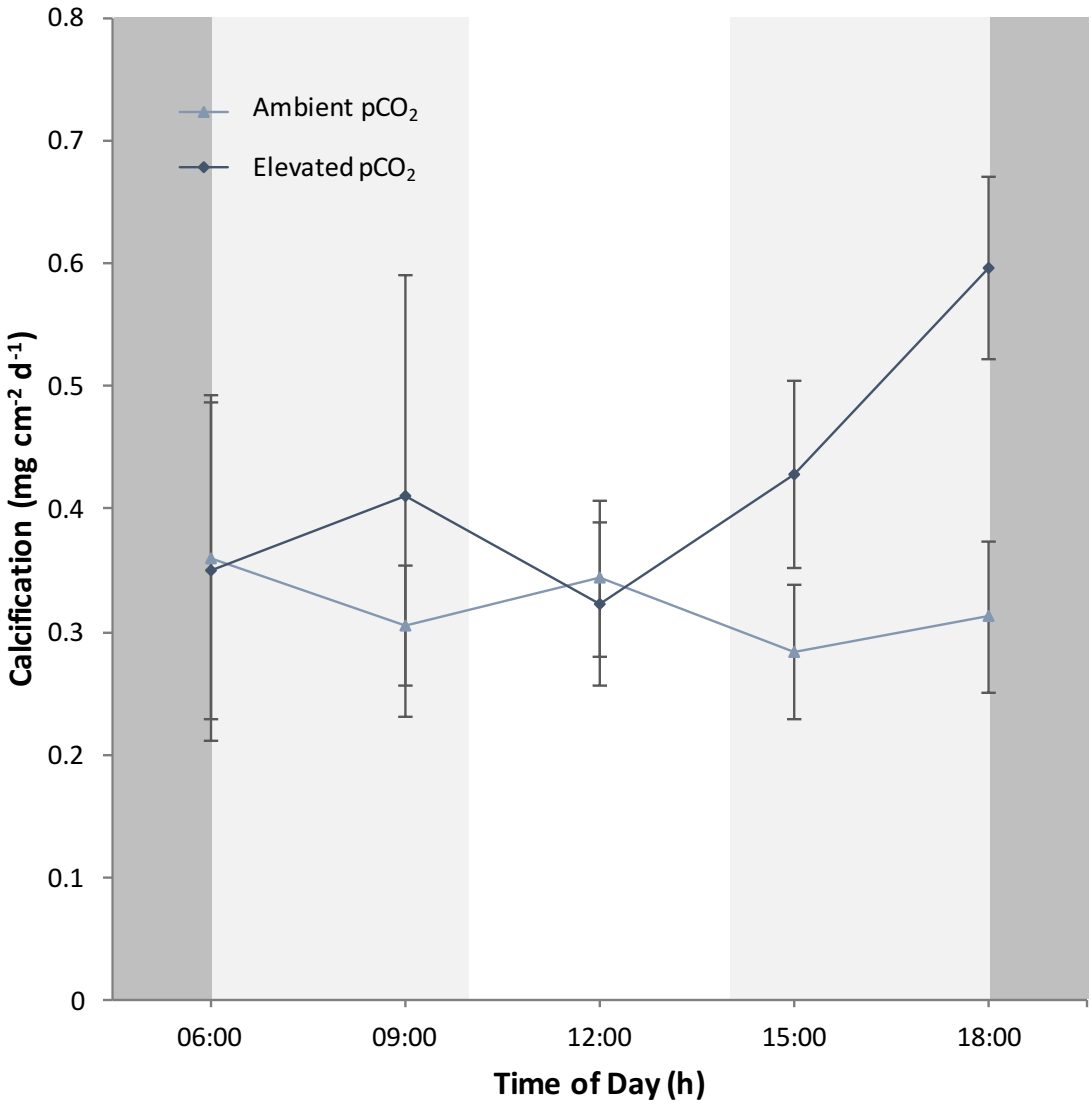

Figure 5. Mean calcification rates ± SE at ~600 µmol quanta m^{-2} s^{-1} (n=3) in experiment 2. Dark gray areas are periods when lights in mesocosms are off, light gray areas are ramp periods, and white area is when lights in mesocosms are fully on.

Chapter 4

Effects of press vs. pulse nutrient addition on tropical crustose coralline algae

Introduction

Coastal marine ecosystems, such as seagrass beds, estuaries, and coral reefs, are among the most valuable ecosystems on the planet, and of these, coral reefs provide the greatest diversity of ecosystem services of any biome on earth (Costanza et al. 2014). They are highly productive and provide goods and services such as coastal protection, fish habitat, and recreational destinations (Moberg 1999). However, despite their ecological and economic importance, increasingly they are threatened by many human-induced impacts. These include global stressors such as rising sea water temperatures and ocean acidification as well as local stressors such as sedimentation and nutrient runoff (Fabricius 2005, Hoegh-Guldberg et al. 2007, Kroeker et al. 2010). Of these stressors, nutrient enrichment is among the potentially most damaging anthropogenic impacts on the ocean (GESAMP 2001).

Coastal development is increasing globally and is leading to the input of nutrients from agricultural runoff, human sewage, urban waste, and industrial effluent to coral reefs worldwide (Smith and Schindler 2009, Jessen et al. 2013). These nutrient additions can come from many different sources and therefore last different lengths of time. In coastal Brazil, nearshore urban locations experience continuously higher levels of nutrients than offshore locations due to sewage, wastewater, and runoff inputs and minimal coastal flushing (Costa et al. 2008). On the Great Barrier Reef, because nutrients largely are added from riverine input, flood plumes (and therefore nutrient inputs) vary

seasonally (Devlin et al. 2001). In Discovery Bay in Jamaica, groundwater outflow contributes greatly to the nearshore nutrient dynamics (Greenaway and Gordon-Smith 2006). There, rain events directly impact the aquifer before the water makes its way into the bay, which means that changes in nutrient concentrations in the coastal waters may be gradual and last longer than the rain events themselves (Greenaway and Gordon-Smith 2006). Around the island of Curaçao, nutrients enter the water through episodic runoff plumes after rain events that last for a few hours and occur with varying frequency depending on the season (den Haan et al. 2016). These examples show just a few of the ways that nutrient inputs vary between different coastal locations. How nutrients are added and the length of time that concentrations are elevated can have greatly differing effects on the coral reef communities that are impacted.

Because of the large role that terrestrial water inputs have on coastal nutrient dynamics, changing rainfall patterns potentially could impact nutrient input on many coral reefs. Equatorial and wet tropical regions are predicted to experience increases in annual mean precipitation and extreme precipitation events under future climate prediction models (IPCC 2014). This increase in precipitation would lead to more runoff and therefore more nutrients entering tropical coastal waters in the future. Additionally, land-use changes can impact the nutrients that enter waters around coral reefs. Deforestation and agriculture both result in nutrients being added to coastal waters due to increased surface runoff and/or the contamination of groundwater (GESAMP 2001). There also is an increasing trend of people moving toward coasts, which leads to nutrients from urban and industrial waste directly entering coastal waters (GESAMP 2001). Taken together these changes likely will increase nutrient enrichment of coral reef

waters into the future, so understanding the effects of this anthropogenic stressor are vital.

One of the most important groups of organisms on coral reefs are crustose coralline algae (CCA). CCA are calcifying algae that reinforce reef structure, provide habitat for other organisms, and contribute to the global carbon cycle (Nelson 2009). However, the impacts of coastal runoff and nutrient addition on CCA physiology and ecology are not fully understood. The processes of photosynthesis, calcification, and nutrient metabolism are connected in CCA (Turpin 1991, Lobban and Harrison 1994, Comeau et al. 2013a). Photosynthesis facilitates calcification by increasing the pH in the cell wall, and photosynthesis uses CO_2 that is produced from carbonic anhydrase after the removal of H^+ from the cell wall to facilitate calcification (Comeau et al. 2013a). Nitrogen is used in the synthesis of amino acids, protein complexes, and phycobilin and chlorophyll pigment molecules (Ryder et al. 1999), and it is integral to photosynthesis and the reduction of nitrate to ammonium through N-containing enzymes, such as RUBISCO and nitrate reductase (Turpin 1991). Nitrogen also can be stored in pigments, proteins, and free amino acids for later use, and this storage could result in delayed physiological responses to nutrient addition (Naldi and Wheeler 1999). Phosphate is used to synthesize ATP (adenosine triphosphate), NADPH (nicotinamide adenine dinucleotide phosphate hydrogen), and RuBP (ribulose 1,5-bisphosphate), all of which are vital to photosynthesis (Turpin 1991), and calcification may serve as a proton source for nutrient uptake (McConnaughey and Whelan 1997). ATP and NADPH also can be used for nutrient uptake, nitrate reduction, and calcification (Turpin 1991, Lobban and Harrison

1994). This interconnectedness among processes means that nutrient addition potentially can impact CCA metabolism in complex ways.

Little previous work has been done on the effects of nutrient addition on CCA physiology. One study found no effect of nutrient addition on calcification in CCA (Björk et al. 1995) and another found no effect on photosynthesis (Russell et al. 2009). However, previous work has shown that nutrient enrichment can lead to increased photosynthetic rates and pigment concentrations in non-calcifying red algae (Chopin et al. 1995, Zheng and Gao 2009). Additionally, the nutrient addition regimes were very different between these experiments. Because of the complex metabolic links between photosynthesis and calcification, it is likely that nutrient addition impacts multiple parts of CCA physiology. Therefore, in order to fully understand the effects of enrichment, changes in photosynthesis should be assessed in response to nutrient addition. Additionally, changes in calcification rate should be assessed due to the interconnectedness of photosynthesis and calcification in CCA metabolism, and photosynthetic pigment content should be measured due to the effects of nutrients on pigment content and the effects of pigments on photosynthetic rate .

The goal of the present study was to understand the effects of different nutrient input scenarios on the photosynthetic and calcification metabolism of *Lithophyllum kotschyanum*, a common species of CCA found on the fringing reefs of Mo'orea, French Polynesia. Fringing reefs experience direct runoff from the island during rain events and therefore often are subject to nutrient enrichment events. Also, these reefs off Mo'orea likely have experienced increased runoff due to land use changes, such as converting upland forest to agricultural land. Three nutrient scenarios were created in the present

study: a long-term press enrichment, a series of short-term nutrient pulses over time, and an ambient treatment with no nutrient addition. Photosynthetic rates were measured to determine the effect of nutrient enrichment on *L. kotschyanum*. Then, calcification over the whole experiment (long-term calcification), calcification over hourly time scales (short-term calcification), and photosynthetic pigment concentrations were measured to help explain the photosynthetic response due to the connectedness of calcification and photosynthesis metabolically and the importance of pigments in photosynthesis. I predicted that press nutrient enrichment would lead to increased photosynthetic rates due to elevated pigment concentrations, and that this also would increase calcification rates. The nutrient pulse treatment was expected to result in a smaller increase in photosynthesis, pigment concentrations, and long-term calcification than the press treatment. However, in the pulse treatment, short-term calcification was not expected to increase on longer time scales than a single pulse length, so I did not predict short-term calcification rates in the pulse treatment to be higher than in the ambient treatment at the end of the experiment. The results of the present study help explain the response of CCA to different nutrient enrichment scenarios and suggest further study to better understand the complexities of nutrient cycling on coral reefs, especially with increased anthropogenic input.

Methods

Sample collection

Forty-five individuals of *Lithophyllum kotschyanum* (Rhodophyta) were collected using hammer and chisel from the fringing reef on the north shore of Mo'orea, French Polynesia at ~1-2-m depth (Fig. 1a). The samples were transported to the Richard B. Gump Research Station where they were cleaned of epibionts, acclimated in a flowing water table for 7 days, and glued to a plastic base using Coral Glue (EcoTech Marine).

Experimental setup

Samples were placed in individual clear polypropylene containers (height: 7.6 cm, diameter: 8.4-11.7 cm base-top, ChoiceHD deli container) to slightly reduce water flow and retain nutrients around the samples (Fig. 1c). Turnover time in the containers varied from ~1.2 L m^{-1} to ~2.4 L m^{-1}. The containers then were attached to cement blocks with cable ties to anchor them on the sea floor and were distributed at least 1 m apart on the fringing reef on the north shore at 1-2-m depth in the same area where the samples were collected (Fig. 1a,b). Light levels (measured with LI-COR LI-1400 data logger, 2Π underwater sensor model LI-192 UWQ 7060) varied between 250-600 μmol quanta m^{-2} s^{-1} on mostly cloudy days and 800-1500 μmol quanta m^{-2} s^{-1} on mostly sunny days at the site.

Samples were divided into three nutrient treatment groups: press, pulse, and ambient. Osmocote® (ScottsMiracle-Gro, USA) plant food (N:P:K 15:9:12) was used to provide nutrient enrichment to the press and pulse treatments. Twenty g of Osmocote® was placed into a nylon sac (L'eggs® pantyhose), and the sac was placed in the clear

container surrounding, but not touching, the algal sample (Fig. 1c). For the press treatment, sacs were replaced halfway through the 6-week experiment. For the pulse treatment, sacs were deployed for 3 days every 1.5 weeks, with the last pulse ending 6 days before the end of the experiment. For the ambient treatment, no sacs were added to the containers. Ambient levels of nitrate + nitrite and phosphate in the lagoon waters of Mo'orea are 0.55 μM and 0.17 μM respectively (Alldredge 2013). The goal of the enrichment in the present experiment was to manipulate the frequency and duration of nutrient addition around the algal samples. Therefore, the enrichments were not intended to achieve a specific concentration of nutrients, but to achieve the same moderately elevated conditions in the press and pulse treatments so that the effects of timing of nutrient addition could be compared. Target elevation of nitrate concentration was to within levels seen in other runoff plumes in tropical waters (Devlin and Brodie 2005, den Haan et al. 2016). All samples and containers were cleaned of algae and sediment every ~4 days throughout the experiment to ensure that the samples were not shaded.

Water samples were taken throughout the experiment and tested for nitrate concentration to determine levels of nutrient enrichment around the algal samples. Water samples from press and pulse treatments were analyzed using an enzyme-based test kit (NECi Superior Enzymes; limits of detection: 3.6 – 71.4 μM Nitrate). Ambient samples and selected replicates were sent to the MSI Analytical Lab (UCSB) for nitrate determination by flow injection analysis with a QuikChem 8500 flow injection analyzer (Lachat Instruments Div., Zellweger Analytics, Inc., Loveland, CO USA, limits of detection: 0.2-300 μM nitrate + nitrite) to confirm nutrient levels.

Response variables

Photosynthesis (measured via oxygen evolution), short-term calcification (calcification measured over hourly time scales), long-term calcification (calcification measured over daily time scales), and photosynthetic pigment concentrations (chlorophyll *a* and phycobilin accessory pigments) of the samples were measured in the lab at the end of the 6-week experiment.

Photosynthesis was measured by incubating samples for 1 h in an acrylic incubation chamber (250-mL volume). The chamber was surrounded by an outer water jacket that was supplied with constant water flow from a circulating bath to maintain a temperature of 27 °C in the chambers during the incubations to mimic austral winter temperatures in the lagoon of Mo'orea where the present experiment was conducted (Edmunds et al. 2010). Within the chambers, a stir bar provided constant water mixing, and a piece of mesh was placed over the stir bar to keep the algal pieces above it. A PreSens (Precision Sensing GmbH, Germany) oxygen optode (calibration: 0% O_2 – sodium dithionite, 100% O_2 – water-saturated air) and temperature probe made measurements in the chambers every second throughout the incubation. Two fiber optic halogen lights (Ace 1, Schott North America, Inc.) were used to supply saturating light (~600 μmol quanta m^{-2} s^{-1}, measured with 2Π sensor, Walz Diving PAM, Germany). PI curves were calculated using a Walz Diving PAM (Germany) to determine saturating light at midday (~200 μmol quanta m^{-2} s^{-1}). Samples were incubated for one hour to allow for a 5-10% change in oxygen concentration, which allowed for accurate determination of oxygen evolution rate by the sample in the chamber over the course of the incubation. Control incubations without samples in the chamber were run, and control values were

subtracted from algal photosynthetic rates. Photosynthetic rate was calculated from the linear slope over time of the dissolved oxygen concentrations in the chambers. The photosynthetic rate was standardized to the surface area of each sample.

Short-term calcification (calcification rate measured over hourly time scales at the end of the experiment) was measured using the total alkalinity anomaly method (Smith and Kinsey 1978). Total alkalinity of the water was measured at the beginning and end of the hour-long photosynthesis incubation for each sample. The change in alkalinity was used to calculate calcification under the model that alkalinity is lowered two units for every one mole of carbonate precipitated (Smith and Kinsey 1978). Calcification in the control incubations was also measured and was subtracted from sample calcification values. Long-term calcification (calcification rate measured over the whole experimental duration) was calculated by buoyant weighing (Mettler Toledo PB303-S balance, resolution: 0.001 g) the samples at the beginning and end of the experiment, using the method of Spencer-Davies (1989).

The algal samples were frozen after incubations to preserve the photosynthetic pigments (Holm-Hansen and Riemann 1978) and transported to California State University, Northridge for pigment processing. Two subsamples (~1 cm^2 each) were taken from each individual, and one was used for chlorphyll *a* extraction and the other was used for phycobilin pigment extraction. Chlorophyll *a* was extracted in 90% acetone, and the phycobilin pigments were extracted in phosphate buffer, both using mortar and pestle (similar to Parsons et al. 1984, Carpentier 2004, and Sudhakar et al. 2014, using the equations of Bennett and Bogorad 1973 [phycobilins] and Jeffrey and Humphrey 1975 [chl *a*]). The solutions of extracted pigments were placed in the refrigerator (4 °C)

for two days and then warmed to room temperature and centrifuged at ~2500 RPM for 5 minutes. The absorbance of the samples was measured in a Beckman Coulter DU-730 spectrophotometer. Absorbances of the acetone slurry samples were measured at 750 nm and 664 nm, and absorbances of the phosphate buffer slurry samples were measured at 750 nm, 652 nm, 615 nm, and 562 nm. These absorbances then were used to calculate the chlorophyll *a*, phycocyanin, allophycocyanin, and phycoerythrin concentrations. The pigment concentrations were standardized to the surface area of the sample piece used for pigment extraction.

Surface area was estimated for each algal sample via the dye-dipping method (Hoegh-Guldberg 1988). Samples were dipped in a solution (400 mL) of methylene blue dye (~0.4 g), detergent (~0.10% Triton X-100), and DI water and then rinsed in a known volume of seawater. The absorbance of seawater solution then was measured on a Shimadzu UV-2450 spectrophotometer at 620 nm, and the absorbance was compared to a standard curve created by dipping pieces of foil with known surface areas into the dye and seawater. The dye then was removed from the samples by scrubbing in seawater before freezing for pigment analysis.

Statistical Analysis

Each response variable was analyzed in R (R 3.2.2 GUI) using a 1-way ANOVA with nutrient treatment as the fixed factor, and Tukey post-hoc tests were run when ANOVA detected significant differences among treatments. All response variables met requirements for normality and homogeneity of variance. Four samples were lost or damaged and were excluded from the analysis. One additional sample was excluded from

the long-term calcification analysis due to loss of branches. This resulted in sample sizes of 15 for ambient, 13 for pulse, and 13 for press (12 for long-term calcification for the press treatment).

Results

Elevated nitrate concentrations in the water surrounding the samples ranged from 1.1 to 16.6 μM, representing a 2-30-fold increase over the ambient seawater. These concentrations varied among algal samples and among sampling periods, and there were not any trends of higher or lower nutrient availability surrounding certain samples over the course of the experiment.

Photosynthetic rates were higher in the press treatment than the ambient and pulse treatments (ANOVA $F_{2,38}$=4.20, p = 0.022; Fig. 2). Photosynthesis increased from (mean ± SE) 0.097 ± 0.010 μO_2 cm^{-2} h^{-1} in the ambient treatment and 0.086 ± 0.010 μO_2 cm^{-2} h^{-1} in the pulse treatment to 0.131 ± 0.014 μO_2 cm^{-2} h^{-1} in the press treatment. Although there was only a significant post-hoc difference between the press and pulse treatments, there was also a clear increase in photosynthetic rate from the ambient treatment to the press treatment. Of the four photosynthetic pigment concentrations that were measured (chlorophyll *a* (chl *a*), phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE)), only PE concentrations differed significantly among treatments (ANOVA $F_{2,38}$=3.69, p = 0.034). Although there were not post-hoc differences among treatments, concentrations were higher in the press treatment (mean ± SE: 0.0010 ± 8.01 x 10^{-5} mg cm^{-2}) than the other two treatments (ambient: 0.0007 ± 9.41 x 10^{-5} mg cm^{-2}, pulse: 0.0007 ± 6.76 x 10^{-5} mg cm^{-2}) (Fig. 3a). Chl *a* followed the same trend as PE with higher concentrations (0.265 ± 0.025 μg cm^{-2}) in the press treatment than the ambient (0.225 ± 0.023 μg cm^{-2}) and pulse (0.226 ± 0.023 μg cm^{-2}) treatments, but was not significantly different among treatments (ANOVA $F_{2,38}$=1.76, p=0.186; Fig. 3b). PC and APC also were not significantly different among

treatments (ANOVA PC: $F_{2,38}$=1.81, p=0.178; APC: $F_{2,38}$=0.24, p=0.791). The mean (±SE) short-term calcification rate was 0.114 ± 0.019 mg cm^{-2} d^{-1}, and there were not differences in rate among treatments (ANOVA $F_{2,38}$=1.43, p=0.252; Fig. 4a). The mean long-term calcification rate (±SE) was 0.114 ± 0.019 mg cm^{-2} d^{-1}, and it also did not differ among treatments (ANOVA $F_{2,37}$=0.70, p=0.502; Fig. 4b).

Discussion

Nutrient runoff is an increasingly common anthropogenic input in coastal ecosystems (GESAMP 2001). It results in increased biomass of phytoplankton and epiphytic algae, changes in macroalgal production, biomass, and species composition, and death and losses of corals (Smith et al. 1999). It has led to macroalgal blooms in some areas (McGlathery 2001, Li et al. 2016), and it is one of the contributing factors in the shift of coral reefs to algal dominated reefs and in the outbreak of crown-of-thorns seastars (Graham et al. 2015, Babcock et al. 2016). In order to better understand the impacts that nutrient addition can have on coral reef communities, the present study looked at the effects of nutrients on an important coral reef functional group, crustose coralline algae (CCA), and the results demonstrate the complex nature of their physiological response to nutrient addition.

The response of *L. kotschyanum* to 6 weeks of nutrient enrichment was increased photosynthetic rates and phycoerythrin (PE) concentrations. Although Russell et al. (2009) did not find an effect of nutrients on photosynthetic yield in temperate CCA, nutrient enrichment has been shown to increase photosynthesis in many other species of algae (Dawes and Koch 1990, Chopin et al. 1995, Martins et al. 2011, Hofmann et al. 2015), so these results are in agreement with some previous studies. Additionally, it is well-known that red algae store nitrogen in pigment proteins, especially PE (Naldi and Wheeler 1999), so increases in these pigments have been shown with nutrient enrichment previously (Martins et al. 2011). Although this increase in pigments may not increase photosynthesis directly if the algae are light-saturated, it is an indication that the algae are nutrient replete. The increase in photosynthesis may be due to the storage of nitrogen as

free amino acids and proteins (Naldi and Wheeler 1999), including RUBISCO which is required for photosynthesis (Turpin 1991). Additionally, despite the fact that tropical coastal waters are typically P limited (Smith 1984), the amount of phosphate provided by the plant food may have been enough to shift the algae to N limitation, and N enrichment in marine environments has been shown to produce a greater growth response than P enrichment (Elser et al. 2007). Alternatively, the simultaneous addition of N and P has been shown to produce greater responses in some marine environments than N or P alone (Elser et al. 2007, Allgeier et al. 2011), so the response observed here could be due to the addition of both N and P via the fertilizer.

In many calcifying algae, photosynthesis and calcification are linked metabolically. Photosynthesis increases the pH in the cell wall, which facilitates calcification (Comeau et al. 2013a). Despite this connection, there was not a statistically significant increase in either short- or long-term calcification in either nutrient addition treatment. It has been suggested by Goreau (1963) that photosynthesis and calcification responses are not as tightly correlated in calcifying algae as it may seem due to their integrated metabolisms. Calcification instead may be more correlated with tissue growth (Goreau 1963). It has also been shown in coccolithophores that calcification is not necessary to maintain high growth or photosynthesis, and therefore these processes do not appear to be tightly linked (Bach et al. 2013). A similar decoupling of these physiological processes could be happening in the CCA in the present study.

Also, the algae may have allocated energy to nitrate uptake and breakdown instead of calcification (Turpin 1991). Osmocote® contains nitrogen as both nitrate and ammonium, which are taken up differently in algae. Nitrate needs to be reduced to

ammonium within the cell in order for it to be incorporated into amino acids and other cellular molecules (Larsson et al. 1985, Lobban and Harrison 1997), so ammonium is taken up more readily (D'Elia and DeBoer 1978, Thomas and Harrison 1985). Nitrate reductase activity can take several days to increase when nitrate concentrations are increased (Thomas and Harrison 1985). In the meantime, nitrate slowly fills nitrate pools and results in decreased nitrate uptake rates (Thomas and Harrison 1985, Pedersen et al. 2004). Additionally, if ammonium is present, nitrate uptake can be inhibited by ammonium uptake due to the differences in energy requirements (Thomas and Harrison 1985). Uptake rates also depend on the concentration of nutrients in the surrounding water and the nutrient history (4-10 days prior in Thomas and Harrison 1985) before addition (Thomas and Harrison 1985, Pedersen et al. 2004). In the present experiment, ammonium could have delayed initial uptake of nitrate and continually been incorporated into amino acids (Thomas and Harrison 1985, Turpin 1991). Then, when nitrate finally was taken up and there was a surplus of nitrogen, it was stored as pigments and enzymes, and this increased photosynthetic rates. However, there may have been a decrease in uptake rates over the course of the experiment due to increasing internal nitrogen concentrations and a changing nutrient history over time.

Finally, in CCA, there may be an even further lag after photosynthesis increases before there is a significant increase in calcification rate, and it is possible that six weeks is not enough time for all of these processes to occur in crustose coralline algae due to their slow carbon fixation rates (Goreau 1963, Comeau et al. 2016b). Therefore, the lack of calcification responses in the present experiment might be due to the length of the experiment. This line of speculation would imply that in order for nutrient enrichment to

impact calcification in CCA on coral reefs, if it does at all, there needs to be press nutrient enrichment for long periods of time (greater than six weeks).

There was no effect of the nutrient pulse treatment on any of the response variables. This finding suggests that the specific nutrient pulse regime that was applied in this experiment did not supply enough nutrients to affect the long-term physiological response of the algae or force long-term storage of nutrients in pigments. Nutrient pulses are highly variable in their frequency and length. Over the time period that the present study was conducted (May-July; dry season), runoff-producing rain events lasted no more than a few hours at a time and occurred irregularly a few times per month. During the rainy season (January), rain events can last for longer periods of time and occur as often as every day for a week. This variability in runoff events produces equally variable nutrient pulses on fringing reefs (den Haan et al. 2016). Because of this variability, it may be difficult to determine the frequency and size of nutrient inputs that significantly affect photosynthesis in *L. kotschyanum* in the same way that the press treatment did. It is possible that three days was long enough to increase the photosynthetic rate and pigment contents, but then one week was enough time for the rates and contents to return to lower levels. Dawes and Koch (1990) found that photosynthetic pigments in two species of red algae increased during a nutrient enrichment period and then decreased over time following the nutrient addition. Den Haan et al. (2016) showed that some species of coral reef algae on the reefs around the island of Curaçao have very high nutrient uptake rates when subjected to a nutrient pulse. These high uptake rates suggest that the algae are able to take advantage of short bursts of nutrients in an otherwise nutrient-poor environment. The algae in the present study may have used the nutrients immediately after they were

added and then returned to baseline metabolic levels when nutrients returned to ambient concentrations. Because photosynthesis and pigment concentrations were only measured at the end of the present experiment in order to estimate the cumulative long-term effects of short-term pulses, the short-term effects of the pulses remain unknown.

Fluctuations in metabolic rates may also explain why there was no effect of increased calcification in the pulse treatment. Short-term calcification rates had returned to baseline levels 6 days after the last pulse ended when this response was measured in the samples, and the pulses were not long enough to result in storage of extra nutrients that could have eventually lead to increased long-term calcification or result in a higher average long-term rate. Because the press treatment did not result in increased calcification, it is not surprising that the pulse treatment did not either.

Overall, the results of the present study show that nutrients can have a stimulatory effect on photosynthesis in *L. kotschyanum*. However, the enrichment needs to last longer than 3 days, and it may not increase calcification rates over the time scales of weeks. This finding also emphasizes the importance of understanding how nutrients are added to a coral reef system. Not all nutrient addition regimes have the same effects, and simulating long-term (a week or longer) enrichment in experiments may not be reflective of the actual nutrient scenario on many coral reefs (i.e., den Haan et al. 2016). In the future, increases in nutrient addition due to an increase in rain events, or elevated nutrient levels due to increased coastal development, could both impact photosynthetic rates in tropical calcifying algae. Further studies are needed to determine whether projected increases in coastal runoff events due to climate change are frequent or large enough to impact these algae. Additionally, in locations where long-term enrichment does occur, studies should

be done to determine what the long-term effects are on calcification. It is important that we more fully understand the effects of anthropogenic nutrient addition on coral reefs, which are increasingly likely to be impacted by it.

(a)

(b)

(c)

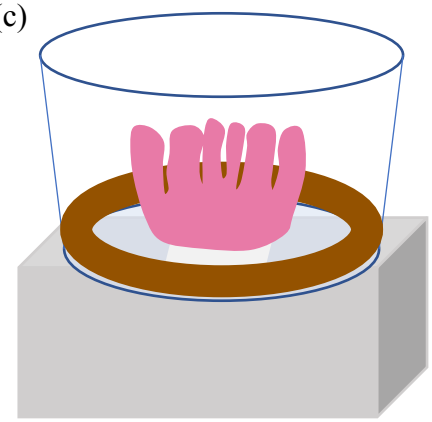

Figure 1. Location of field site on the north shore of Mo'orea, French Polynesia (a), photo of experiment in the field at 1-2m depth (b), and diagram of sample setup with nutrient sac surrounding algal sample in clear plastic cup (c; not to scale, actual cup dimensions – height: 7.6 cm, diameter: 8.4-11.7 cm base-top).

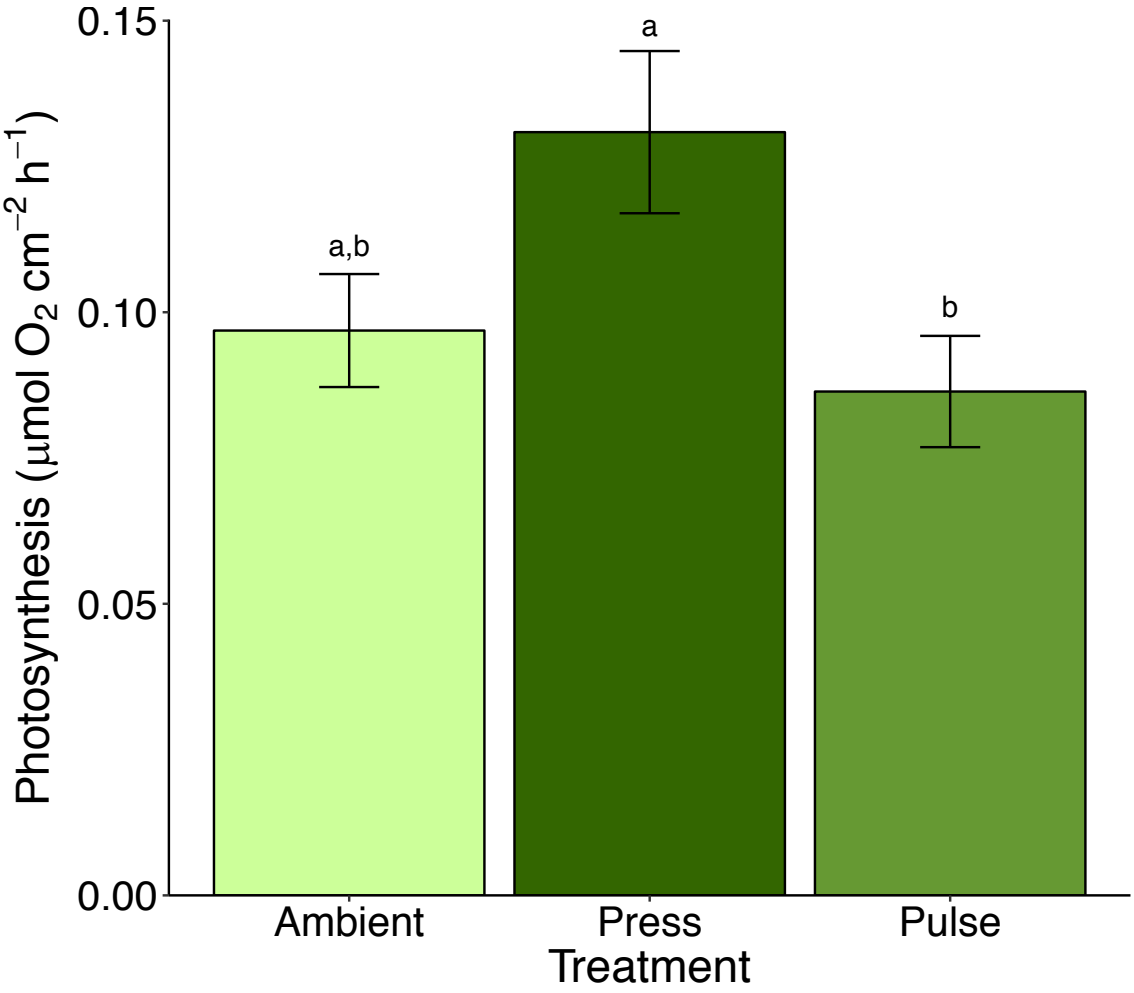

Figure 2. Rates of photosynthesis (means ± SE, n=15 in ambient and n=13 in press and pulse) normalized to surface area for each of the nutrient treatments. Letters represent Tukey post-hoc tests, and different letters show significantly different treatments.

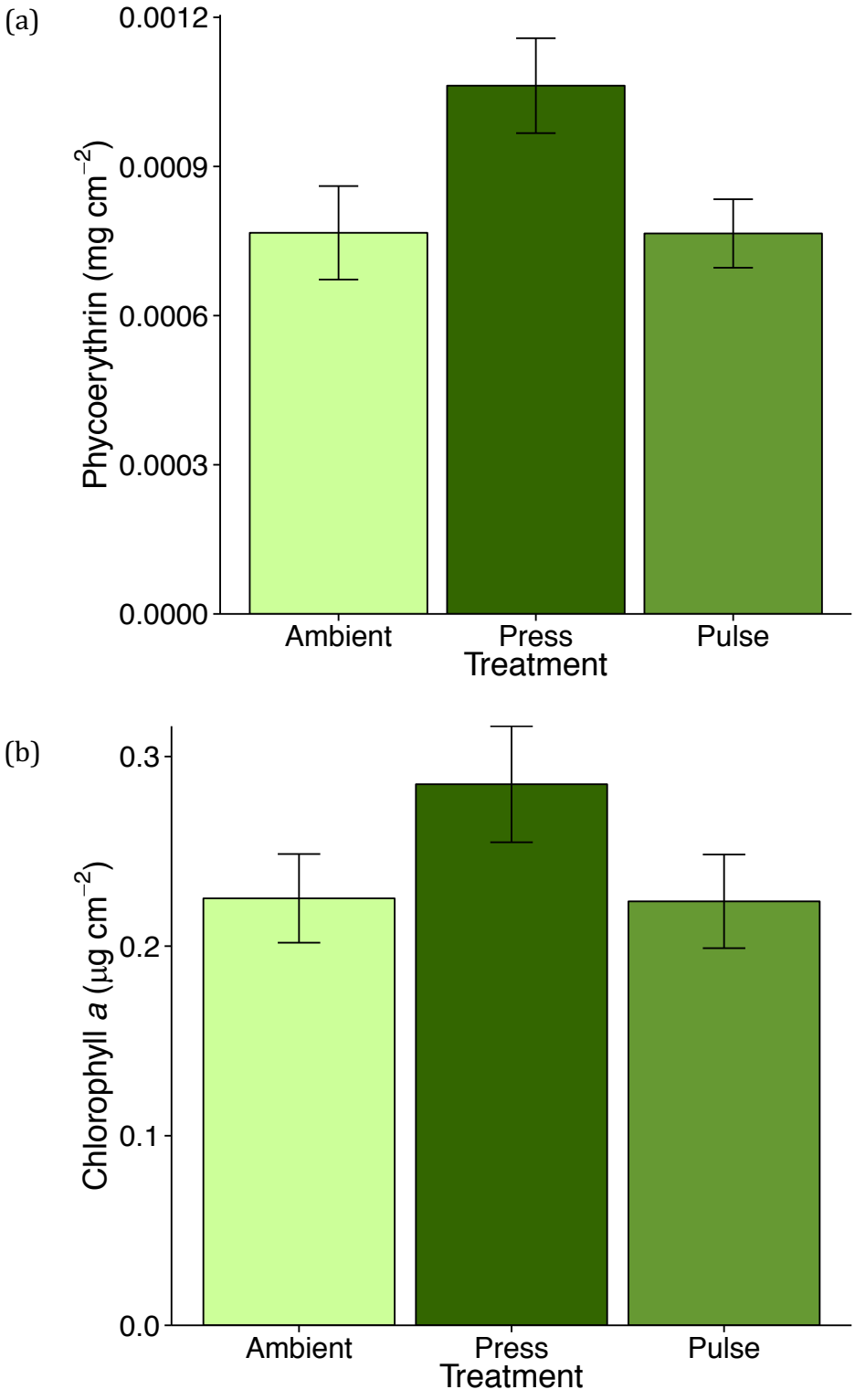

Figure 3. Phycoerythrin (a) and chlorophyll *a* (b) concentrations (means ± SE, n=15 in ambient and n=13 in press and pulse) normalized to surface area for each of the nutrient treatments. There were no post-hoc differences between treatments for phycoerythrin, and there were no significant differences between treatments for chlorophyll *a*.

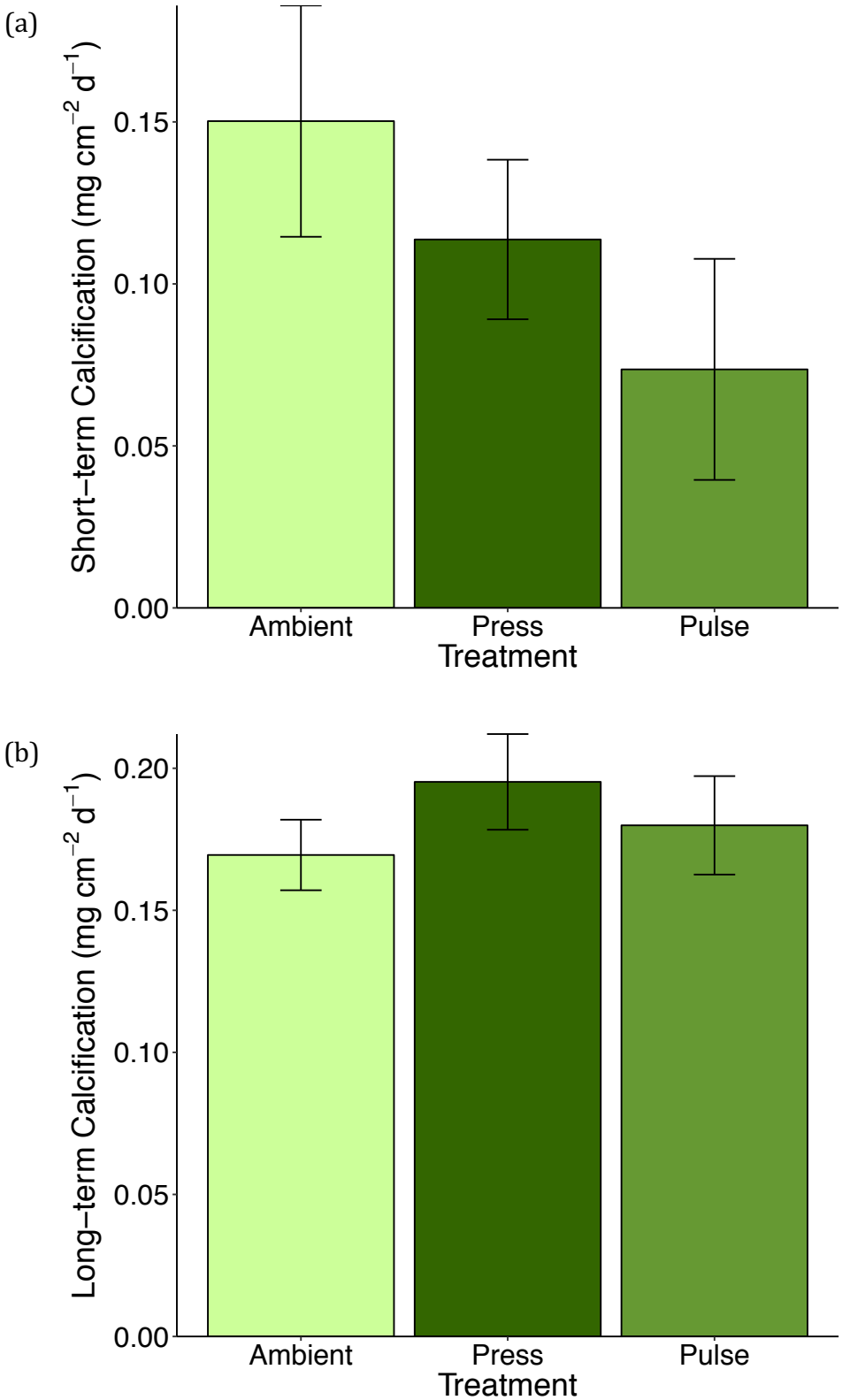

Figure 4. Rates of short- (a, n=15 in ambient, n=13 in press and pulse) and long-term (b, n=15 in ambient, n=12 in press, and n=13 in pulse) calcification (means ± SE) normalized to surface area for each of the nutrient treatments. There were no significant differences between treatments.

Chapter 5

Conclusions

Humans continue to impact coastal marine ecosystems, such as coral reefs, in many ways, and the impacts are increasing as coastal development increases (GESAMP 2001, IPCC 2014). One of these impacts, ocean acidification (OA), is a global problem that is projected to increase past the end of the century and has negative effects on the calcifying organisms that make up coral reefs (Hoegh-Guldberg et al. 2007, Kroeker et al. 2010). Another impact, nutrient addition from coastal runoff, occurs on short timescales that often correspond to rain events or rainy seasons, and these pulses of nutrients can have positive effects on coral reef algal growth (Smith et al. 1999, den Haan et al. 2016). One of the most important groups of algae on coral reefs are crustose coralline algae (CCA), which are calcifying algae that serve as reef builders and habitat for other organisms (Nelson 2009). These algae can be impacted by both OA and nutrient addition, especially as the likelihood of these disturbances occurring at the same time increases. This thesis sought to better understand the physiological responses of *Lithopyllum kotschyanum*, a common species of CCA found on the fringing reefs of Mo'orea, French Polynesia, to these two common coral reef disturbances, both individually and synergistically.

When combined, OA and nutrient addition have interactive effects on photosynthetic rates of *L. kotschyanum*. The results of Chapter 2 showed that when nitrogen and phosphorus were added, any differences in photosynthetic rates due to differences in pCO_2 disappeared. Additionally, 10 days after nutrient addition ended, the

patterns in photosynthetic response changed, suggesting an interaction between metabolic processes as the algae returned to an environment of lower nutrients. Interactive effects of OA and nutrients also were observed in the calcification response of *L. kotschyanum*. Calcification rates were lower under elevated pCO_2, but only when nutrients were added. Additionally, the response was not consistent among treatments. Although the lack of calcification response in some of the treatments may be due to the experiment not lasting long enough to detect a response, the remaining responses suggest some interactive effects of OA and nutrients after a nutrient pulse ends. The experiments in Chapter 2 where there were interactive effects showed the potential for nutrient pulses to have lag effects that are different from the impacts that nutrients can have while addition is occurring. These lag effects emphasize the need to differentiate between short- and long-term nutrient addition, as was investigated in Chapter 4. Additionally, the Chapter 2 experiments showed that OA and nutrients can interact in unanticipated ways on the metabolic response of CCA.

Nutrient addition alone can have different effects depending on the length and frequency of the addition. Chapters 2 and 4 showed that nutrient addition can lead to increases in photosynthesis, but only under certain enrichment regimes. In Chapter 2, photosynthesis only increased when both nitrogen (N) and phosphorus (P) were added. Then, after addition of N and P stopped, photosynthetic rates dropped by 27% under ambient pCO_2 and 41% under elevated pCO_2. These results are consistent with the results of Chapter 4, which showed that 6 weeks of nutrient addition, including both N and P, lead to increased photosynthesis. However, the addition of four short pulses of nutrients over 6 weeks did not result in an increase in photosynthesis. These results together

suggest that photosynthetic rates can respond rapidly to both the increase and decrease of nutrients in the water. They also show that the specific nutrient scenario that a reef experiences will determine how it is affected, so it is important to take into account the runoff and nutrient input patterns that influence a reef when assessing the impacts of coastal development.

The first experiment in Chapter 3 attempted to quantify the hourly response of *L. kotschyanum* to a short-term nutrient pulse under two different pCO_2 treatments. This study lead to the discovery of a diurnal pattern of light-saturated photosynthesis, which was quantified in the second experiment of that chapter. This strong pattern of increased maximum photosynthesis in the middle of the day under a constant light treatment is similar to circadian patterns seen in some other algae (Samuelsson et al. 1983, Roenneberg and Mittag 1996, Sorek et al. 2013). This diurnal pattern of light-saturated photosynthesis has important implications for the effects of short nutrient pulses on these algae. The effect of a nutrient pulse on CCA photosynthesis could change depending on what time of day it occurs and how long it lasts throughout the day or night. Additionally, this photosynthetic pattern needs to be taken into account when measuring photosynthetic rates in the lab because the time of the measurement could impact the measured rate.

Finally, the results of Chapters 2, 3, and 4 show that the photosynthetic and calcification responses of CCA to OA and/or nutrients may not be tightly correlated. In all of the studies, an increase in photosynthesis did not necessarily correlate to a change in calcification rate. Specifically, in Chapter 2, the complicated patterns of photosynthetic responses to the varying treatments did not match the calcification responses. In the second experiment of Chapter 3, there was a diurnal pattern in light-saturated

photosynthesis, but not in light-saturated short-term calcification rate. In Chapter 4, six weeks of nutrient addition increased photosynthetic rates, but not long-term calcification rates. This disconnect between two seemingly connected metabolic processes suggests that there is another part of CCA metabolism that is being overlooked. Bach et al. (2013) showed that in coccolithophores calcification is not necessary to maintain high growth or photosynthesis, and therefore these processes do not appear to be tightly linked. Additionally, Goreau (1963) suggested that calcification may be more correlated with tissue growth than photosynthesis in calcifying algae. These unexpected patterns show the need for further study of the intricacies of the metabolism of CCA in order to allow better predictions of how these algae will respond to OA and nutrient addition in the future.

Overall, this work has shown that two of the most common and impactful anthropogenic stressors on coral reefs, ocean acidification and nutrient addition, can have complex effects on one of the most important coral reef organisms, crustose coralline algae. As humans continue to affect coastal oceans in varying ways, these algae will be impacted, too. Changes in photosynthetic rates will modify the overall metabolism of coral reefs, and changes in calcification rates could affect the ability of these algae to cement the reef together and defend themselves against herbivory. Because of the complexity of coral reef ecosystems, there are potential impacts of changes in CCA metabolism that are still unknown. Although stressors like OA will continue to affect coral reefs for centuries to come, work like this can help us anticipate the effects of certain scenarios on reef communities and work to reduce local impacts, such as nutrient

addition. Every effort should be made to better understand these valuable ecosystems so that their resources and ecosystem services can be protected and preserved into the future.

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