Responses of native Hawaiian calcifying macroalgae to naturally occurring ocean acidification conditions

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DEDICATION

I dedicate my graduate thesis to the Pacific Ocean, which faces many changes.

ACKNOWLEDGEMENTS

I express my sincere gratitude to my thesis advisor, Karla McDermid, and my committee members, Steven Colbert and Tracy Wiegner. I would like to extend my thanks to my family and Jack Carson for their continuous support throughout my graduate schooling. With the help of these individuals, I have successfully completed a Masters in Tropical Conservation Biology and Environmental Sciences. I feel I have developed the skills needed in academia and research. I hope to achieve a meaningful position in applied research in climate change. In addition, I thank Jason Adolf, Becky Ostertag, Marta deMaintenon, Tara Holitzki, Erik Johnson, Linn Montgomery, Ron Kittle, Susan Cordell, and field volunteers: Michelle Smith, John Venrick, Devon Aguiar, Rose Hart, and Kailey Pascoe.
ABSTRACT

Rising atmospheric carbon dioxide (CO\textsubscript{2}) levels from anthropogenic emissions have elevated oceanic CO\textsubscript{2} concentrations through air-sea exchange, which lowers pH and decreases calcium carbonate (CaCO\textsubscript{3}) saturation, including aragonite saturation (one of the mineral forms of CaCO\textsubscript{3}). These conditions, named ocean acidification (OA), impair the ability of calcifying marine algae to form CaCO\textsubscript{3} and maintain physiological structures essential for growth, reproduction, and survival. In the Hawaiian Islands, submarine groundwater discharge (SGD) has high partial pressure of CO\textsubscript{2} (pCO\textsubscript{2}), average concentration 3000 ppm. This study used natural variations in pCO\textsubscript{2} caused by SGD to examine effects of lowered aragonite saturation on Native Hawaiian calcifying macroalgae: Halimeda macroloba (Chlorophyta), Padina australis (Phaeophyta), Dichotomaria marginata (Rhodophyta), and Galaxaura rugosa (Rhodophyta). Temperature, salinity, pH, total alkalinity, total CO\textsubscript{2}, pCO\textsubscript{2}, and aragonite saturation states were quantified at four sites on Hawai‘i Island. Four calcifying algal species were transplanted within and between sites with differing pCO\textsubscript{2} and aragonite saturation states. Percent CaCO\textsubscript{3} content and photosynthetic activity were assessed before, during, and after transplantation. After ten days, all algal species showed a greater change in percent CaCO\textsubscript{3} content at the experimental site, the site representative of OA conditions including high pCO\textsubscript{2}, low pH, and low aragonite saturation, than the corresponding control sites. Padina australis experienced the greatest percent change in CaCO\textsubscript{3}, -60\% (± 63\% propagation of error); whereas, G. rugosa experienced the least change, -4\% (± 5\% propagation of error). Photosynthetic activity in H. macroloba and G. rugosa had no significant change during transplant experiments; however, P. australis did
have a significant change in photosynthetic activity at the experimental site, and thalli were dead by day ten. *Halimeda* and *Galaxaura* may be more resistant to OA than *Padina*. Different responses among the algal species may be related to differences in their morphology and anatomy. Results suggest that OA has the potential to shift nearshore macroalgal community structure and reduce biodiversity in the Hawaiian Islands.
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**ABREVIATIONS**

CaCO$_3$: calcium carbonate  
Ca$^{2+}$: calcium ion  
CH$_4$: methane  
CO$_2$: carbon dioxide  
CO$_2$$_{aq}$: aqueous CO$_2$ in seawater  
$[CO_2]_{atm}$: atmospheric carbon dioxide concentration  
CO$_3^{-2}$: carbonate ion  
DIC: dissolved inorganic carbon  
DOC: dissolved organic carbon  
GtCO$_2$ eq yr$^{-1}$: Total annual anthropogenic greenhouse gas gigatonnes of CO$_2$ – equivalent per year  
GHG: Greenhouse gases  
H$_2$CO$_3$: carbonic acid  
HCO$_3^{-}$: bicarbonate  
IPPC: International Panel of Climate Change  
Ma: million years ago  
mol kg$^{-1}$: moles per kilogram  
mmol kg$^{-1}$: millimoles per kilogram  
µmol kg$^{-1}$: micromoles per kilogram  
N$_2$O: nitrous oxide  
NaHCO$_3$: sodium bicarbonate  
OA: ocean acidification; the shift in ocean chemistry that includes reductions in pH and carbonate ion availability  
pCO$_2$: partial pressure of CO$_2$  
ppm: parts per million  
ppt: parts per thousand  
rpm: revolutions per minute  
SEM: scanning electron microscope  
SGD: submarine groundwater discharge  
TA: total alkalinity  
TCO$_2$: total carbon dioxide  
UW: underwater  
Ω: CaCO$_3$ saturation  
$\Omega_{carbonate}$: CO$_3^{-2}$ saturation  
$\Omega_{arg}$: aragonite saturation
INTRODUCTION

The total anthropogenic greenhouse gas (GHG) emissions from 1970 to 2010 were the highest in human history, reaching 49 GtCO₂ – eq yr⁻¹ in 2010 (International Panel of Climate Change (IPCC) 2014). Emissions are primarily attributed to fossil fuel combustion, industrial processes, and land use changes (IPCC 2014). GHG emissions have caused the Earth to exceed the maximum atmospheric carbon dioxide concentrations ([CO₂]ₚ) experienced in the last 740,000 years (Augustin et al., 2004). Between 1750 and 2011, 2,040 ± 310 GtCO₂ were emitted into the atmosphere. Of these emissions, 40% has remained in the atmosphere, the rest taken up by land plants and the ocean. The ocean absorbs 30% of emitted CO₂, which lowers the ocean’s pH and decreases calcium carbonate (CaCO₃) saturation (Doney et al., 2009). There has been an average pH reduction of 0.1 and a decline in carbonate [CO₃⁻²] by about 210 µmol kg⁻¹ (Hoegh-Guldberg et al., 2007), a condition referred to as ocean acidification (OA) (Caldeira & Wickett, 2003; Ragazzola et al., 2012). As CO₂ dissolves into the ocean, the concentration of CO₃⁻² is reduced (Fabry et al., 2008), due to the consumption of CO₃⁻² during the formation of bicarbonate (HCO₃⁻). CO₃⁻² ions are necessary for marine calcifying organisms that produce CaCO₃ for biological structures. The degree of the impact on these organisms depends on the CaCO₃ saturation state (Ω). A decrease in CO₃⁻² concentration causes a decrease in Ω for both forms of CaCO₃: aragonite and calcite. Many marine organisms precipitate aragonite, including corals, pteropods, and some calcifying macroalgae, all of which are impacted by OA. Several investigators have suggested that CaCO₃-forming marine organisms may have difficulty in maintaining calcification in response to decreased Ω (e.g. Leclercq et al., 2000; Langdon et al.,...
2000; Orr et al., 2005). Most calcifying organisms studied have shown reduced calcification in response to increased partial pressure of CO₂ ($p$CO₂), decreased Ω, and lowered pH (Fabry et al., 2008). However, little research has been conducted on responses of calcifying macroalgae to OA conditions.

The effects of seawater carbonate chemistry on calcifying macroalgal species’ responses to OA may depend on CaCO₃ content within the organism; photosynthetic rate; CO₂ uptake during calcification; species’ fecundity, metabolism, and physiology, and ultimately, how species can adapt or shift distributions. Macroalgae can acquire CO₂ for photosynthesis from seawater as CO₂ and/or HCO₃⁻. The form used can alter metabolism and growth rates of calcifying and non-calcifying macroalgae (Harley et al., 2012), and thus, impact their sensitivity to OA. Although the responses of calcifying macroalgae to nutrient enrichment and sedimentation are well documented (Delgado and Lapointe, 1994; Nelson, 2009), the impact of OA on specific species of calcifying macroalgae in different localities and environments is not well-studied (Harley et al., 2012; Wernberg et al., 2012). Laboratory and fieldwork have shown decreased calcification of branched calcified macroalgae and crustose coralline algae in a variety of locations, including the Mediterranean Sea, Southern Great Barrier Reef in Australia, Florida, and Hawai’i (Jokiel et al., 2007; Martin & Gattuso 2009; Sinutok et al., 2011; Campbell et al., 2016; Niedermeyer, unpubl. data). Other studies have reported persistent calcification in calcifying macroalgal species despite OA conditions (Johnson et al., 2012; Vogel et al., 2015). Gaps in knowledge about physiological thresholds for OA stress in calcifying macroalgae still exist, particularly in the Hawaiian Islands.

In the Hawaiian Islands, the rich seaweed flora (500+ species), 58 of which are calcareous macroalgae, and the diversity of marine habitats, including areas of naturally elevated $p$CO₂, are a model
system to study responses of macroalgae to OA. Submarine groundwater discharge (SGD) and volcanic
CO₂ seeps in marine environments can increase $pCO_2$ two to five or more times that of the atmosphere
(Allan & Castillo, 2007), alter CO$_3^{2-}$ saturation, decrease $\Omega_{\text{arg}}$, and create habitats that can be used to
conduct in situ studies on the effects of naturally occurring OA conditions on calcifying marine
organisms. SGD into oceanic systems is common on Hawai`i Island, and thus, it is an ideal location to
test the responses of calcifying marine species to OA conditions.

The goal of this study was to examine the potential effects of OA on calcifying
macroalgae using a field approach. The responses of aragonite calcifying Hawaiian macroalgae
were measured in transplant experiments. Specific objectives were as follows: (1) to determine
macroalgal community structure at study sites located on east and northwest Hawai`i Island; (2)
to measure environmental parameters at each site, including water temperature, salinity, pH, and
total alkalinity (TA); (3) to calculate total CO₂ (TCO₂), $pCO_2$, and $\Omega_{\text{arg}}$ at study sites; (4) to
determine aragonite calcifying macroalgal species’ responses to exposure to variations in $\Omega_{\text{arg}}$ in
terms of survival, CaCO₃ content, and photosynthetic activity via in situ transplants; (5) to
determine calcifying macroalgal species’ responses to variations in salinity under constant TA
and pH; and lastly, (6) to develop a map using ArcGIS 10.7 depicting a geo-visualization of the
study sites. The hypotheses were OA conditions affect percent CaCO₃ content and
photosynthesis of calcifying macroalgal species (*Halimeda macroloba*, *Padina australis*,
*Dichotomaria marginata*, and *Galaxaura rugosa*), and salinity does not affect percent CaCO₃
content of calcifying macroalgal species.

MATERIALS AND METHODS
Study sites

Three sites on Hawai‘i Island: Onekahakaha Beach Park (Onekahakaha), Richardson Ocean Park (Richardson), and Kawaihae Harbor (Kawaihae) were selected for their similar environmental parameters (i.e., water temperature, salinity, pH) and presence of specific calcifying macroalgal species (Fig. 1). These sites were the control sites during transplant experiments. *Halimeda macroloba* Decaisne (Fig. 2a) was found at Kawaihae, *Padina australis* Hauck (Fig. 2b) at Richardson, *Dichotomaria marginata* (J. Ellis et Solander) Lamarck (Fig. 2c) at Onekahakaha, and *Galaxaura rugosa* (Ellis et Solander) Lamouroux (Fig. 2d) at Richardson. These species use aragonite as their biogenic form of CaCO₃. A fourth site, Kuhio Kalani‘ana‘ole Park (Kuhio), was selected as the experimental site because its environmental parameters represented predicted OA conditions.

Onekahakaha (19° 44’ 18.35” N, 155° 2’ 20.32” W) and Richardson (19° 44’ 13.20” N, 155° 0’ 43.66” W) are located on the windward side of Hawai‘i Island. Onekahakaha and Richardson receives 3,284 mm and 3,267 mm annually, respectively (Giambelluca et al., 2013). Both sites have springs near the shoreline that discharge brackish groundwater. Substratum is dominated by basalt rock with a marine community, including: invertebrates, fish, corals (i.e., *Porites*, *Pocillopora*, and *Montipora* species), as well as a variety of calcifying and non-calcifying algal genera (i.e., *Dictyota*, *Asteronema*, *Jania*, *Padina*, and *Dichotomaria*).

Kuhio (19° 43’ 32.45” N, 155° 3’ 39.74” W) is an east-facing inlet inside Hilo Bay, located on the windward side of Hawai‘i Island. Kuhio receives 3,320 mm of rainfall annually (Giambelluca et al., 2013). This site was selected for its high SGD from the Wailoa River flowing into Hilo Bay. The Waiakea Pond feeds the Wailoa River, which is the largest source of groundwater into Hilo Bay (M & E Pacific, Inc., 1980). The nearshore area is dominated by a
basalt substratum, with high water stratification within the water column (M & E Pacific, Inc., 1980). Algal cover is low and primarily filamentous and turf, with patches of *Ahnfeltiopsis concinna*, a fleshy red alga.

Kawaihae (20° 1’ 58.32” N, 155° 49’ 44.23” W) is a northwest-facing harbor, on the leeward side of Hawai‘i Island. Average annual rainfall is only 255 mm (Giambelluca et al., 2013). From the shoreline to 112 m offshore, coral (i.e., *Porites* and *Pocillopora*) and sand dominate the benthic substratum. A calcareous green alga, *H. macroloba*, is abundant.

**Site characterization**

Benthic surveys were conducted to assess species composition and abundance of calcifying vs. non-calcifying macroalgal species using standard transect and point-intercept quadrat methodology. Three 25-m transects were laid on the substratum perpendicular to the shoreline within each study area. Five 0.25-m² quadrats were placed every five meters, and benthic substratum type was recorded at 12 intercept points within each quadrat (60 points per transect). Intercept data were converted into percent cover by summing the number of intercepts with macroalgal cover (calcifying or non-calcifying) and dividing by the total number of intercepts.

Water samples were collected from the control and experimental sites to characterize site conditions at the beginning and end of each transplant experiment. Water was collected at three stations per site with two replicates at each station (six samples per site), placed in clean, rinsed biological oxygen demand (BOD) glass bottles, and stored on ice during transport (1-2 h) to the laboratory. Temperature and salinity were measured using a handheld multi-parametric probe (YSI Model) *in situ*. Samples were analyzed for water carbonate chemistry including: pH, TA,
TCO$_2$, $p$CO$_2$ and $\Omega_{\text{arg}}$. At the laboratory, titrations were conducted using a Thermo Electron Orion (model 900A) and a Thermo Orion Sage dispensing system, with a 0.01 N HCl titrant (US EPA method 310.1) and pH standards 4.00 and 7.00. Data output from the titrations was inputted into a CO2Calc program (CO2CalcNet Application, Version 1.0.3) to calculate TCO$_2$, $p$CO$_2$, and $\Omega_{\text{arg}}$. Dissociation constants used were Mehrbach et al. (1973) as refit by Dickson and Millero (1987), KHSO$_4$ of Dickson (1990), total boron of Uppstrom (1974), NBS pH scale (mol kg$^{-1}$ H$_2$O), and gas exchange rates by Wanninkhof (1992).

Additional water samples were collected to assess nutrient concentrations at each site at the beginning and end of the transplant periods. Samples were collected in situ using clean, acid-washed syringes and filtered (GF/F, Whatman™ International Ltd), placed in a cooler, and stored frozen until analyzed within one month of date of collection. Samples were analyzed for sum of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) [detection limit (DL) 0.07 µmol L$^{-1}$, USEPA method 353.4], phosphate (PO$_4^{3-}$) [DL 0.03 µmol L$^{-1}$, USEPA method 365.5], silica (H$_4$SiO$_4$) [DL 1.00 µmol L$^{-1}$, EPA method 366], ammonium (NH$_4^+$) [DL 0.36 µmol L$^{-1}$, USEPA method 349.0], and total dissolved nitrogen (TDN) [DL 5.00 µmol L$^{-1}$, USGS 1-4650-03]. Dissolved nutrients were analyzed on a Lachat QuikChem 8500. Samples were analyzed in UH Hilo Analytical Laboratory.

Transplant experiment
Whole adult thalli, including basal holdfasts, of *H. macroloba, P. australis, D. marginata*, and *G. rugosa* were collected from wild populations. At the start of each experiment, 32 thalli of a species were collected. Thalli were selected for similar size and biomass. On the same day as collection, the selected calcifying macroalgal thalli were experimentally transplanted within and between sites that differed in $pCO_2$. Four thalli of a species were placed into each of eight mesh bags. Four bags were transplanted to Kuhio, a high $pCO_2$ site, and four bags were transplanted back into their original collection site, as controls. The mesh bags were tied onto a rock at the experimental and control sites, and placed securely at the same depth as the original collection (Fig. 3). These methodologies optimized macroalgal transplantation success, and minimized possible stress to the macroalgae during the transplantation procedure. Experiments lasted ten days. On days 0 (initial), 3, 5, 7, and 10 (final), one mesh bag was collected from the experimental and control sites. A mesh bag represented one sample. *In situ* photosynthetic activity was assessed before, during, and after transplantation with a Pulse Amplitude Modulated fluorometer (DIVING-PAM, WALZ) and Shutter Fluorometer Sensor (Aquation) with AquationDirect V2.3.2 software. Three measurements were taken at different locations on each of the thalli within each sample bag collected from Kuhio and the control sites. In the laboratory, each thallus was rinsed briefly with fresh water to remove epiphytic species, and then measured for size. Thalli were analyzed for percent CaCO$_3$ content using a gravimetric acid wash method. Each thallus was blotted dry with a paper towel, weighed, dried in an oven at ~ 60 °C until no further weight loss (8 to 16 hours, depending on species), reweighed to determine initial dry weight, soaked in 10% HCl for 2 h until bubbling ceased, dried again in oven at ~ 60 °C until dry for 8-16 h, weighed again to determine final mass of the decalcified dried thallus.
Mass of CaCO$_3$ in a thallus was calculated using the following formula:

\[
\text{Mass of CaCO}_3 = \text{Initial thallus dry wt.} - \text{Decalcified thallus dry wt.} \tag{1}
\]

To standardize characteristics of thalli within a species, percent CaCO$_3$ was calculated:

\[
\% \text{CaCO}_3 = \left(\frac{\text{Mass of CaCO}_3}{\text{Initial thallus dry wt.}}\right) \times 100\% \tag{2}
\]

In order to compare changes in percent CaCO$_3$ during the transplants among species with different initial CaCO$_3$ content, percent change in CaCO$_3$ content for a species was calculated:

\[
\% \text{Change in CaCO}_3 = \left[\frac{(\text{Mean } \% \text{CaCO}_3 \text{ on Day 0} - \text{Mean } \% \text{CaCO}_3 \text{ on Day 10})}{(\text{Mean } \% \text{CaCO}_3 \text{ on Day 0})}\right] \times 100\% \tag{3}
\]

Photosynthetically Active Radiation (PAR) light level measurements were taken underwater (UW) at the surface, inside the sample bags used for transplants, and in ambient air using a LICOR light meter (LI-COR 250 and LI-92 Underwater Quantum Sensor). Measurements were taken at the beginning and end of each transplant experiment. Percent light transmittance UW and inside the sample bags were calculated based on the PAR in air (designated as 100%) for that site.

A SEM (Hitachi 3400-II SEM) provided further analysis of surface calcification on random subsets of the transplanted algal samples. Subsets were kept as vouchers, pressed onto herbarium paper, and stored until examined. Images of macroalgal surfaces were taken of each sample to use as a visual representation of algal response to OA conditions. Examination required cutting small apical segments from each specimen and mounting the pieces onto aluminum stubs with adhesive tape. Images were taken across the thallus at 2.00 µm to 1.00 mm to compare algal surface structure from Day 0 to Day 10 of each transplant.
Salinity experiment

Because salinity varied among experimental and control sites due to differing amounts of SGD, a controlled laboratory experiment was designed to determine how differing salinity concentrations affect CaCO$_3$ content in calcifying macroalgal species, while keeping TA and pH constant. Five different salinity concentrations were prepared using Instant Ocean Seasalt (Aquarium Systems, Inc), mixed with deionized water. Solutions were mixed until they achieved oxygen/CO$_2$ equilibrium with the surrounding atmosphere. Fifteen 400 mL Erlenmeyer flasks were used for the five salinities (35, 27, 23, 18, and 14) with three replicates for each salinity. One thallus of each of the three species of calcifying macroalgae, *H. macroloba*, *P. australis*, and *G. rugosa*, was placed in each flask. Flasks were shaken at a medium speed (~176 rpm) to ensure flasks were continuously mixed. Thalli were exposed to natural day light from windows. The experiment ran for ten days, the same time period as the transplant experiments in the field. Twice a day, morning and late afternoons, measurements of pH, salinity, and temperature were made and recorded to monitor for constant pH and TA. Small amounts of sodium bicarbonate (NaHCO$_3$) were added to buffer the solutions if pH values drifted. Initial and final percent CaCO$_3$ content for each species was measured. Initial and final water samples collected from the five salinity treatments were analyzed for TA, pH, and $\Omega_{\text{arg}}$.

Data analysis and statistics
Percent change in CaCO₃ content in each species was calculated from samples collected at Kuhio and compared to the samples collected at each species’ corresponding control site. Data were tested for normality and equal variances. If assumptions for parametric analyses were met, one-way analysis of variance (ANOVA) and Tukey pairwise comparison were used to test for significant differences ($\alpha = 0.05$). Log₁₀ transformations were used when needed. If assumptions of normality and equal variance were not met, the non-parametric Kruskal-Wallis test was used ($\alpha = 0.05$). A Pearson correlation was used for each algal species to assess possible associations between photosynthetic activity and CaCO₃ content. Statistical analyses were conducted using Minitab 17.

**RESULTS**

*Site & benthic characterization*

Macroalgal cover and community composition varied among sites (Table 1). Richardson had the greatest species abundance of calcifying (mean percent cover = 22%, ± 21 SD) and non-calcifying macroalgae (mean percent cover = 41%, ± 8 SD). Percent cover of calcifying macroalgae at Onekahakaha and Kawaihae were lower, 16% and 7%. Turf or low-lying filamentous algae were the major non-calcifying macroalgae at Onekahakaha and Kuhio. At Kuhio, percent cover of calcifying macroalgae was very low (mean percent cover = 4%, ± 4 SD), and was comprised only of crustose coralline algae restricted to shallow depths of 0.3 - 0.6 m.

The physical characteristics and inorganic carbon parameters differed significantly among sites: salinity ($p < 0.01$, $F = 777.69$, df = 3), temperature ($p < 0.01$, $F = 364.50$, df = 3), pH ($p < 0.01$, $F= 81.73$, df = 3), TA ($p < 0.01$, $F = 632.84$, df = 3), $pCO_2$ ($p < 0.01$, $F= 48.17$, df = 3), TCO₂ ($p < 0.01$, $F = 252.73$, df = 3), and $\Omega_{\text{arg}}$ ($p < 0.01$, $F = 151.98$, df = 3) (Table 2 & Fig.)
4). Kuhio, the experimental site, had the lowest TA, lowest pH, lowest $\Omega_{\text{arg}}$, and highest $pCO_2$ in comparison to the control sites, Onekahakaha, Richardson, and Kawaihae. Water temperature at Kuhio was ~ 0.5°C lower than the control sites. Notably, the salinity at Kuhio was half that of the control sites. Total alkalinity at Kuhio was about 50% lower compared to TA at the control sites, and $\Omega_{\text{arg}}$ was below one, representative of undersaturated levels of aragonite. Surface UW percent light transmittance values at Kuhio ranged from 55 to 88, and inside the mesh bags from 25 to 59, well within the range at the control sites. Measurements varied due to local weather conditions, and water clarity; however, PAR values were similar among sites.

Nutrient concentrations ($NO_2^- + NO_3^-$, $PO_4^{3-}$, $H_4SiO_4$, $NH_4^+$, and TDN) were similar at Richardson and Kawaihae ($p > 0.01$) (Table 3). However, the concentrations of these parameters at Kuhio were significantly higher, except $NH_4^+$. $NH_4^+$ concentrations were similar among all sites.

**Transplant experiments**

During transplant experiments, pH, TA, $TCO_2$, and $\Omega_{\text{arg}}$ were consistently lower, while $pCO_2$ was consistently higher at Kuhio, compared to control sites (Table 4). The four algal taxa showed differences in mean initial percent $CaCO_3$ content, ranging from 60% (Padina) to 84% (Dichotomaria) (Fig. 5).

$CaCO_3$ content of *H. macroloba* and *P. australis* were affected by OA conditions at Kuhio; *D. marginata* and *G. rugosa* did not show significant loss of $CaCO_3$. In the first transplant experiment (October to November 2015), percent $CaCO_3$ in *H. macroloba* had declined by Day 10 at Kuhio (~ 46%), but not at the control site (Kawaihae) (Fig. 6a). In
contrast, during the second transplant experiment with *H. macroloba* (January to February 2016), there was a significant difference in percent CaCO$_3$ among samples at Kawaihae (p = 0.003, F = 11.68, df = 3) and at Kuhio (p = 0.001, F = 15.35, df = 3) (Fig. 6b). For *P. australis*, in a preliminary transplant experiment (June 2015), percent CaCO$_3$ declined by Day 10 at Kuhio. The thalli had completely disintegrated. Percent CaCO$_3$ of *Padina* declined from a mean of 48% CaCO$_3$ to 16% CaCO$_3$ at Kuhio, whereas, percent CaCO$_3$ of *Padina* samples at the control site declined slightly, from 48% CaCO$_3$ to 44% (Fig 7a). In a second transplant experiment (September to October 2015), percent CaCO$_3$ in *P. australis* had dropped significantly by Day 7 at Kuhio (p = 0.045, F = 3.63, df = 4) (Fig. 7b). In contrast, no significant change in percent CaCO$_3$ was observed at the control site. No significant change in percent CaCO$_3$ was observed in *D. marginata* (March to April 2016), nor in *G. rugosa* (January to February 2016) at their corresponding control sites (Fig. 8 & 9). Although there was some decrease in percent CaCO$_3$ in some thalli of *D. marginata* and *G. rugosa* transplanted to Kuhio, it was not significant.

When comparing the percent change in CaCO$_3$ content among species, all species experienced a greater percent change (loss) in their CaCO$_3$ content when transplanted to Kuhio, where pH, TA, and $\Omega_{\text{arg}}$ were lower (OA conditions) (Fig. 10). At Kuhio, transplanted *P. australis* experienced the greatest decline in CaCO$_3$ (-60 %) in comparison to the other species: -24% in *H. macroloba*, -8% in *D. marginata*, and -4% in *G. rugosa*. *Padina australis* also showed the greatest difference in percent change of CaCO$_3$ between the experimental and control sites (56%), followed by *Halimeda* (22%), and *Galaxaura* (3%).
Photosynthetic activity in the four algal species showed fluctuations during the transplant experiments. Photosynthetic yield among samples of *H. macroloba* in the first and second transplant experiments were not significantly different at Kuhio, nor at Kawaihae (Fig. 11a & 11b). Photosynthetic yield among samples of *P. australis* at Kuhio were significantly different (*p* < 0.05, *F* = 69.43, *df* = 3), but not at Richardson (*p* = 0.744, *F* = 0.42, *df* = 3). At Kuhio, by Day 10, the thalli showed no photosynthetic activity, and were disintegrating (Fig. 11c).

Photosynthetic yield among samples of *G. rugosa* was significantly different at Kuhio (*p* = 0.003, *F* = 6.36, *df* = 4), but not at Richardson (*p* = 0.855, *F* = 0.33, *df* = 4) (Fig. 11d).

*Dichotomaria marginata* was not measured for photosynthetic activity, due to lack of the fluorometer during this transplant experiment. Photosynthetic activity and percent CaCO$_3$ were not correlated for *H. macroloba* (*p* = 0.749) (Fig. 12a), *P. australis* (*p* = 0.284) (Fig. 12b), or *G. rugosa* (*p* = 0.855) (Fig. 12c).

Little to no changes in CaCO$_3$ content were observed from SEM scans for *H. macroloba*, *D. marginata*, and *G. rugosa* due to their internal inter- and intracellular aragonite deposition. However, changes in the specimens’ morphology were noted on Day 10 (i.e., thalli seemed amorphous) (Fig. 13a, 13c, 13d). For *P. australis*, CaCO$_3$ could be visualized with the SEM due to *Padina’s* characteristic surface deposition of aragonite. Comparison of initial and final samples of *P. australis* indicated that CaCO$_3$ decreased by Day 10 (i.e., fewer CaCO$_3$ crystals were evident) (Fig. 13b).

**Salinity effect on CaCO$_3$ content experiment**

Because the experimental site (Kuhio) and control sites differed significantly in salinity, a controlled laboratory experiment was conducted to test the effects of salinity on percent CaCO$_3$
of the macroalgae studied here. Salinity did not significantly affect the percent CaCO$_3$ content of any of the calcifying macroalgal species tested: *H. macroloba*, *P. australis*, and *G. rugosa* (Fig. 14). TA and pH were not significantly different among salinity treatments and were maintained during the experiment. However, $\Omega_{\text{arg}}$ did vary among salinity treatments ($p = 0.013, H = 12.70, df = 4$) (Table 5 & Fig. 15), but all values were indicative of supersaturation of $\Omega_{\text{arg}}$ (range = 6.82 to 3.86).

**DISCUSSION**

In some parts of the ocean, SGD, volcanic seeps, and some upwelling events create conditions of naturally high $p$CO$_2$, allowing for *in situ* experiments on the effects of OA on marine organisms (Paquay et al., 2007; Feely et al., 2008; Vogel et al., 2015). On Hawai‘i Island, SGD occurs in many nearshore locations, e.g. Hilo Bay (Mead & Wiegner, 2010), Kiholo Bay, Honokohau Harbor, Kahaualoa Bay, and Makako Bay (Peterson et al., 2009). In this study, Kuhio, a SGD site in east Hawai‘i, served as an experimental site to determine responses of native calcifying macroalgae to OA conditions. Carbonate chemistry parameters at Kuhio were significantly different from the control sites (Onekahakaha, Richardson, and Kawaihae). In fact, $p$CO$_2$ at Kuhio was twice as high as at control sites, and mimicked OA conditions related to anthropogenic CO$_2$ emission predictions for the year 2100 (RCP scenario RCP4.5, IPCC 2014).

Calcified *H. macroloba*, *P. australis*, *D. marginata*, and *G. rugosa* showed the greatest decline in percent CaCO$_3$ after ten days of transplantation at Kuhio. Based on the results from the laboratory salinity experiment, this decline in percent CaCO$_3$ in thalli at Kuhio cannot be attributed to lower salinity, but to OA conditions. Other calcifying macroalgal species have
shown similar responses to OA conditions, including reduced CaCO\(_3\) content (Jokiel et al., 2007; Martin & Gattuso, 2009; Sinutok et al., 2011; Niedermeyer, unpubl. data).

In the current study, no drop in photosynthetic activity in the four species was observed relative to percent CaCO\(_3\). These results are similar to those of Vogel et al. (2015), in which photosynthetic activity of six *Halimeda* species did not decline under OA conditions at volcanic seeps; however, Sinutok et al. (2011) reported reduced photosynthetic efficiency in *Halimeda* thalli with loss of calcification. The current study could not corroborate the conclusion that some organisms may be able to compensate for changes in carbonate chemistry by increasing photosynthesis (Kroeker et al., 2010; Johnson et al., 2012; Campbell et al., 2016).

Different tolerances to OA conditions among calcifying algal species may be explained by each species’ specific morphology, anatomy, as well as, seasonal effects on growth or physiology. In this study, *H. macroloba, D. marginata*, and *G. rugosa* seemed more resistant to OA conditions than *P. australis*. *Padina australis* may be more susceptible to OA conditions because it has a thin blade that is only two-cell layers thick (Abbott & Huisman, 2004), and its CaCO\(_3\) is deposited on the upper and lower surfaces of the blade. In comparison, *H. macroloba* is a massive, pseudo-parenchymatous thallus of densely woven filaments with CaCO\(_3\) deposited between cells throughout the thallus. *Halimeda’s* CaCO\(_3\) is not directly exposed to ambient OA conditions, as is *Padina’s* CaCO\(_3\) on the thallus surface. Like *Halimeda*, *G. rugosa* and *D. marginata* are multi-branched macroalgae; however, these two calcifying reds sequester CaCO\(_3\) within their cell walls. Thick branches with internal, inter- or intra-cellular CaCO\(_3\) deposits appear to make macroalgal species more resistant to OA conditions compared to those who deposit CaCO\(_3\) on the surface of their blades.
Although OA is known to have significant negative effects on many marine organisms’ survival, calcification, growth, and reproduction (Kroeker et al., 2010), studies on specific taxa of calcifying macroalgae have demonstrated nuanced responses to OA when subjected on different temporal scales or experimental conditions. For instance, in two-week long field experiments, *Halimeda* species (Chlorophyta) increased calcification in OA conditions (Vogel et al., 2015); whereas, in five-week long controlled laboratory experiments, *Halimeda* species showed lowered CaCO$_3$ content (Sinutok et al., 2011), and in a four-week mesocosm experiment, calcification in *H. incrassata*, *H. opuntia* and *H. simluans* declined 41% under low pH conditions (Campbell et al., 2016). In the current study, in ten-day long field experiments, *H. macroloba* showed mixed responses to OA conditions: CaCO$_3$ content declined in winter months (January and February), but not in fall (October to November). Notably, *H. macroloba* had greater initial CaCO$_3$ content in the winter (~ 70-90% CaCO$_3$ content) than in the fall (~ 50-80% CaCO$_3$). Such seasonal patterns of calcification may cause different responses to OA conditions at different times of year.

The brown calcifying macroalgae, *Padina pavonica* and *P. australis*, lost calcification, but persisted with no CaCO$_3$ content in areas of high $p$CO$_2$ at volcanic seeps in the Mediterranean Sea and in Papua New Guinea (Johnson et al., 2012), perhaps due to the lack of grazers in the area and the natural ability of some *Padina* species to survive in an uncalcified *Vaughniella*-stage (Abbott & Huisman, 2004). In contrast, the current study and another on Hawai‘i Island (Niedermeyer unpubl. data) found that percent CaCO$_3$ content, and thallus integrity, declined within eleven days, when *P. australis* was transplanted to an area with low pH and high $p$CO$_2$. Perhaps, differences in responses are due to environmental differences among
the geographical locations, or to genetic or physiological differences among the *Padina*
populations.

In OA studies on calcifying red algae, crustose coralline algae (Rhodophyta), which
deposit calcite within cell walls, declined in growth (Jokiel et al., 2007; Martin & Gattuso, 2009).
Similarly, *Jania*, a branched, articulated coralline alga (Rhodophyta) decreased in abundance in
OA conditions (Porzio et al., 2011). In contrast, in the current study, *D. marginata* and *G.
rugosa* showed no decline in CaCO$_3$ after transplantation to high $p$CO$_2$, low pH, and low $\Omega_{\text{arg}}$
conditions. Differences in responses among red algae may be due to differences in growth form,
as well as the solubility of the CaCO$_3$ deposited by the alga: calcite ($K_{sp} = 10^{-8.48}$) in Corallinales
vs. aragonite ($K_{sp} = 10^{-8.34}$) in Nemaliales (Morse & Mackenzie, 1990).

Under OA conditions, reduced calcification in calcifying organisms may give other
species a competitive advantage (Fabry et al., 2008). Decreased $\Omega_{\text{arg}}$ stimulated a transition from
a calcifier-dominated system to a fleshy algal-dominated system in a multiple mesocosm tank
experiment conducted on O‘ahu, HI (Kuffner et al., 2008). Jokiel et al. (2007) predicted that
reduced recruitment and survival of crustose coralline red algae in high $p$CO$_2$ conditions could
cause shifts in macroalgal community composition, as well as negative consequences for
carbonate reef structure. Similarly, a study on another crustose coralline alga, *Lithophyllum
cabiochae*, in the Mediterranean concluded that OA will have consequences for biodiversity and
biogeochemistry within marine ecosystems (Martin & Gattuso, 2009). At CO$_2$ vents (pH 7.8) on
Ischia Island, Italy, 95% of the macroalgal species, both calcifying and non-calcifying, were able
to tolerate greater and more rapid diel fluctuations in pH than what is expected for OA (Porzio et
al., 2011). *Hydrolithon cruciatum*, a crustose coralline alga, was able to out-compete other
calcifying species living farther from the vents, suggesting that macroalgal species more tolerant of elevated CO$_2$ levels or acclimated to pH fluxes may replace those that are sensitive to these conditions (Porzio et al., 2011). In the current study, the four species of calcifying macroalgae responded differently to short-term exposure to naturally occurring OA conditions. Hawaiian $H$. *macroloba*, *D. marginata*, and *G. rugosa* may have a competitive advantage over *P. australis* in OA conditions. These results imply that future changes in algal community composition and function in Hawai‘i are possible with OA. Macroalgal species showing tolerance to high $p$CO$_2$, low pH, low TA, and reduced CaCO$_3$ saturation may dominate nearshore ecosystems when OA conditions become prevalent.
TABLES

Table 1. Mean percent cover (± SD) of substratum at study sites on Hawai‘i Island, HI.

<table>
<thead>
<tr>
<th>Site</th>
<th>Calcifying algae</th>
<th>Non-calcifying algae</th>
<th>Coral</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onekahakaha</td>
<td>6 (7)</td>
<td>49 (2)</td>
<td>7 (4)</td>
<td>37 (8)</td>
</tr>
<tr>
<td>Richardson</td>
<td>22 (21)</td>
<td>41 (8)</td>
<td>3 (3.47)</td>
<td>34 (22)</td>
</tr>
<tr>
<td>Kawaihae</td>
<td>16 (12)</td>
<td>0</td>
<td>37 (17)</td>
<td>48 (8)</td>
</tr>
<tr>
<td>Kuhio</td>
<td>4 (4)*</td>
<td>24 (7)</td>
<td>0</td>
<td>72 (11)</td>
</tr>
</tbody>
</table>

Note: Asterisk "*" = Crustose coralline algae was present only at 0.3-0.6 m depth.
Table 2. Mean (± SD) values of physical characteristics and inorganic carbon parameters at each site. Onekahakaha, Richardson, and Kawaihae were control sites. Kuhio, the site representative of OA conditions, was the experimental site for transplants. Letters indicate results of a Tukey pairwise test. Values that share the same letter are not significantly different. α = 0.05

<table>
<thead>
<tr>
<th>Sites</th>
<th>Salinity</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>TA (µmol kg(^{-1}))</th>
<th>TCO(_2) (µmol kg(^{-1}))</th>
<th>pCO(_2) (µatm)</th>
<th>Ω(_{\text{arg}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onekahakaha</td>
<td>33.75 (0.89)(^a)</td>
<td>25.27 (1.04)(^b)</td>
<td>8.37 (0.04)(^a)</td>
<td>2228 (68)(^b)</td>
<td>1819 (34)(^b)</td>
<td>224 (19)(^c)</td>
<td>4.49 (0.51)(^a)</td>
</tr>
<tr>
<td>Richardson</td>
<td>33.87 (0.46)(^a)</td>
<td>25.60 (0.25)(^b)</td>
<td>8.29 (0.08)(^a)</td>
<td>2298 (9)(^a)</td>
<td>1931 (60)(^a)</td>
<td>300 (66)(^bc)</td>
<td>4.11 (0.56)(^ab)</td>
</tr>
<tr>
<td>Kawaihae</td>
<td>34.60 (0.62)(^a)</td>
<td>27.65 (0.27)(^a)</td>
<td>8.18 (0.10)(^b)</td>
<td>2250 (26)(^ab)</td>
<td>1933 (45)(^a)</td>
<td>409 (117)(^b)</td>
<td>3.58 (0.66)(^b)</td>
</tr>
<tr>
<td>Kuhio</td>
<td>17.23 (1.78)(^b)</td>
<td>20.56 (0.37)(^c)</td>
<td>7.90 (0.09)(^c)</td>
<td>1480 (82)(^c)</td>
<td>1438 (66)(^c)</td>
<td>681 (152)(^a)</td>
<td>0.74 (0.21)(^c)</td>
</tr>
</tbody>
</table>

Note: TCO\(_2\), pCO\(_2\), and Ω\(_{\text{arg}}\) were calculated values.
Table 3. Mean (± SD) values of nutrient concentrations measured at each site. Onekahakaha, Richardson, and Kawaihae were control sites. Kuhio was the experimental site for transplants. Statistics indicate an ANOVA test.

<table>
<thead>
<tr>
<th>Sites</th>
<th>NO$_2^-$ + NO$_3^-$</th>
<th>PO$_4^{3-}$</th>
<th>H$_2$SiO$_4$</th>
<th>NH$_4^+$</th>
<th>TDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onekahakaha</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Richardson</td>
<td>1.18 (0.13)</td>
<td>0.14 (0.03)</td>
<td>10.37 (3.70)</td>
<td>1.82 (2.78)</td>
<td>12.41 (3.53)</td>
</tr>
<tr>
<td>Kawaihae</td>
<td>1.95 (0.59)</td>
<td>0.25 (0.04)</td>
<td>2.27 (0.76)</td>
<td>1.29 (0.27)</td>
<td>9.19 (0.93)</td>
</tr>
<tr>
<td>Kuhio</td>
<td>27.65* (5.41)</td>
<td>1.41* (0.19)</td>
<td>316.46* (60.73)</td>
<td>1.33 (0.82)</td>
<td>29.21* (5.23)</td>
</tr>
</tbody>
</table>

Note: Asterisk “*” = significant difference among sites relative nutrient type, (α= 0.05).
Table 4. Mean (± SD) values for water carbonate chemistry parameters and light transmittance measured at the start and end of each transplant experiment. Light measurements were collected underwater (UW) at the surface, UW inside the mesh bags containing the algal samples, and out of water at sea surface (air). Percent light transmittance was based on air as 100%. NA = Not available

<table>
<thead>
<tr>
<th>Transplant</th>
<th>Date</th>
<th>Site</th>
<th>Salinity</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>TA (µmol kg⁻¹)</th>
<th>TCO₂ (µmol kg⁻¹)</th>
<th>pCO₂ (µatm)</th>
<th>Ω&lt;sub&gt;arg&lt;/sub&gt;</th>
<th>% Light transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Halimeda macroloba</em></td>
<td>26-Oct-2015 to 10-Nov-2015</td>
<td>Kuhio</td>
<td>17.45 (3.31)</td>
<td>21.57 (1.03)</td>
<td>8.03 (0.11)</td>
<td>1505 (162)</td>
<td>1426 (117)</td>
<td>496 (106)</td>
<td>1.09 (0.47)</td>
<td>76 (NA) 25 (NA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kawaihae</td>
<td>35.07 (0.53)</td>
<td>27.93 (0.98)</td>
<td>8.29 (0.02)</td>
<td>2311 (18)</td>
<td>1912 (28)</td>
<td>292 (15)</td>
<td>4.45 (0.21)</td>
<td>58 (41) 20 (16)</td>
</tr>
<tr>
<td><em>Halimeda macroloba</em></td>
<td>27-Jan-2016 to 5-Feb-2016</td>
<td>Kuhio</td>
<td>21.81 (1.65)</td>
<td>22.36 (1.31)</td>
<td>8.22 (0.04)</td>
<td>1680 (42)</td>
<td>1529 (30)</td>
<td>320 (35)</td>
<td>1.84 (0.16)</td>
<td>55 (NA) 59 (41)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kawaihae</td>
<td>33.82 (0.42)</td>
<td>25.92 (0.21)</td>
<td>8.32 (0.01)</td>
<td>2308 (22)</td>
<td>1923 (20)</td>
<td>276 (10)</td>
<td>4.31 (0.11)</td>
<td>76 (1) 18 (12)</td>
</tr>
<tr>
<td><em>Padina australis</em></td>
<td>21-Sep-2015 to 1-Oct-2015</td>
<td>Kuhio</td>
<td>14.37 (4.41)</td>
<td>21.60 (0.79)</td>
<td>7.92 (0.16)</td>
<td>1383 (183)</td>
<td>1339 (136)</td>
<td>659 (224)</td>
<td>0.77 (0.44)</td>
<td>88 (NA) 33 (18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Richardson</td>
<td>33.35 (0.61)</td>
<td>27.12 (0.26)</td>
<td>8.39 (0.04)</td>
<td>2225 (26)</td>
<td>1795 (36)</td>
<td>216 (27)</td>
<td>4.78 (0.33)</td>
<td>75 (5) 42 (12)</td>
</tr>
<tr>
<td><em>Galaxaura rugosa</em></td>
<td>27-Jan-2016 to 5-Feb-2016</td>
<td>Kuhio</td>
<td>20.30 (0.99)</td>
<td>22.36 (1.31)</td>
<td>8.22 (0.04)</td>
<td>1680 (42)</td>
<td>1529 (30)</td>
<td>320 (35)</td>
<td>1.84 (0.16)</td>
<td>60 (NA) 43 (26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Richardson</td>
<td>34.05 (0.60)</td>
<td>25.18 (0.65)</td>
<td>8.39 (0.09)</td>
<td>2303 (6)</td>
<td>1866 (79)</td>
<td>224 (63)</td>
<td>4.82 (0.80)</td>
<td>80 (3) 58 (12)</td>
</tr>
<tr>
<td><em>Dichotomaria marginata</em></td>
<td>22-Mar-2016 to 1-Apr-2016</td>
<td>Kuhio</td>
<td>21.33 (1.77)</td>
<td>21.22 (0.74)</td>
<td>8.19 (0.05)</td>
<td>1726 (99)</td>
<td>1577 (67)</td>
<td>350 (42)</td>
<td>1.82 (0.38)</td>
<td>74 (25) 43 (30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Onekahakaha</td>
<td>33.75 (0.89)</td>
<td>25.27 (1.04)</td>
<td>8.37 (0.04)</td>
<td>2228 (68)</td>
<td>1819 (34)</td>
<td>224 (19)</td>
<td>4.49 (0.51)</td>
<td>87 (6) 45 (NA)</td>
</tr>
</tbody>
</table>

Note: TA, TCO₂, pCO₂, and Ω<sub>arg</sub> were calculated values.
Table 5. Mean (± SD) values of total alkalinity (TA), pH, and aragonite saturation ($\Omega_{\text{arg}}$) measured at the end of the salinity experiment.

<table>
<thead>
<tr>
<th>Salinity Group</th>
<th>TA (µmol kg$^{-1}$)</th>
<th>pH</th>
<th>$\Omega_{\text{arg}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>3192 (197)</td>
<td>8.46 (0.03)</td>
<td>6.92 (0.13)</td>
</tr>
<tr>
<td>27</td>
<td>2699 (89)</td>
<td>8.49 (0.02)</td>
<td>5.46 (0.28)</td>
</tr>
<tr>
<td>23</td>
<td>2524 (279)</td>
<td>8.47 (0.04)</td>
<td>4.42 (0.27)</td>
</tr>
<tr>
<td>18</td>
<td>2694 (382)</td>
<td>8.41 (0.09)</td>
<td>3.86 (0.36)</td>
</tr>
<tr>
<td>14</td>
<td>2880 (178)</td>
<td>8.54 (0.05)</td>
<td>4.67 (0.06)</td>
</tr>
</tbody>
</table>

p-value: p = 0.121, p = 0.131, p = 0.013
F-value: H = 7.30, H = 7.10, H = 12.70
df-value: df = 4, df = 4, df = 4

Note: “H” denotes a Kruskal-wallis test, ($\alpha$ = 0.05).
FIGURES

Figure 1. Sites in Hawai‘i Island where calcifying macroalgal transplants were conducted.
Figure 2. The four calcifying macroalgal species used in study. a) *Halimeda macroloba* (Phylum: Chlorophyta, Order: Bryopsidales), b) *Padina australis* (Phylum: Phaeophyta, Order: Dicyotaes), c) *Dichotomaria marginata* (Phylum: Rhodophyta, Order: Nemaliales), and d) *Galaxaura rugosa* (Phylum: Rhodophyta, Order: Nemaliales).
Figure 3. Flow chart of experimental transplant design conducted between and within control sites (Onkahakaha, Richardson, and Kawaihae) and experimental site (Kuhio) on Hawai‘i Island.
Figure 4. Mean (± SD) values of physical characteristics and inorganic carbon parameters for each site. Onekahakaha, Richardson, and Kawaihae were control sites. Kuhio, the site representative of OA conditions, was the experimental site for transplants. Statistics indicate an ANOVA test, (α= 0.05). Letters indicate results of a Tukey pairwise test; values that share the same letter are not significantly different.
Figure 5. Mean (± SD) initial percent CaCO$_3$ content of *H. macroloba*, *P. australis*, *D. marginata*, and *G. rugosa*. *Halimeda* and *Dichotomaria* are heavily calcified species.
Figure 6. Mean (± SD) percent CaCO$_3$ content in *Halimeda macroloba*, during the first (a) and second (b) transplant experiments. Statistics indicate an ANOVA test, ($\alpha = 0.05$).
Figure 7. Mean (± SD) percent CaCO₃ content in *Padina australis* during the first (a) and second (b) transplant experiments. Statistics indicate an ANOVA test, (α= 0.05).
Figure 8. Mean (± SD) percent CaCO$_3$ content in *Dichotomaria marginata* during transplant experiment. Statistics indicate an ANOVA test, ($\alpha=0.05$).

Figure 9. Mean (± SD) percent CaCO$_3$ content in *Galaxaura rugosa* during transplant experiment. Statistics indicate an ANOVA test, ($\alpha=0.05$).
Figure 10. Mean percent change (± propagation of error) in CaCO$_3$ content for each species during transplant experiments. Note: “NA” = not available, due to lost data during transplant.
Figure 11. Mean (± SD) photosynthetic yields measured during transplant experiments, a) *Halimeda macroloba* in first transplant, b) *Halimeda macroloba* in second transplant, c) *P. australis*, and d) *G. rugosa*. Note: *D. marginata* photosynthetic yield was not measured. Statistics indicate an ANOVA test, ($\alpha = 0.05$).
Figure 12. Mean (± SD) percent CaCO₃ vs. mean photosynthetic yield measured during transplant experiments, a) *H. macroloba*, b) *P. australis*, and c) *G. rugosa*. Statistics indicate a Pearson correlation, (α = 0.05).
Figure 13. SEM images taken of initial (left) and final (right) samples from transplants using a) *H. macroloba*, b) *P. australis*, c) *D. marginata*, and d) *G. rugosa* samples.
Figure 14. Mean (± SD) percent CaCO$_3$ content of thalli incubated in five different salinities, a) *Halimeda*, b) *Padina*, and c) *Galaxaura*. The dashed line represents the initial CaCO$_3$ content for each algal species. Statistics indicate an ANOVA test, ($\alpha = 0.05$).
Figure 15. Mean (± SD) (a) TA, (b) pH, and (c) $\Omega_{\text{arg}}$ recorded at the end of the ten day experiment. Statistics indicate a Kruskal-wallis test, ($\alpha = 0.05$).
APPENDIX A: SUPPLEMENTAL BACKGROUND INFORMATION

Since the Industrial Revolution in the late 1800’s, the rise of both \([\text{CO}_2]_{\text{atm}}\) and global temperature is 100-1000 times faster than at any point in the last 420,000 years, when most extant marine species evolved (Hoegh-Guldberg et al., 2007; Harley et al., 2012). The ocean absorbs atmospheric \(\text{CO}_2\), which lowers seawater pH, and sets in motion a cascade of carbonate chemistry reactions. The magnitude of environmental change (e.g. increasing temperatures and OA) caused by this increase in \([\text{CO}_2]_{\text{atm}}\) poses serious physiological challenges to marine species (Harley et al., 2012). The effects of climate change on ecosystems, their biodiversity, community structure, and ecological processes should be assessed now, in order to protect marine species in the future. Research is limited on the potential impacts climate change will have on seaweed-dominated ecosystems (Harley et al., 2012; Wernberg et al., 2012). Marine macroalgae are natural resources that provide goods and services for humans including food, medicine, pharmaceuticals, and shoreline protection (Ronnback et al., 2007; Harley et al., 2012). Developing an interdisciplinary understanding of species-specific, as well as ecosystem responses, to climate change is critical to developing conservation and management plans.

Three major abiotic climate change trends resulting from rising atmospheric \(\text{CO}_2\) are OA, sea temperature increase, and sea-level rise that directly and indirectly affect biogeochemical processes critical to macroalgae, and can alter species’ physiology, growth, reproduction, and survival (Harley et al., 2012). Direct and indirect ecological factors have been shown to affect the ecological balance within seaweed communities, including interactions between competing species, top-down herbivory, primary productivity, seaweed biodiversity, and species resilience to stress (Harley et al., 2012). How the three climate change trends will affect macroalgal distribution worldwide is unknown (Harley et al., 2012). Calcifying macroalgae may respond to climate change through adaptation, acclimation, distribution shifts, or extinction (Harley et al., 2012).

The responses of calcifying macroalgae to OA are influenced by the carbonate chemistry in the ambient seawater. Dissolved inorganic carbon (DIC) is the sum of inorganic carbon species. DIC exists in three major forms, including 1) carbonic acid \((\text{H}_2\text{CO}_3)\), 2) bicarbonate ion
(HCO$_3^-$), and 3) carbonate ion (CO$_3^{2-}$) (Libes 2009). Each form of DIC contains one atom of carbon; therefore total DIC (TDIC or TCO$_2$) is defined by the following mass balance equation:

$$TCO_2 = [CO_2] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$$  \hspace{1cm} (1)

Interaction of CO$_2$ in seawater causes a change in marine carbon chemistry and affects which form of CO$_2$ dominates the carbonate system, which can affect how a marine organism will utilize CO$_2$ physiologically.

As anthropogenic CO$_2$ dissolves into the ocean, the concentration of CO$_3^{2-}$ ions is reduced (Fabry et al., 2008). CO$_3^{2-}$ ions are necessary for marine calcifying organisms that produce CaCO$_3$ for biological structures. The magnitude of the impact on these organisms depends on the CaCO$_3$ saturation state ($\Omega$). A decrease in CO$_3^{2-}$ ion concentration causes a decrease in both forms of CaCO$_3$: aragonite and calcite. The saturation state is determined by the relationship between total calcium (Ca$^{2+}$) and CO$_3^{2-}$ concentrations and their solubility product in situ, K$^*_sp$. Ocean temperature, salinity, pressure, and the mineral phase of CaCO$_3$ affect the solubility product.

$$\Omega = [Ca^{2+}_{in\,\text{situ}}][CO_3^{2-}_{in\,\text{situ}}]/ K^*_sp$$  \hspace{1cm} (3)

Ca$^{2+}$ is nearly constant in the ocean system, and about 30 to 50 times greater than the concentration of CO$_3^{2-}$; the $\Omega$ is primarily controlled by the abundance of CO$_3^{2-}$. The rate at which water circulates through and within the ocean is faster than the process that significantly alters the concentration of the major ions, like Ca$^{2+}$, which explains why [Ca$^{2+}$] is nearly constant. If $\Omega >1$, ocean water is supersaturated with CaCO$_3$, if $\Omega < 1$, ocean water is undersaturated with respect to CO$_3^{2-}$ ions (Andersson et al., 2005).

Alkalinity is the concentration of a negative charged ions in a solution that can be titrated by a strong acid, such as hydrogen chloride (HCl) (Libes 2009). CO$_3^{2-}$ and HCO$_3^-$ contribute the majority of this negative charge in seawater. Total alkalinity (TA) is the alkalinity contributed CO$_3^{2-}$ species and other titratable charges (i.e. HCO$_3^-$), minus the in situ hydrogen ion (H$^+$) concentration. The portion of TA that is attributed to CO$_3^{2-}$ species is referred to as CO$_3^{2-}$ alkalinity (Libes 2009).
Reduced dissolved \( \text{CO}_3^{2-} \) ion concentrations, occurring through OA, are causing a decline in aragonite saturation (\( \Omega_{\text{arg}} \)). Additionally, dissolution of marine carbonates, including aragonite (from corals and pteropods), magnesium calcites (from coralline algae), and calcite (from coccolithophorids and foraminifera), neutralizes anthropogenic \( \text{CO}_2 \) and increases TA (Feely et al., 2004). Temperature, pH, and depth (pressure) are environmental parameters that affect \( \Omega_{\text{arg}} \). Higher temperatures correspond with higher \( \Omega_{\text{arg}} \), and lower pH levels. Lower \( \Omega_{\text{arg}} \) may affect the growth and/or survival of calcifying macroalgae.

Decreased pH levels and \( \text{CO}_3^{2-} \) saturation levels can cause an undersaturation of \( \text{CaCO}_3 \) precipitated by calcifying macroalgae. Interaction of \( \text{CO}_2 \) in seawater causes a change in carbonate speciation, which determines how a marine organism will utilize \( \text{CO}_2 \) physiologically with respect to what form of DIC is available. Decreased [\( \text{CO}_3^{2-} \)] reduce the ability of \( \text{CaCO}_3 \)-forming marine species to calcify, a process referred to as calcification. Marine calcifying organisms extract \( \text{Ca}^{2+} \) ions and \( \text{CO}_3^{2-} \) ions from seawater and combine to form crystalline structures of \( \text{CaCO}_3 \). Low pH can alter \( \Omega \). These changes impact calcifying marine fauna and flora that utilize \( \text{CaCO}_3 \) in their shells, cell walls, or other biological structures (Doney et al., 2009). Researchers (Kleypas et al., 1999; Feely et al., 2004; McNeil et al., 2004; Andersson et al., 2005) have concluded that \( \text{CaCO}_3 \)-forming marine organisms may have difficulty in maintaining calcification. According to Fabry et al. (2008), most calcifying organisms show reduced calcification in response to increased \( p\text{CO}_2 \), decreased \( \Omega \), and lower pH. (Gattuso et al., 1998; Langdon et al., 2000, 2003; Riebesell et al., 2000).

Macroalgae can acquire \( \text{CO}_2 \) for photosynthesis from seawater as \( \text{CO}_2 \) and/or \( \text{HCO}_3^- \) through the use of a carbon-concentrating mechanism (CCM) (Giordano et al., 2005). Macroalgae with CCMs generally are not carbon-limited under most environmental conditions (Giordano et al., 2005). Depending on the carbonate chemistry within the environment, the use of \( \text{CO}_2 \) or \( \text{HCO}_3^- \) can alter metabolism and growth rates of calcareous and noncalcareous macroalgae (Harley et al., 2012). The method by which an algal species acquires \( \text{CO}_2 \) may predict that species’ sensitivity to OA. Different macroalgal species would be expected to respond differently to OA due to their physiological capabilities to acquire \( \text{CO}_2 \). The impact of
OA is dependent on species-specific physiology, as well as water quality, carbonate chemistry, and community composition.

A few species of calcifying macroalgae have been studied to determine the effects of increased temperature and CO$_2$ (Harley et al., 2012). In tank experiments on Oahu, HI, Jokiel et al. (2007) found that elevated $p$CO$_2$ treatments reduced coralline algal recruitment rates and percent cover, suggesting that in nature OA will shift marine ecosystem structure. Similarly, studies on red coralline algae (*Lithophyllum cabiocha*) in Mediterranean coastal ecosystems predicted that OA will have consequences for biodiversity and biogeochemistry within marine ecosystems (Martin & Gattuso, 2009). However, other studies suggest contrasting results for calcifying macroalgae in response to OA conditions. In the Mediterranean and Papua New Guinea, *Padina australis* (Hauck) and *Padina pavonica* (Linnaeus) persisted despite decalcification in areas of high CO$_2$, and actually increased abundance under acidic conditions if grazers were absent (Johnson et al., 2012). Preliminary field experiments with *Padina australis* on Hawai‘i Island, HI, found a reduction in CaCO$_3$ content of the thalli after being transplanted from an area with low $p$CO$_2$ (308.8 ±76.9 ppm) to an area with high $p$CO$_2$ (437.6 ±130.7 ppm) (Niedermeyer, unpubl. data). Vogel et al. (2015) investigated the impacts of low pH and high $p$CO$_2$ on six different *Halimeda* species at a tropical volcanic CO$_2$ seep in Milne Bay Province, Papua New Guinea. Rates of calcification of *H. digitata* and *H. opuntia* were higher in light than in the dark, at the volcanic CO$_2$ seep. In contrast, Sinutok et al. (2011) found that the rate of calcification decreased in *H. macroloba* and *H. cylindracea*, when exposed to low pH conditions between 7.7 to 7.4 and elevated temperature (34°C). However, when comparing these two studies, it is important to note the different experimental designs that were implemented, *in situ* (Vogel et al., 2015) versus controlled laboratory experiments (Sinutok et al., 2011). Gaps in knowledge still exist in identification of calcifying macroalgal physiological thresholds for OA stress, particularly in the Hawaiian Islands.

In addition to volcanic CO$_2$ seeps as sites to study the responses of marine organisms to elevated CO$_2$ conditions (i.e. Vogel et al., 2015), SGD into the ocean also provides an ideal location for opportunities to test the response of calcifying marine species to OA conditions. Sites of SGD into the ocean can have effects on CO$_3$-2 saturation, increasing $p$CO$_2$ and decreasing $\Omega$$_{arg}$, where outgassing of CO$_2$ is unable to eliminate all CO$_2$ brought into the water system via respiration from organic matter off the land. Therefore, $p$CO$_2$ is two to five or more times than that of the atmosphere (Allan & Castillo,
As a result, SGD, high in CO$_2$, that flows into marine ecosystems, affect the surrounding community structure and function.

Calcereous macroalgae are important primary producers in tropical, temperate, and polar waters, existing in marine habitats from intertidal shores to the deep zones of the euphotic zone. These species play critical ecological roles, such as recruitment substratum for benthic organisms, reef cementation, and habitat structure (Nelson, 2009), now and in ancient seas.

Fossil evidence of calcereous green algae is best known in the order Dasycladales, whose paleontological record includes more than 175 fossil genera that have been described back to the Precambrian-Cambrian strata (ca. 570 million years ago), but today this order is reduced to only eleven extant species (Lee 1999). Records of calcereous red algae exist from the Phanerozoic period (ca. 542 Ma), as CaCO$_3$-skeletons in reef formations (Lee 1999). These deposits are closely analyzed for petroleum deposits, thus receiving much attention (Lee 1999). Solenopores (Solenoporaceae) were the first calcereous red algae that arose in the Ordovician Period (ca. 443 Ma) and became extinct during the Jurassic Period (ca. 145 Ma) (Lee, 1999). Similar to today, calcifying red macroalgae were key species in reef formation and structure. Modern members of the order Corallinales appeared in the Jurassic period (ca. 201 to 145 Ma) and are tolerant of invertebrate herbivory, perhaps explaining their ecological success (Tappan 1980). In the Division Phaeophyta, there is little fossil evidence due to the small number of species that produce hard parts or resistant spores. However, deposits of the order Dictyotales have been identified from the Miocene epoch (ca. 23 to 5.3 Ma) (Parker & Dawson, 1965). Padina and Newhousia (order Dictyotales) are the only two extant brown genera that calcify.

In the Hawaiian Islands, of the rich seaweed flora (500+ species) 58 are calcereous macroalgae. Four common calcifying macroalgal genera in nearshore waters of the Hawaiian Islands are Halimeda Lamourex (Chlorophyta), Padina Adanson (Phaeophyta), Dichotomaria Lamarck (Rhodophyta), and Galaxaura Lamouroux (Rhodophyta).

Seven species of Halimeda exist in the Hawaiian marine flora (Abbott & Huisman, 2004). Halimeda thalli can grow 10 – 20 cm tall and are composed of catenate calcifying segments. Thallus segments are usually pendant or erect, but can grow sprawling or suspended along steep inclined surfaces. Halimeda thalli attach to the substratum by a holdfast or multiple holdfasts. Substratum type includes coral rubble, rock, and/or sandy, muddy bottoms (Abbott & Huisman, 2004). Remains of ancient Halimeda (order Bryopsidales) thalli form mounds of
sediment (bioherms) in the form of CO$_3^{2-}$ throughout the Bahamas, Great Barrier Reef, continental shelf of India, and reef flats or lagoons of Pacific atolls and islands (Payri, 1988; Pxxxx Rao, et al., 1994; Rees et al., 2007).

Seven species of *Padina* are reported in the Hawaiian Islands. *Padina* are fan-shaped calcifying macroalgae, often growing in small groups, ranging from 3-15 cm tall. Blades are erect and calcifying, that often split with age. Some species have an alternate developmental stage that is uncalcifying, the *Vaughniella*-stage. *Padina* is attached to the substratum by a conical holdfast of rhizoids. *Padina* species are found usually on coral rubble, rock, or sandy bottoms. Size of thalli may depend on substratum and water depth (Abbott & Huisman, 2004.)

*Dichotomaria* and *Galaxaura* are both calcifying members of the red order Nemaliales. *Dichotomaria* was previously classified within the genus *Galaxuara*, but was resurrected as an independent genus in 2004 (Huisman et al., 2004; Wang et al., 2005). Six species of the red calcifying genus *Galaxaura* grow in the Hawaiian Islands, and only one of *Dichotomaria*. These species are multiaxial. Axes can be terete to flattened, with subdichotomously divided branches. Depending on species, epidermal cells may have pigmented assimilatory hairs (Abbott 1999). Species are common in tidepools and shallow subtidal areas, attached to coral rubble or rock.

Algal-dominated ecosystems provide trillions of dollars of ecosystem resources and services yearly (Costanza et al., 1997; Harley et al., 2012). Loss of these unique ecosystems will lead to grave consequences for human societies (Harley et al., 2012). The impacts of climate change, on macroalgae, including OA, global warming, and sea-level rise, have implications for marine communities and food webs as a whole. Changes in the abundances of common calcifying macroalgae, *Halimeda, Padina, Dichotomaria*, and *Galaxaura*, caused by OA might cause changes in nearshore community function and structure in the Hawaiian Islands.
Figure 16. Detailed geospatial representation of research study sites using ArcMap 10.7.
Figure 17. a) Salinity experiment setup and b) CaCO₃ content analysis from Salinity experiment.


