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The effects of ocean acidification on fluorescence in Cnidarians: a potential non-invasive proxy of health

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by

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The Thesis of Jenny Tu is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

For their continued support, this thesis is dedicated to the following people:

Pei-Lin Tu

Su-John Tu

Ellen Tu

Clifford Swall

Dimitri Deheyn

Martin Tresguerres

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LIST OF ABBREVIATIONS

BME – beta-mercapto-ethanaol

BSA – bovine serum albumin

CA – carbonic anhydrase

CCM – carbon-concentrating mechanism

DIC – dissolved inorganic carbons

DMSO – dimethyl-sulfoxide

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

GFP – green fluorescent protein

HRP – horseradish peroxidase

IHC – immunohistochemistry

OA – ocean acidification

PB – phosphate buffer

PBS – phosphate buffer saline

pH_i – intracellular pH

PBS-TX – phosphate buffer saline with Triton-X

PhosSTOP – phosphatase inhibitor

PVDF – polyvinylidine fluoride

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOD – superoxide dismutase

TBST – tris-buffered saline with Tween-20

VHA – vacuolar proton ATPase

XO – xanthine oxidase

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Martin Tresguerres, Chair
Professor Kaustuv Roy, Co-Chair

Currently, the only way to identify the extent of coral stress is through visually observable decreases in coral coloration as a product of coral bleaching. However, by the time bleaching has been initiated, the coral may already be in such a deleterious state that it may be difficult for it to recover if or when the stress factors are removed. Having the ability to identify when the coral is in the early stages of stress would be beneficial in managing and, eventually, restoring coral reefs.

Fluorescent proteins make up roughly 20% of the total soluble proteins in corals but, despite the abundance of fluorescent proteins in corals, their physiological roles are still not well understood. However, they have previously been hypothesized to function as photo-protective ROS scavengers, photo-enhancers, and light-driven proton pumps.

This study aims to quantify changes in fluorescence in corals exposed to CO_2-

induced ocean acidification (OA) conditions over the course of 31 days with three realistic pH conditions under controlled laboratory settings. This study shows that the intensity of green fluorescence declined in the coral's chronic response to decreased pH (increased CO_2), while the color under bright field did not pale. Towards the end of the experiment, fluorescence in the corals under OA treatment showed no increase in fluorescence and did not reach the level of the control corals. With regards to their calcification, it was found that corals exposed to OA were able to grow just as well as the corals kept in control conditions for most of the experiment, suggesting that corals have mechanisms to cope with short-term OA. However, growth did decline after a one-month of exposure to OA. This study also found that SOD activity increased rapidly and consistently maintained a high level of scavenging in response to OA. On the other hand, exposure to OA was not correlated to the overall abundance of VHA, an enzyme proposed to be involved in carbon-concentrating for zooxanthellae photosynthesis. This may imply that corals are able to buffer the effects of OA for their zooxanthellae in order to maintain their symbiotic relationship.

Overall, this study highlights the effectiveness of green fluorescence in corals as an early proxy to stress levels and an indicator to potential long-term physiological effects.

INTRODUCTION

Human activity has many global impacts on the environment, which include but are not limited to rising seawater levels, increase seawater temperatures, and decreasing oceanic pH. The ocean is a carbon sink—meaning that increased anthropogenic CO_2 emissions in the atmosphere also lead to an increase in the CO_2 concentration in the ocean. As CO_2 dissolves into the ocean, it combines with water to form carbonic acid, which then readily dissociates into hydrogen ions and bicarbonate ions. This increase in hydrogen ions lowers pH; this process is known as ocean acidification (OA) and can be detrimental to calcifying organisms because the dissolved hydrogen ions can freely combine with carbonate ions in the seawater to form bicarbonate. The equation is as follows:

$$H^+ + CO_3^{2-} \rightleftarrows HCO_3^- \qquad \text{Eq. 1}$$

Le Chatelier's Principle states that if a chemical system at equilibrium experiences a change in concentration, then the equilibrium will shift to counteract the imposed change in order to re-establish a new equilibrium. Thus, increasing the concentration of hydrogen ions drives Eq. 1 to the right towards the formation of bicarbonate ions and the disappearance of carbonate ions that corals need to form aragonite, a stable form of calcium carbonate that is the main component in skeletons (Fig. 1). As the pH decreases, so does the aragonite saturation. However, the mechanisms for calcification and how carbonate is transported to the site of calcification are not known.

Average surface pH has already decreased approximately 0.1 pH units since the pre-industrial period. This corresponds to a 33% increase in hydrogen ion concentration due to the logarithmic nature of pH. It has been predicted by the Intergovernmental Panel

on Climate Change (IPCC) to decrease by another 0.4 pH units before the end of the century. This can be especially detrimental to calcareous organisms, such as corals. Research has shown that coral reef coverage has dramatically declined in recent years. Analysis of time series data on reef conditions have identified a loss of 50.7% of initial coverage over the past two decades due to human activity and climate change (De'ath, et al. 2012). Although coral reefs only cover 0.2% of the ocean floor, scientists have estimated that, of the 2.2 million predicted species in the ocean, approximately one million species of fish, invertebrates, and algae can be found in this complex habitat (Weier, et al. 2001). This ecosystem drives species diversity by allowing multiple niches to evolve.

Corals share a mutualistic relationship with their symbionts. The zooxanthellae photosynthesize and transfer glucose, glycerol, amino acids, and oxygen to the coral and, in return, the coral provides protection and the compounds necessary for photosynthesis. The coral uses the products provided by the zooxanthellae to produce proteins, fats, carbohydrates, and energy for skeleton formation (Levinton, 1995; Sumich, 1996; Barnes and Hughes, 1999). In fact, the corals may obtain as much as 95% of their energy from the zooxanthellae, who can transfer up to 90% of the organic material produced during photosynthesis to the coral (Muscatine, 1990; Georgii, 2009). These two organisms have developed a relationship in which both parties benefit from their association.

When the symbiotic relationship between the zooxanthellae and coral host is broken down, the zooxanthellae either leaves or is expelled from the host; this process is known as bleaching because the coral host loses its coloration in the process (the brown coloration of corals is mainly derived from their zooxanthellae content). Currently, the

only way to identify the extent of coral bleaching is by visually observable changes in coral coloration. This is problematic because this method is highly subjective and variable from one viewer to another and thus only a good assessment of coral health once bleaching is already well underway. However, by the time bleaching has been initiated, the coral may already be in such a deleterious state that it may be difficult for it to recover if conditions return to optimal. Because this method of evaluating organism health is fundamentally measuring death rates, it is not advantageous towards assisting coral reef recovery. Therefore, having the ability to identify when the coral is in the early stages of distress will prove to be very beneficial in managing and eventually restoring coral reefs.

There are currently no available methods to determine coral reef physiology except by destructive sampling. However, coral reefs have already been in such a dramatic decline that it would not be beneficial to damage and remove these animals from their environment. This process would quickly deplete the already-declining coral reef coverage. Thus, there is a great demand for an *in situ* non-invasive method to determine coral reef health remotely.

Light production has evolved independently approximately 40 different times, providing strong evidence that it is beneficial for survival and propagation of genetic lines (Rees, et al 1998). Fluorescent proteins make up a large portion, roughly 20%, of the total proteins in corals (Roth and Deheyn, 2013). Roth has shown that green fluorescent protein concentrations and, thus fluorescence, decrease with declining coral health, even prior to bleaching initiation. Healthy coral were brightly fluorescent but after the introduction of stressful environmental conditions—in Roth's study, temperature

shocks—fluorescence dropped sharply before either recovering back to normal conditions or surpassing basal levels as the zooxanthellae leave the coral and thereby decrease the shading of the GFP. Therefore, both healthy and severely bleached corals are brightly fluorescent whereas stressed corals show a much dimmer fluorescence pattern. Despite the abundance of fluorescent proteins in corals, their physiological roles are still not well understood. Because of these factors, fluorescence was chosen as a potential method of health assessment.

Green fluorescent proteins have many hypothesized functions that include photo-enhancement, photo-protection, and ROS scavenging. Photo-enhancement is the ability of GFP to concentrate light for use in photosynthesis by the zooxanthellae. Photo-protection is the ability of the coral to shield itself from UV radiation. ROS scavenging, on the other hand, is the ability of the protein to repair damages that would otherwise be a caused by UV radiation. (Roth et al, 2010)

Using spectral emission analysis of Caribbean coral tissue extracts, fluorescent protein concentrations and antioxidant capacity were identified to have a positive correlation (Palmer et al, 2009). In an independent study, spectrophotometric assays identified GFP as a mediator of superoxide radicals, similar to that of superoxide dismutase (SOD) (Bou-Abdallah et al, 2006).

In addition, GFP was identified via x-ray crystallography to possess the potential to function as a proton channel (Agmon, 2005). As the GFP accepts an electron, a proton is emitted in the excited state through a switchable exit pathway. This allows GFP to function as a light-driven proton pump. They also determined that GFP has similar structural motifs to that of carbonic anhydrase II (CA-II), further solidifying the idea that

GFP could be involved in maintaining intracellular pH (pH_i) (Shinobu and Agmon, 2009).

To study whether the additional task of calcification impairs the ability of organisms to survive under OA conditions, two model organisms were selected for this study. *Acropora* is one of the two genera of corals that contribute the most to reef formation because of their fast-growing ability. *Acropora spp* are most sensitive to changes in environmental conditions and thus was selected as one of the model organisms for this study. The *Anemonia majano,* like the *Acropora yongei,* is a symbiotic sea anemone that also lives in the tropics. Both of these organisms contain a green fluorescent protein with an emission spectrum that peaks at 519nm and zooxanthellae with a chlorophyll a emission spectrum around 684nm or 688nm. One key difference between the *A. majano* and the *A. yongei* used in the study is that the *A. yongei* is a calcareous species, while the *A. majano* is not.

The questions proposed in this thesis serve to link all the aforementioned functions of GFP together by exploring the following topics: the relationship of GFP to ROS scavenging activity, carbon-concentrating mechanisms, and the task of calcification in growth under OA. Do OA conditions induce the same change in fluorescence as do other environmental stressors? What are the physiological changes correlated with OA? The final purpose of this thesis was to evaluate whether fluorescence can be used as a non-invasive proxy to health levels in cnidarians in response to ocean acidification.

MATERIALS AND METHODS

This experimentation was conducted two independent times and, each time, the same preparation of samples was followed. The difference between the two rounds of experimentation was mainly in relation to the types of analysis applied to the samples once sacrificed. The numerical one (1) and two (2) are indicated at the end of each heading to specify from which round of experimentation the samples (and data to be presented) were acquired.

Acquiring and Rearing Biological Materials:

Both *A. yongei* and *A. majano* were obtained from the Birch Aquarium in La Jolla, CA. An *A. yongei* colony was obtained by Birch Aquarium from a wholesaler in 1995 and then later split into several fragments. Forty of those fragments were allocated to SIO in April of 2008 and have been maintained in the experimental aquarium since. These corals have been grown over time under four Tek 44 lights (Sunlight Supply Inc). In each lighting unit, two 54 watt T-5 Powerchrome Midday bulbs and two 54 watt T-5 Powerchrome Aquablue+ bulbs were used. The Powerchrome Aquablue+ lights are specifically designed for reef aquariums, mimicking sunlight in seawater depth from one to twenty meters. The lighting units are connected to an automatic timer that is set on a 12-hour day:night cycle. The flow-through system uses seawater that originates off the coast of La Jolla, CA, which is pumped up and flows down the length of the Scripps Pier, where it then passed through a filtration system and warmed to approximately 25-27˚C and distributed to various experimental aquaria.

A. majano were obtained a week prior to experimentation from Birch Aquarium and were maintained in the same conditions and on the same schedules as *A. yongei*.

S*electing Individuals:*

A. yongei fragments used in the experiment all came from the same colony, and therefore they are genetically identical to one another. Corals that were 3 cm or more and possessed an apical tip were clipped using bone cutters and glued down to 2x2 inch ceramic tiles with gel super glue.

Sea anemones <2 cm across were carefully plucked from the rocks in which they originally resided using a metal spatula. The sea anemones were then transferred to hand made cages prior to experimentation. The cages were made of six well plates that were poked 15 times on each side in order to allow water flow through.

Building the pH Stat System:

Building and optimizing the system took 8 months. During this time, water flow rates and CO_2-bubbling rates were optimized so that deviation from the designated pH were minute and oscillation times were short, thereby ensuring the organism does not spend long periods of time in the upper (highly basic conditions) or lower (highly acidic conditions) range.

Warm water arrives into the aquarium via a PVC pipe with three valves, each of them discharging into sump tanks. Each sump tank is then maintained at a different pH using a pH stat system; there was a control tank of approximately pH 8.05 and two experimental tanks where the pH is acidified to 7.6 and 7.9 with the addition of CO_2. The

water from each of these tanks would then flow out by way of gravity into PVC pipes and into the different organism tanks at a rate of 500mL/min. There were six organism tanks—three coral tanks and three sea anemone tanks—one tank for each pH condition (Fig. 2).

The tanks were aligned into two rows, one for each species. Each row of tanks had their own light source but both were controlled by the same timer, set on a 12-hour day:night cycle. This is to ensure that should any inconsistencies occur, drifting clocks/timers for example, this would occur in both systems, allowing direct comparisons to be made regarding their nutrient sources.

The IKS-Aquastar is a pH stat monitoring system that I used to maintain pH in these experiments. One pH electrode was located at the out flow opening for each sump tank and a temperature electrode monitored the temperature of the control tank. Each electrode was set to monitor the pH and adjust it accordingly. The desired experimental pHs were programmed into the IKS-Aquastar, which would control CO_2 bubbling in the sump tanks. When the pH in the sump tank hit the upper limit, a solenoid valve would click open, thus bubbling CO_2 into the sump tank and lowering the pH. On the other hand, when the pH hit the lower limit, the solenoid valve would close and cease bubbling. It does take some time after the solenoid valve closes for the system to equilibrate so the pH does drop a bit (approximately 0.02 pH units) below the designated average.

The system was calibrated every Monday, Wednesday, and Friday to ensure that the pH electrodes were working properly. The electrodes were calibrated using a pH 4.0 and pH 7.0 buffer (that had previously been sitting in a water bath to acclimate it to the same temperature as experimental waters).

The pH of the sump tanks were measured continuously and were recorded every two minutes by the IKS-Aquastar system. The information was stored in a database until downloading and exporting to excel, which was done at the time of calibration.

The system was run for approximately 3 days before the start of the experiment and the organisms were exposed to their respective conditions. At the conclusion of the experiment, the system was left on for an additional 3 days to show that the system took consistent measurements throughout the duration of the experiment.

Water Chemistry:

Due to the logarithmic scale of pH, a small change in pH can lead to a very drastic change in hydrogen ion concentrations. Thus it is especially important to confirm that the pH stat system used during the course of this experiment was measuring and regulating the seawater pH both accurately and precisely. The electrodes used in the IKS-Aquastar system are calibrated every other day using standard buffers of pH 4.0 and 7.0 that are warmed to the correct temperature using a water bath. Glass electrodes are generally known to be precise but not always accurate; thus is it particularly challenging to determine the accuracy of the system without water chemistry to confirm the pH.

To validate the electrode pH measurements, discrete water samples were collected for chemical analysis. During this process, sterile 500mL bottles were dunked inside the sump tank and then two drops of mercuric chloride were added to kill any bacteria that were present in the water, in order to prevent them from respiring and changing the carbonate chemistry. The bottles were then sealed using a stopper coated in grease and then the whole bottle was rubber-band shut and stored in a dark, dry environment until

the time of measurement, following the instructions of the testing laboratory (Dickson lab at SIO). The water samples were then analyzed for DIC, alkalinity, and salinity. These values were then used to calculate pH using CO_2CalcNet with the following settings: Lueker et al, 2000 for CO_2 constants, Dickson, 1990 for $KHSO_4$, Lee et al, 2010 for total boron, and NBS scale (mol/kg-H_2O) for pH scale. During the first round of experimentation, water samples were collected at the end of the day cycle and at the end of the night cycle from all three sump tanks, coral tanks, and anemone tanks. During the second round of experimentation, water samples were only collected from the sump tank during each day a set of organisms were removed from the experimental conditions and sacrificed. This data was used to ensure that no abnormalities occurred that would induce an acute shock and thus alter the physiology of the organisms at the time of sampling.

Sample Imaging (2):

A total of 114 corals and 114 sea anemones were imaged using an epifluorescence microscope (Nikon SMZ 1500 equipped with a Retiga 2000R digital camera) and a hyperspectral PARISS® imager (Lightform Inc.) prior to and after exposure to acidification or control conditions. Six fixed exposures in bright field and six fixed exposures in fluorescence were taken in addition to the two auto-exposures for each individual organism on the stereoscope. In addition, a green balance, a white balance, and a fluorescent balance were used to identify any shifts in the camera's sensitivity.

Hyperspectral measurements were taken both in the middle and in the tip of the *A. yongei* fragments due to the uniqueness of the growing, apical tip compared to the base of the coral. Fluorescence in the tip fluctuated greatly and sometimes did not represent the

intensity of the coenosarc in the middle of the coral accurately; thus it was important for two separate measurements to be taken. In *A. majano,* the hyperspectral measurements were taken from the tentacles. The unique spectrum from each individual's hyperspectral acquisition was then identified and processed in order to determine the intensity at two given wavelengths. These wavelengths were selected based on the peaks with the highest intensity within the spectrums. These values obtained were then normalized for exposure and then against the initial measurement for that individual animal to determine any changes in fluorescence intensity.

The corals and sea anemones were then exposed to control or OA conditions. On days 0, 3, 5, 7, 13, 21, and 31, three corals and three sea anemones from each condition were reimaged and then sacrificed. Samples were flash frozen with liquid nitrogen and stored at -80˚C during the first round of experimentation. During the second round, the samples were fixed in paraformaldehyde, decalcified if necessary, and then stored in ethanol.

Sample Homogenization (1):

For western blot analysis and ROS quantification, coral samples were removed from -80˚C, weighed using a balance, and the tissue was immediately airbrushed off the skeleton using phosphate buffer at pH 7.0 with Sigma P1860 protease inhibitor and PhosSTOP (Roche). The coral skeleton was weighed again and the amount of coral tissue removed was determined by subtracting initial and final weights. The mucus produced by the coral was broken down by using a 5 mL syringe with a 21G1 gage needle. Homogenate was stored as 500 µL aliquots. 1 mM EDTA, 70 mM sucrose, and 210 mM

mannitol was added to one of the fractions to stabilize the SOD enzyme. In another fraction, 1 mM of EDTA was added to prevent the degradation of catalase. The other homogenates receive no additional add-ons. The homogenates were stored frozen at -80˚C, processing of coral sample took approximately 20 minutes.

Western Blot Analysis of Samples (1):

When all the coral fragments had been homogenized, the homogenates were thawed and then a Bradford Assay was performed on the crude homogenate to determine the protein concentration to ensure loading of equal protein concentrations into the gels. 2µg of protein was pipetted into a fresh Eppendorf tube, where it was combined with equal volumes of 95% laemmli buffer and 5% β mercapto-ethanol. Samples were heated at 70˚C for 20 minutes. Afterwards, the homogenates were centrifuged and run through a 10% SDS-PAGE gel in order to separate the proteins by size. The gel was run at 60 V for approximately 20 minutes until the front reached the separating gel, and then at 200 V until the front ran off the gel, approximately 30-45 minutes later. Proteins were transferred from the gel into a PVDF membrane using a semi-wet Biorad transfer system at 25 V and 1.0 amps for 30 minutes. PVDF membranes were incubated in 10mL of 5% non-fat powdered milk in TBS-T for one hour on a rocker for blocking. At that time, the solution was replaced with 10mL of a 1:2,500 VHA subunit B rabbit primary antibody in blocking buffer solution and the whole cassette was transferred to a rocker at 4˚C for overnight incubation. PVDF membranes were rinsed with three sequential washes of 10mL of TBS-T for 10 minutes each on a rocker at room temperature to remove the remaining antibodies not bound to the membrane. Goat anti-rabbit chemiluminescent

secondary antibody bound to with horseradish peroxidase (HRP) was then added to the membrane (1:10,000 in TBS-T) and incubated for an hour at room temperature on a rocker. Afterwards, membranes were, again, washed three times with 10mL of TBS-T for 10 minutes each. Membranes were then placed inside a Biorad imager, 1.5mL of ECL development solution was added, and chemiluminescent signal quantified.

Immunohistochemistry (2):

After the coral fragments and sea anemones were imaged using the epifluorescence microscope and the hyperspectral imager, they were fixed in 3% paraformaldehyde in S22 buffer overnight. Anemones were placed in 50% ethanol on a rocker at 4˚C for 5 hours and then transferred to 70% ethanol for storage at 4˚C until further processing. The corals fragments, however, had to be decalcified first by incubation in 5mL of 0.5M EDTA in Calcium-free S22 buffer on a rocker in 4°C. The solution was changed daily until the skeleton dissolved. When the skeleton had fully disintegrated, coral fragments underwent the same dehydration process as the sea anemones—incubated in 50% ethanol for 5 hours and then stored in 70% ethanol in 4˚C.

Once all the samples were collected, the samples were fully dehydrated using a sequentially increasing concentration of ethanol (95% then 100%), followed by xylene washes. The samples were then placed in a cassette, where they were washed with paraffin in a warm bath to keep the paraffin from solidifying. Finally, samples were transferred to a different cassette, embedded by filling with paraffin and allowed to harden for two days.

Paraffin-embedded samples were then sliced into 5 micron sections using a microtome (KEDEE 1508B). Two consecutive sections were then placed two to each slide, then floated on a small volume of water and heated at 40˚C for one minute on a hotplate to allow the paraffin sections to flatten out. The hotplate was turned down to 30˚C to adhere the paraffin sections to the slides overnight.

Paraffin was removed from the sections and were rehydrated by reversing the dehydration process. Sections were then blocked using 80µL of a 0.2% PBS-TritonX, normal goat serum, and hemocyanin solution mixture for one hour at room temperature. One of the two sections was stained with a VHA subunit B rabbit primary antibody while the other remained incubated in the blocking buffer. These slides were allowed to incubate overnight at 4˚C. The following day, both sections were stained with a goat anti-rabbit secondary antibody of 555nm excitation wavelength and a Hoerscht stain (1:10,000) to label to nuclei. Slides were then imaged using a Zeiss inverted microscope and a PARISS® hyperspectral imager using a 470 nm and 535 nm filter excitation long-pass filter.

ROS Quantification (1):

Frozen homogenates were thawed and tested for superoxide dismutase abundance using the Arbor Assay Superoxide Dismutase (SOD) Colorimetric Activity Kit. 50 µL of the substrate preparation was first added to all the wells using a multi-channel pipette. Then 10 µL of either the BSA standard or the coral homogenate was added to each well, followed by 25 µL of axanthine oxidase preparation; the plate was then incubated at room temperature for 20 minutes. During the incubation time, the plate is spun at 3221g

for 3 minutes to remove any bubbles. The absorbance of the plate was read using a Spectromax I3 (Molecular Devices) at 450 nm.

Statistics:

Analysis of the hyperspectral acquisitions were processed using the PARISS® program, in which the spectrum at a specified strip number was then exported to Excel. Using a self-generated script on Bash, the name, exposure, and intensities at defined wavelengths were then extracted and transformed into a separate Excel file where further calculations took place.

ImageJ was used for linear elongation measurements. A scale bar was imaged and measured to identify the amount of pixels per unit. For each individual organism, a line was drawn from the tip of the coral towards a unique landmark on the coral on both the initial day as well as on sacrificing day. The length of the line on the initial day of experimentation was subtracted from the length of the line during the sacrificing day in order to determine how much the coral had grown.

ImageJ was also used for western blot quantification. First, each lane of the blot was identified and then a 0.25x0.25 box was drawn around each of the bands. The program then provided a lane plot, which resembles a bell curve and shows the intensity of the band. The bell curve was then closed off using the program and the area under the curve identified. The area under the curve for each sample was then normalized against a day 0 sample to determine changes in VHA abundance.

All empirical data were processed using Excel (Microsoft Corp.). Statistical analysis was performed by applying a two-way ANOVA using StatView.

RESULTS

Two rounds of experiments were successfully completed with no major technical issues, thus providing confidence that the data acquired were comparable and complementary between rounds.

Water chemistry:

During the first round of experimentation, samples for water chemistry were collected from all three sump tanks, the three anemone tanks, and the three coral tanks at the end of the night cycle and at the end of the day cycle (Fig. 2). The water samples were then analyzed for DIC, alkalinity, and salinity. These results were then used to back-calculate the pH of the seawater and check the accuracy of the pH stat system.

The water chemistry data showed a slight variation from the readings taken by the IKS-Aquastar system but the differences were insignificant (Table 1). Both readings are slightly below the designated average but their standard deviations, at 0.0075 and 0.0061 were almost negligible. The average pH in the 7.9 condition was 0.042 pH units below the set pH and the pH 7.6 condition was 0.023 units too low. Considering the difficulties associated with measuring pH, this is considered relatively accurate (Dickson, 2011).

As expected, the pH of the tanks holding the organisms were lower at the end of the night cycle compared to the end of the day cycle. This is because during the day cycle, photosynthesis occurs, which then uses up the available CO_2 from the water thereby raising the pH of the seawater. On the other hand, during the night cycle, respiration from the animals results in an increase in CO_2, which then drives the pH down.

pH was relatively stable during the course of the experiment (Table 2). Again, pHs calculated by the CO_2CalcNet method were slightly below the set average; the average pH 7.9 condition was roughly 0.0375 under the designated pH and the pH 7.6 condition was 0.0155 lower than anticipated. Again, this is considered relatively accurate (Dickson, 2011).

Unfortunately, water samples were not collected from the system during day 7 but a water sample collected off of Scripps Pier six days after sampling showed an increased pH of 8.19 due to a red tide that moved to shore (Susan Kram, SIO, personal communication). Due to the dynamic ability of the pH stat system to maintain seawater pH within their set range, this increased pH should not have altered the pH of the acidification conditions (only the control tank) and thus the animals exposed to OA conditions would not have felt this increase in pH.

Fluorescence:

In the month-long acclimation studies, corals and sea anemones were exposed to three different pH conditions—a control pH which hovers around 8.07, and two acidification conditions of pH 7.9 and 7.6. During the course of this study, the individual organisms were imaged using both a stereoscope and a hyperspectral imager. With the stereoscope, images were taken at fixed exposures to ensure a direct comparisons could be made between the initial fluorescence and the fluorescence at sacrificing day as well as at auto-exposure settings for identification of physical and anatomical changes (Fig. 3).

It was discovered that endogenous green fluorescence intensity varied greatly in

response to ocean acidification in corals (Fig. 9). The control corals showed a continuous increase in fluorescence, while the corals exposed to OA conditions showed no overall increase in fluorescence over time. Differences between pH conditions were statistically significant ($p=0.01$), as was the factor of time ($p=0.001$) and the combination of pH and time ($p=0.05$). The change in fluorescence between the two OA conditions were not significantly different from one another ($p=0.66$) but were both significantly different from the control condition (pH 7.6: $p=0.01$ and pH 7.9: $p=0.005$). Fisher's post hoc test concluded that no significant changes were detected between the control and acidification condition corals in the acute phase, however, the acidified corals showed a drastic decrease in green fluorescence in the chronic response. The tip of the coral was much more variable, which could be an artifact of ever-changing growth rates in the tip, and was not found to be statistically significant between pH or across time.

Red fluorescence intensity did not change between pH conditions (Fig. 10). While it does appear that the corals exposed to OA have slightly more zooxanthellae in the middle of the coral than those in the control condition in the chronic phase, the overall change was not found to be significant across pH conditions ($p=0.1645$). In fact, it was shown that time accounts for the variance shown ($p=0.01$). Fisher's post hoc test identified statistically significant differences between the red fluorescence intensity at day 0 and day 21 ($p=0.003$) but was not found to be significant between day 0 and day 31 ($p=0.40$). Again, the corals showed high variability in the red fluorescence intensity of the tip and this was not found to be statistically significant (pH: $p=0.66$; time: $p=<0.0001$). The terminal polyps in *Acropora* do not possess zooxanthellae so the

specific location at which the hyperspectral acquisition was taken greatly influences the abundance of zooxanthellae and thus red fluorescence intensity. For the purposes of this study, the top 1 cm of the coral was always considered as the tip of the coral; while measurements were taken as close as possible to the apical tip, due to focal length restrictions, acquisitions were sometimes taken further down the coral tip. Similar to the green fluorescence, significant changes could be identified both in the middle and tip of the coral between the acute and chronic treatment but not within each category.

Lastly, reflectance did not show any difference between pH conditions (Fig. 11). The factor of pH was not found to significantly contribute to the variance ($p=0.12$) in the middle of the coral; most of the variance was due to the factor of time ($p=<0.0001$). This is unsurprising as no observable bleaching was detecting during the course of the experiment. In the tip of the coral, however, variations in reflectance were found to be significant across both pH ($p=0.03$) and time ($p=0.0005$). However, as stated earlier, the reflectance would greatly depend on the brown coloration provided by the coral's zooxanthellae content and thus would also vary with the location of hyperspectral acquisition.

In the *A. majano*, no differences in green fluorescence were detected in neither the acute nor the chronic phase (Fig. 12). The green fluorescence was not found to be significant across pH ($p=0.59$) but, instead, time accounted for the variability seen within their response ($p=0.04$).

Unexpectedly, control anemones showed a sharp increase and decreased in red fluorescence, and thus zooxanthellae concentration, in the acute response potentially

implying they had a hard time acclimating to the system (Fig. 13). However, the control anemones were able to maintain and even increase their zooxanthellae content in the chronic response. This did not hold true for the anemones under OA stress, who experienced an overall decrease in zooxanthellae abundance. Change in red fluorescence intensity was found to be significant across both pH ($p=0.0002$) and time ($p=0.04$). The combination of the two showed a strong tendency towards correlation with a p value of 0.0583 but was likely not significant due to the small sample numbers ($n=3$ at each time point for each condition). Similar to the corals, these changes in the anemone were found to be significant between acute and chronic reactions but not within each category.

Lastly, no change was detected between pH conditions in the reflectance of the sea anemone ($p=0.72$) (Fig. 14). The variability seen within this data set can be accounted for by time, which showed a significant impact with a p value of 0.0002. These changes in reflectance were also only significant between acute and chronic reactions but not within each group. The day 14 acted as a dividing point between the two categories and any pairing involving day 14 samples yielded statistically significant p values, as identified by Fischer's post hoc test.

In an analysis of green and red fluorescence, it was determined that green fluorescence and red fluorescence had an inverse relationship—as the green fluorescence increased, the red fluorescence decreased (Fig. 15). It is important to note that while a trend is apparent in this graph, it has yet to be normalized for the quantum efficiency number of the camera; that is to say, that changes in the sensitivity of the camera have yet to be taken into account. However, normalization for the QEN number can only intensify

the correlation rather than diminish it.

Measurement of the growth tip via elongation analysis using ImageJ on the auto-exposure images taken on the stereoscope indicate that all three pH conditions experienced similar growth rates, with R^2 values of 0.93, 0.95, and 0.98 for pH 7.6, 7.9, and 8.1, respectively, until the last day. The final day of experimentation showed decreased growth in the acidified corals, while the control coral maintained normal growth functions (Fig. 16).

ROS Quantification:

The resting physiological state of organisms results from a fine balance between antioxidants and oxidants. When this balance is disrupted, reactive oxygen species are often generated; this especially occurs during times that are highly stressful for the organism. The mercurial nature of these ROS causes it to become potentially damaging to nuclei acids, proteins, and lipids. Antioxidants are compounds that are able to mediate these volatile electrons and organisms will attempt to generate increased amounts in order to limit the damaging effects of ROS. One of the most prominent antioxidants produced as a first-defense response against ROS is superoxide dismutase (SOD). This antioxidant is very good at scavenging O_2^- and thus is able to mediate the damaging effects of ROS.

Due to the inherent difficulties with measuring ROS directly, SOD enzyme activity was measured in its place. Corals exposed to OA conditions showed higher SOD enzyme activity, and thus higher ROS scavenging capability, than the control corals (Fig. 17). The control corals maintained low levels of SOD enzyme activity throughout most of

the entire experiment, while the acidified corals did not. The coral in OA conditions showed an increase in scavenging capacity through day 7. After this time, the SOD and SOD-like enzyme activity decreases but still showing a much more pronounced activity than that of the control corals.

Carbon-concentrating Mechanism:

Because *A. yongei* is not a species that acquires its zooxanthellae from their parents, known as vertical transmission, they must uptake their symbionts from the surrounding environment. This process is known as horizontal transmission and is beneficial to the coral because the *Symbiodinium* in the surrounding waters are already adapted to those local conditions; the clades that are not suitable for that environment are present at lower concentrations in the seawater. The corals uptake the zooxanthellae through their mouth, where it is then sorted and endocytosed by corals cells in the coelenteron (Weis et al, 2001). The symbiosome membrane surrounds the zooxanthellae inside the coral host cells. However, a problem then arises when the coral and its symbionts need to transfer nutrients to one another.

Fortunately, the coral host and their symbionts have evolved a method to concentrate carbons for photosynthesis. Unpublished research at the Tresguerres Lab at the Scripps Institution of Oceanography has found that the enzyme vacuolar proton ATPase (VHA) is present in the symbiosome membrane where it pumps hydrogen ions into the symbiosome (Fig. 4). The hydrogen ions then combine with bicarbonate inside the symbiosome and, with the help of the enzyme carbonic anhydrase, the two ions are

converted into carbon dioxide and water. The carbon dioxide can then diffuse through the zooxanthellae membrane for use in photosynthesis.

In figures 5 and 6, the VHA in the symbiosome was immunolabeled. The primary antibody recognizes an epitope in the B subunit of VHA that is 100% conserved across organisms. The secondary antibody has an emission spectrum of 555nm, which appears red in color. In the figures, the green shown is from the endogenous green fluorescence of the coral and the blue labels the nuclei. The red signal is a combination of the endogenous red fluorescence from the chlorophyll of the zooxanthellae and the secondary antibody staining; the dense, bright red that appears in the image is due to the staining from the antibodies. The control corals show very strong staining in the symbiosome of (Fig. 5) compared to the negative control. The staining is not as strong in the 7.6 samples (Fig. 6).

Because immunohistochemistry is primarily used for localization purposes and is an inadequate method to quantify concentration, western blots were also performed on the coral homogenates in order to determine VHA abundance. The same concentration of crude homogenate was loaded into each well of the six western blots, which were performed on the same day in order to ensure continuity of the control homogenates. The same three day 0 coral homogenates were loaded across all six blots so that they could be used to normalize the subsequent samples for quantification purposes. All the homogenates were kept on ice in order to prevent protein degradation and repeated freeze/thaw cycles. The gels were offset in groups of two (one acute and one chronic gel) into three sets (Fig. 7). The blots were incubated in the primary antibody for the same

amount of time (20 hours), to ensure continuity between blots for comparison purposes, before secondary antibody incubation and imaging. Western blots analysis showed no statistically significant change in overall VHA abundance between pH conditions (pH: p=84; time: p=0.08).

FIGURES

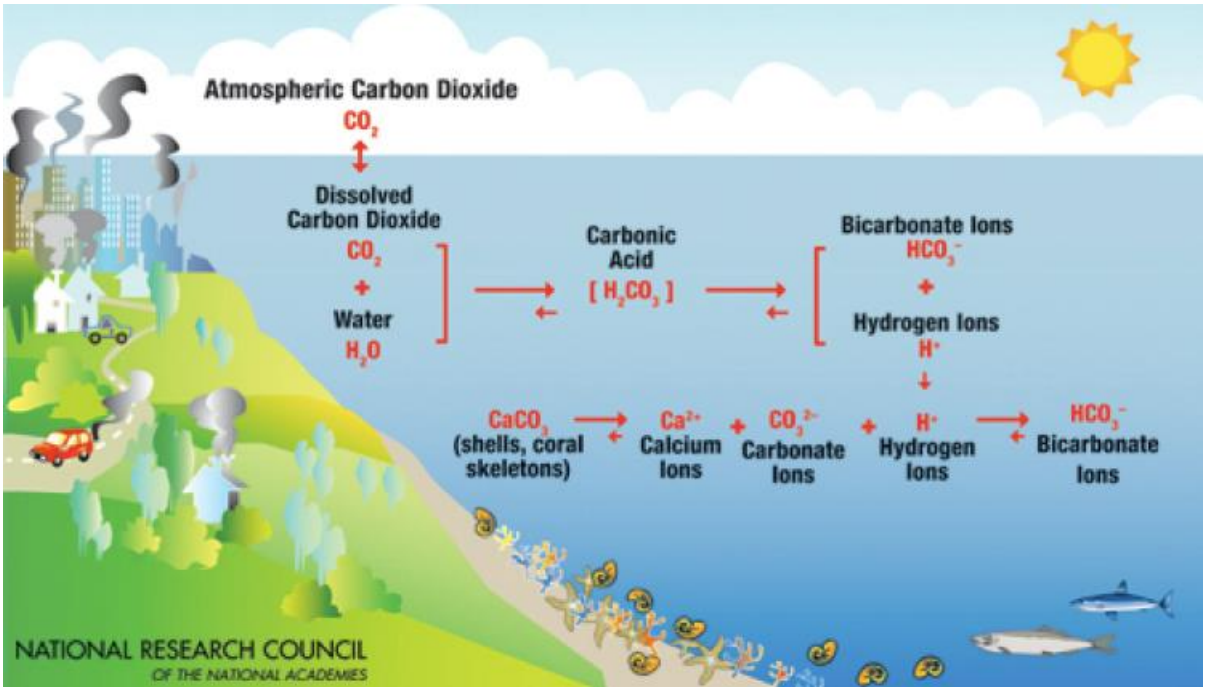


FIGURE 1: CARBONATE CHEMISTRY

This figure shows the flow of carbon dioxide from anthropogenic CO_2 emission into the ocean and the subsequent decrease in pH and aragonite saturation that follows. This decrease in the available carbonate for aragonite formation may be detrimental to calcareous organisms, such as corals, as aragonite is the main component in bones and skeletons.

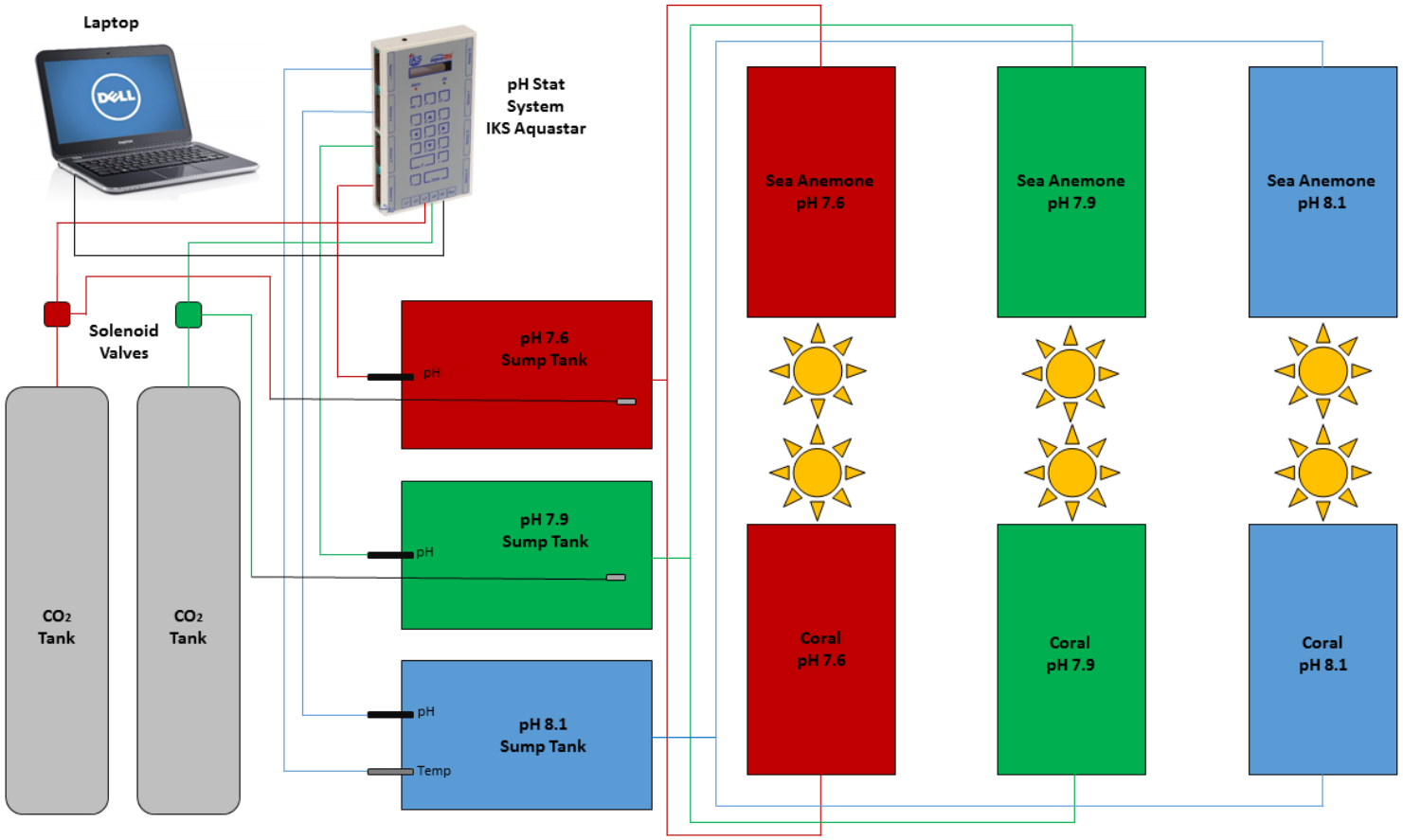
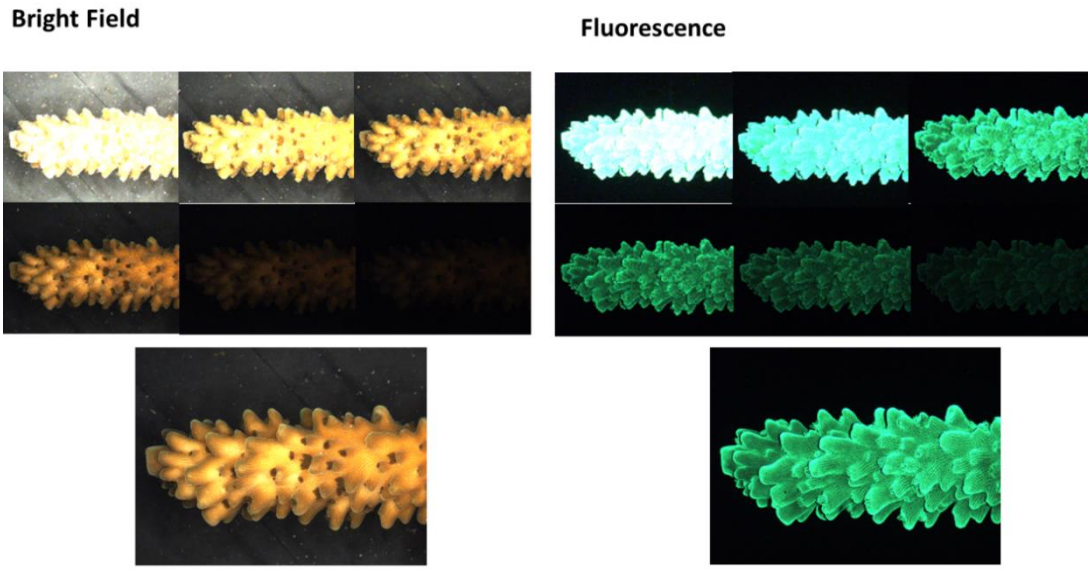


FIGURE 2: EXPERIMENTAL AQUARIA SET UP TO SIMULATE OA

Illustrated here is a schematic diagram of the experimental aquaria set up built to simulate OA for the purpose of this study. The IKS-Aquastar pH stat system used is connected to a laptop that continually monitors and records the data measured by the pH and temperature electrodes. The pH stat system controls the opening and closing of the solenoid valves based on readings made by the pH electrodes. The solenoid valves then regulate the bubbling of CO_2 into the sump tanks. The seawater then flows from the sump tanks into the tanks that house the organisms. The suns in the diagram represent the light source that is set on a 12:12 day:night cycle.

A.



B.

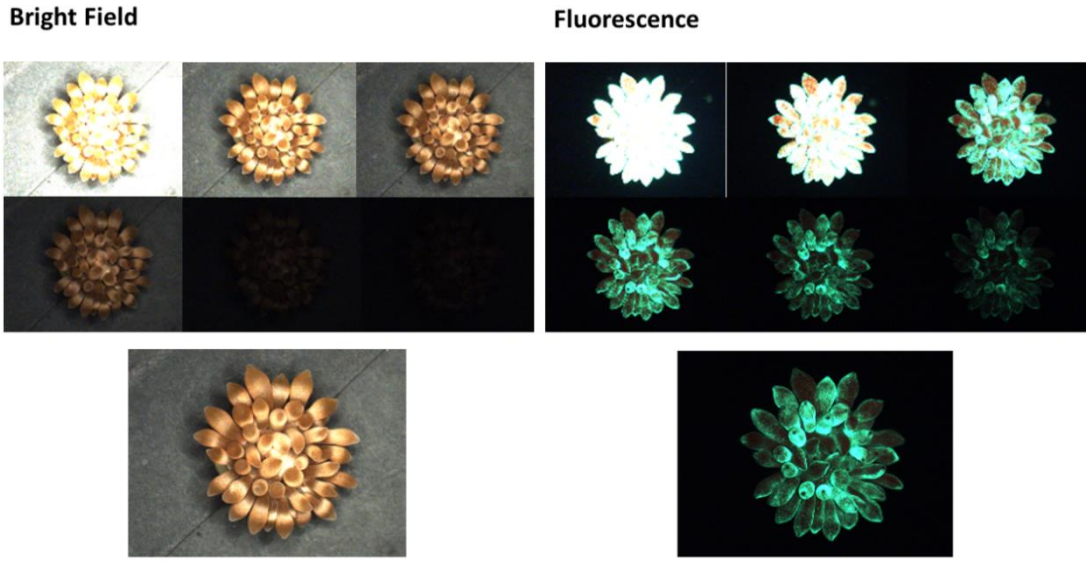


FIGURE 3: STEREOSCOPE IMAGES OF CORALS AND SEA ANEMONES
In the example images shown above, six fixed exposures in bright field and six fixed exposures in fluorescence, as well as two auto-exposures—one in bright field and one in fluorescence—are taken at the start of acclimation and at the time of removal. The six fixed bright field exposures (50ms, 25ms, 10ms, 5ms, 1ms, 0.5ms) and the six fixed fluorescence exposures (5000ms, 2500ms, 1000ms, 500ms, 250ms, and 100ms) are used for direct comparisons between the start and end sampling dates. By comparing the same exposures, the change in intensity can be visually observed. The two auto-exposure images are used to visualize any physical and anatomical changes that occur—such as the growth of a polyp or a change in zooxanthellae density (bleaching).

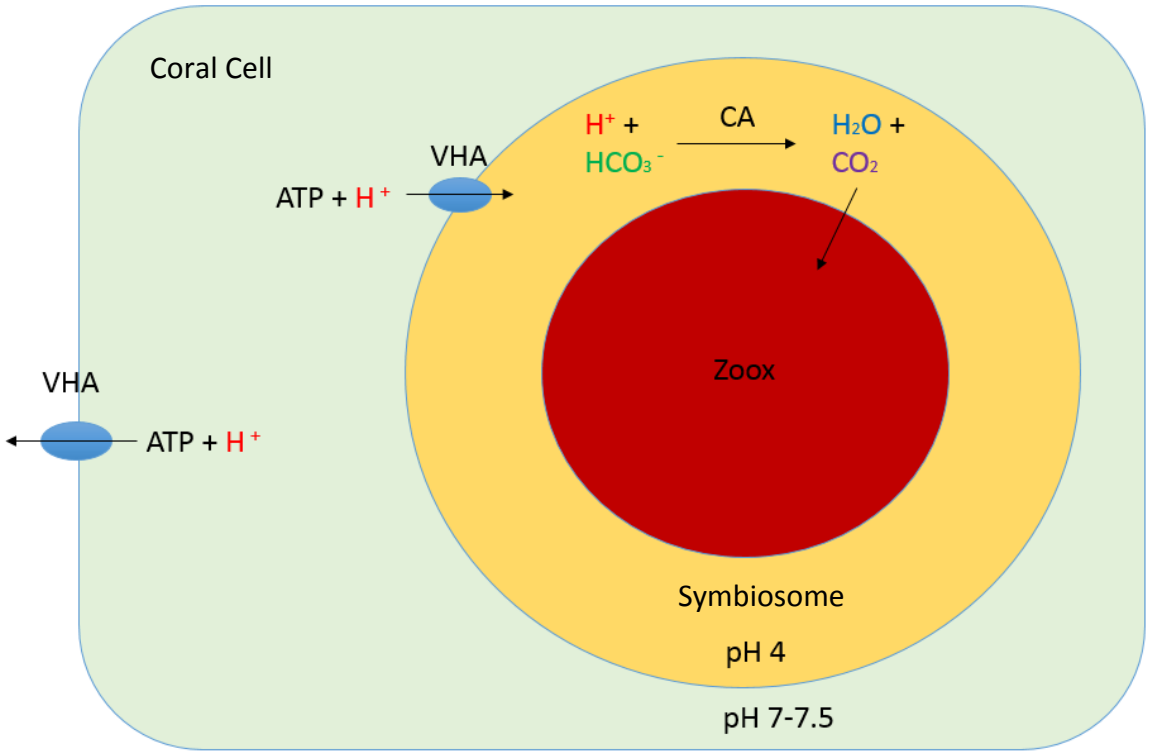
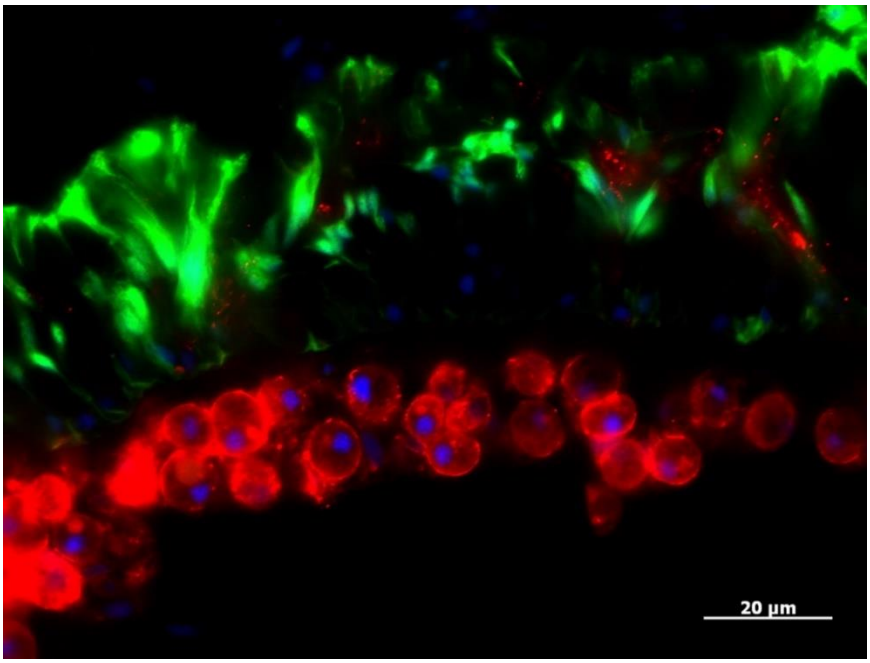


FIGURE 4: TRANSLOCATION OF PROTONS INTO THE SYMBIOSOME VIA VHA
Figure 4 shows the hypothesized movement of hydrogen ions from the cytoplasm of the coral cell into the symbiosome, where it can combine with bicarbonate to form water and carbon dioxide with the help of the enzyme carbonic anhydrase. The carbon dioxide can then diffuse through the zooxanthellae membrane and be used for photosynthesis. The symbiosome is considerably more acidic than the pH of the cytoplasmic space and this has been demonstrated in the literature by Venn, et al, 2009.

A.



B.

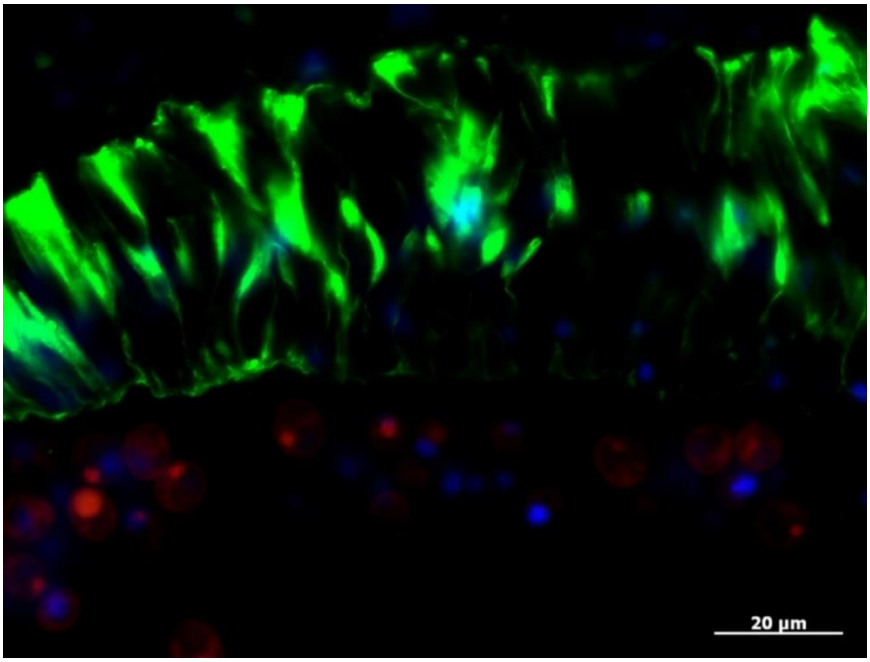
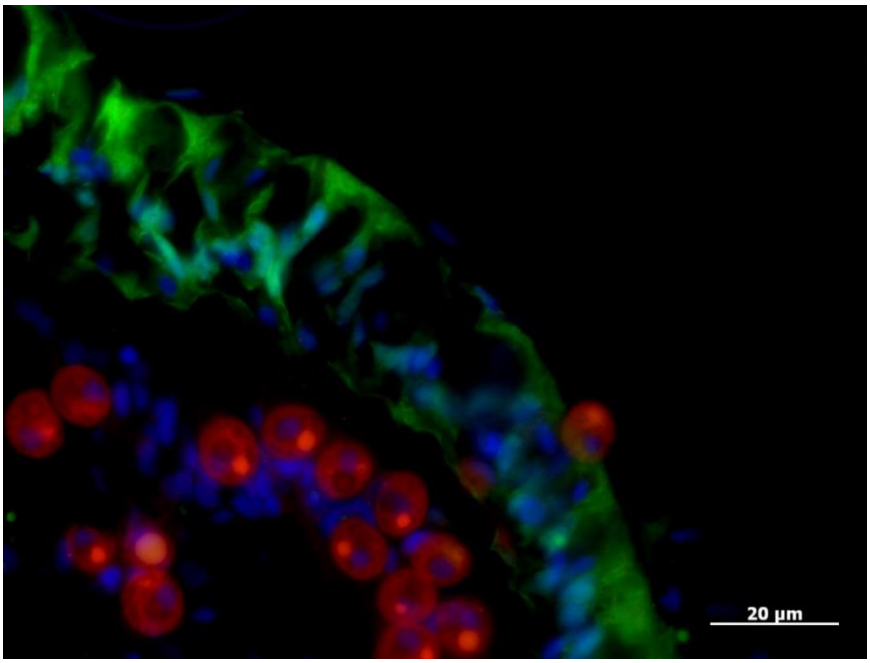


FIGURE 5: IMMUNOHISTOCHEMISTRY OF pH 8.1 *A. YONGEI* AT 40X OBJ. Figure 5A depicts the presence of VHA in the symbiosome membrane of control corals by means of fluorescent labeling (binding of VHA subunit B epitope) as compared to the negative control (without primary antibody staining) in 5B. The green that appears above is the endogenous green fluorescence of the coral and the blue staining labels nuclei. The red shown above is a combination of both the endogenous red fluorescence of the zooxanthellae as well as the emission of the secondary antibody; the bright red staining of the symbiosome is a product of immunolabeling. All three channels in the two images shown above were taking at the same exposure. This image is taken with a 40x objective.

A.



B.

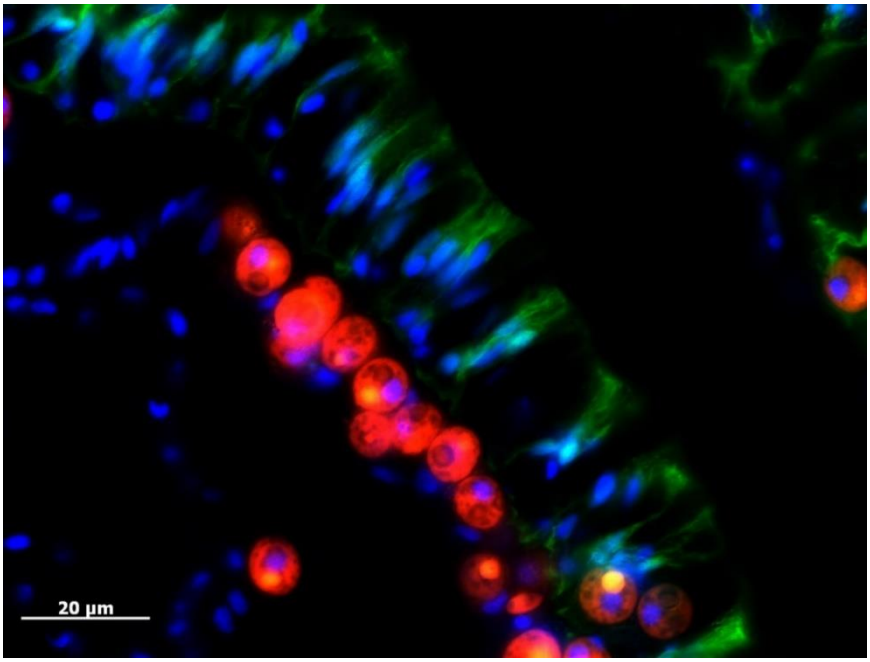
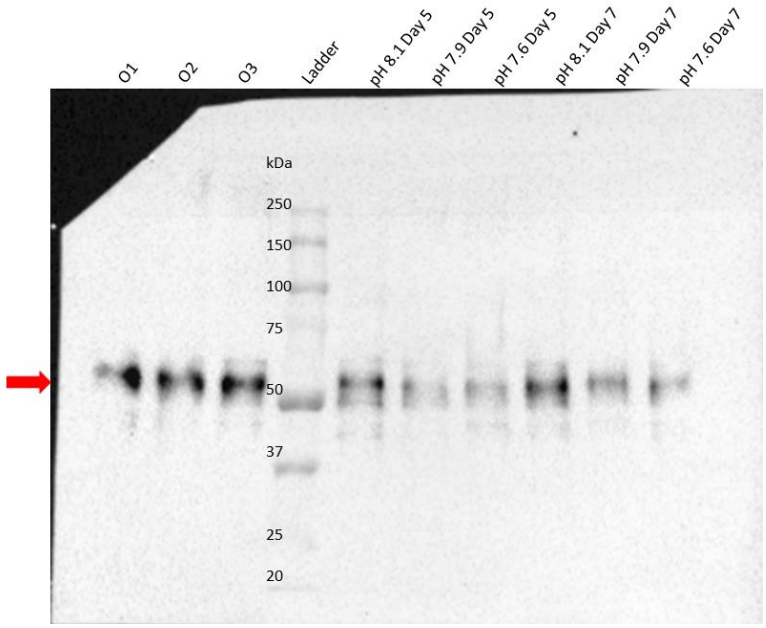


FIGURE 6: IMMUNOHISTOCHEMISTRY OF pH 7.6 *A. YONGEI* AT 40X OBJ. Figure 6 shows no difference in fluorescence labeling of the symbiosome membrane between the sample (5A) and negative control (5B) given the same exposure time. This image was captured using a 40x magnification objective.

A.



B.

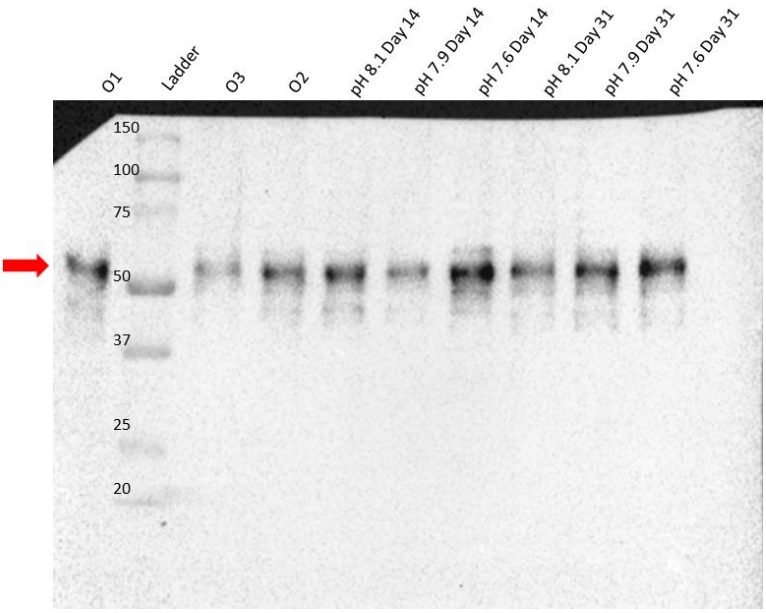
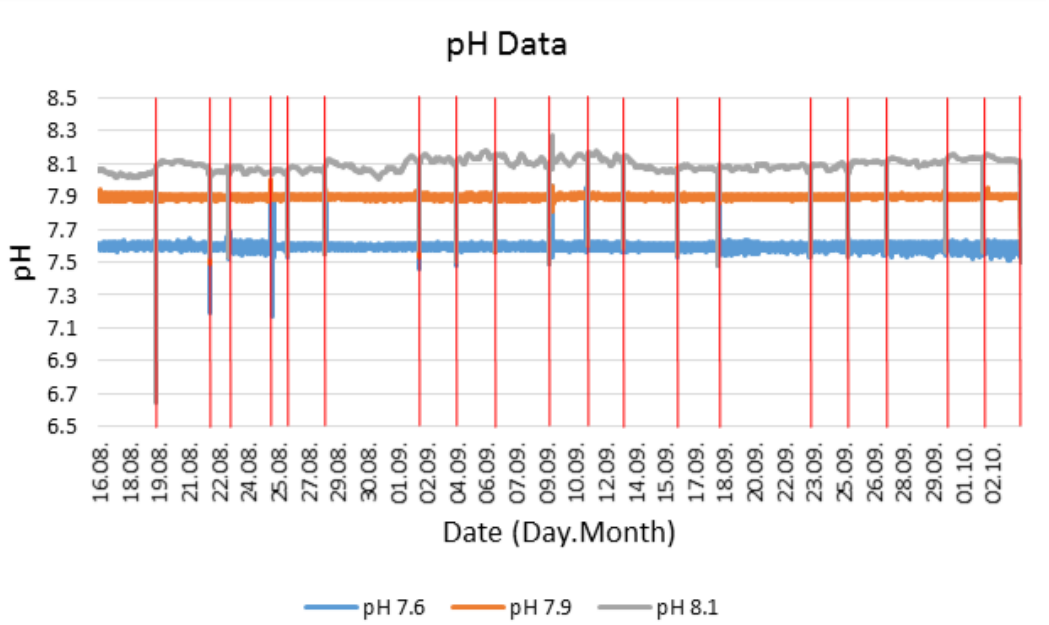


FIGURE 7: VHA ABUNDANCE IN BOTH ACUTE AND CHRONIC PHASES
A. This western blot shows the change in VHA abundance and is one of the three blots obtained depicting the acute response. The O1-3 samples show the baseline abundance of corals before they are introduced into the experimental system. The Day 5 and Day 7 samples constitute the acute response.
B. This western blot is one of the three western blots showing the change in VHA abundance in the chronic response. The O1-3 samples are the same coral homogenates as loaded in the acute response gels. Days 14 and 31 constitute the acute response for the purpose of this VHA measurement.

A.



B.

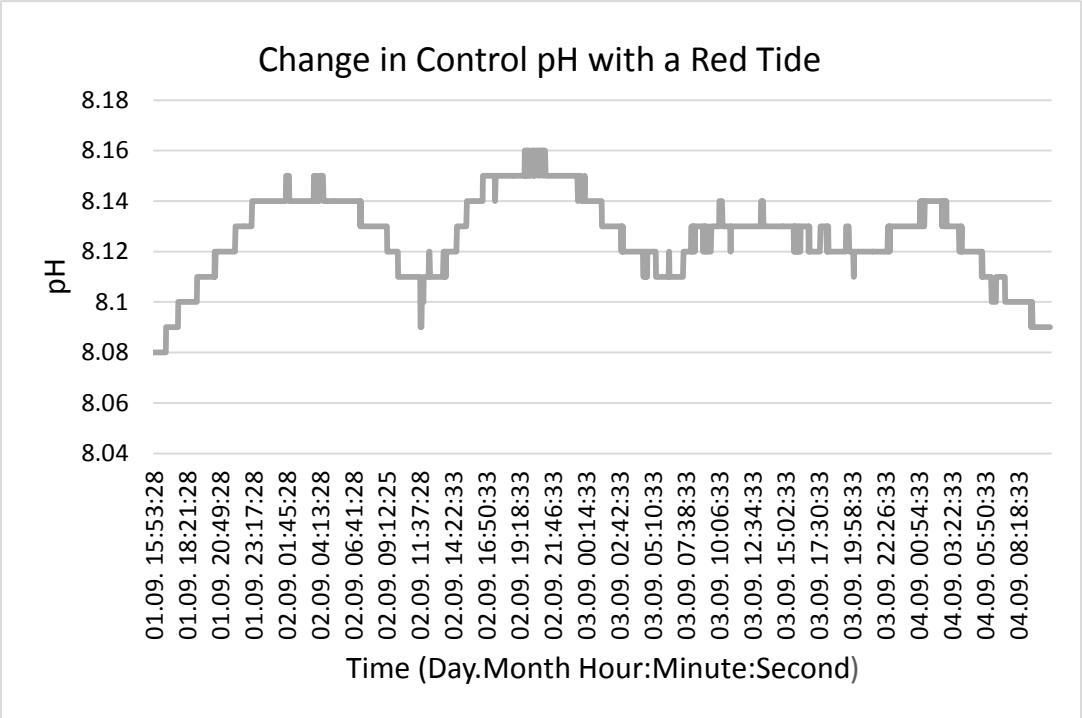
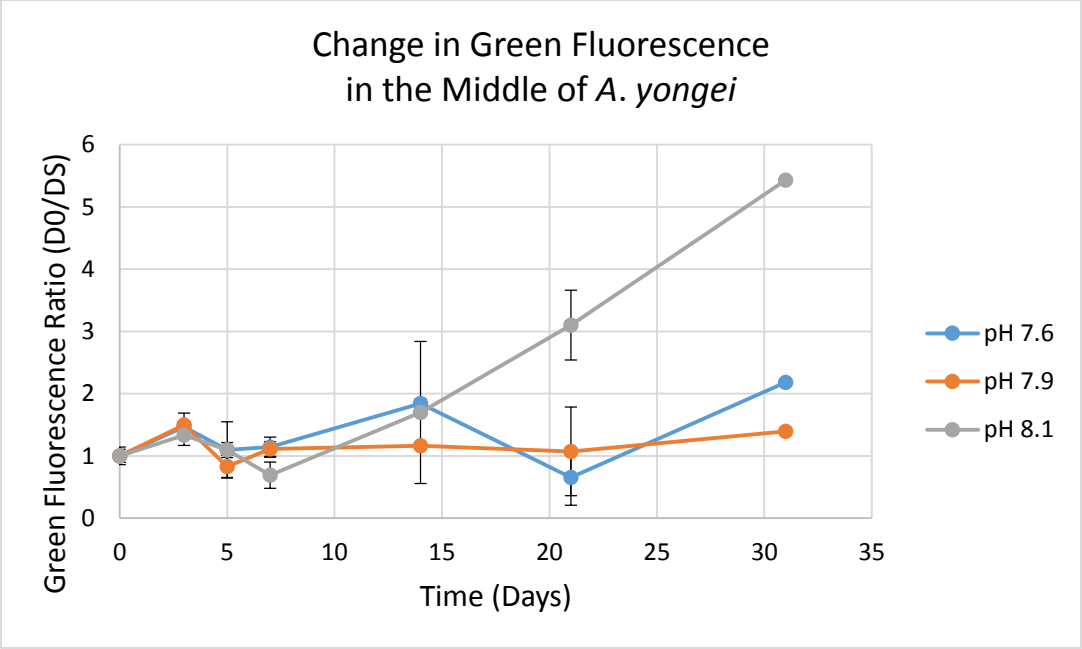


FIGURE 8: STABILITY OF pH MEASUREMENTS OVER TIME

Figure 9A shows the stability of pH readings for a period of 49 days. The two OA conditions are tightly regulated while the control is not manipulated. The red lines indicate times of calibration, where the electrode is out of seawater for precision testing. Figure 9B shows the change in pH, as measured by the IKS-Aquastar, for the control tank during the red tide. The pH stat system identified a high of 8.16 while water chemistry analysis provided a pH of 8.19. Both of these values are considerably higher than the pH 8.07 average seen throughout the rest of the experiment.

A.



B.

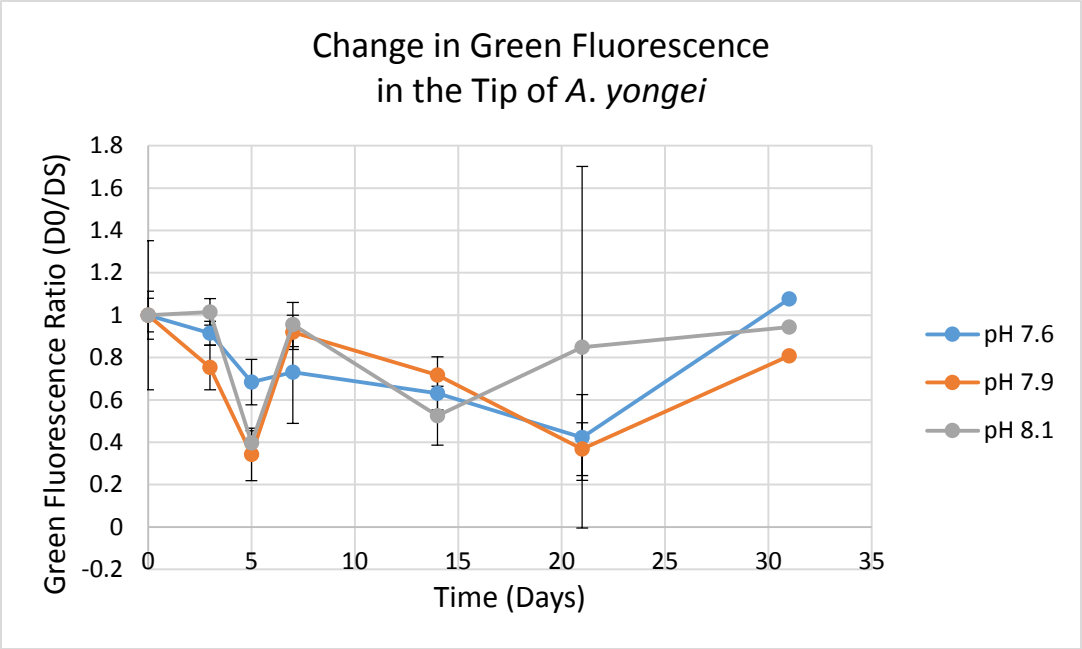
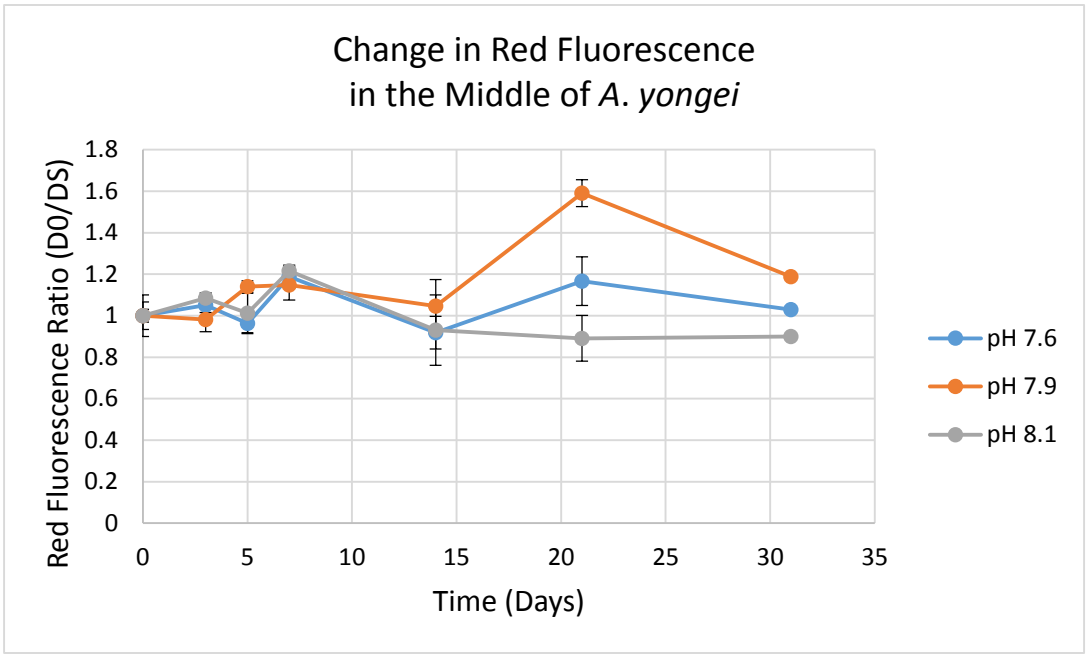


FIGURE 9: HYPERSPECTRAL ANALYSIS OF GREEN FLUORESCENCE IN CORALS

Figure 9A show the chronic increase of green fluorescence in control conditions while the green fluorescence in the two acidification conditions remain roughly the same throughout the entire duration of the experiment. Treatment was determined to be significant across pH conditions ($p = 0.01$), days ($p = 0.001$), and a combination of both ($p = 0.05$). Figure 9B shows the variability in the apical tip of the coral fragments. No significant differences were found between pH treatments (pH: $p=0.30$, time: $p=0.08$). Standard deviations are indicated by the bars.

A.



B.

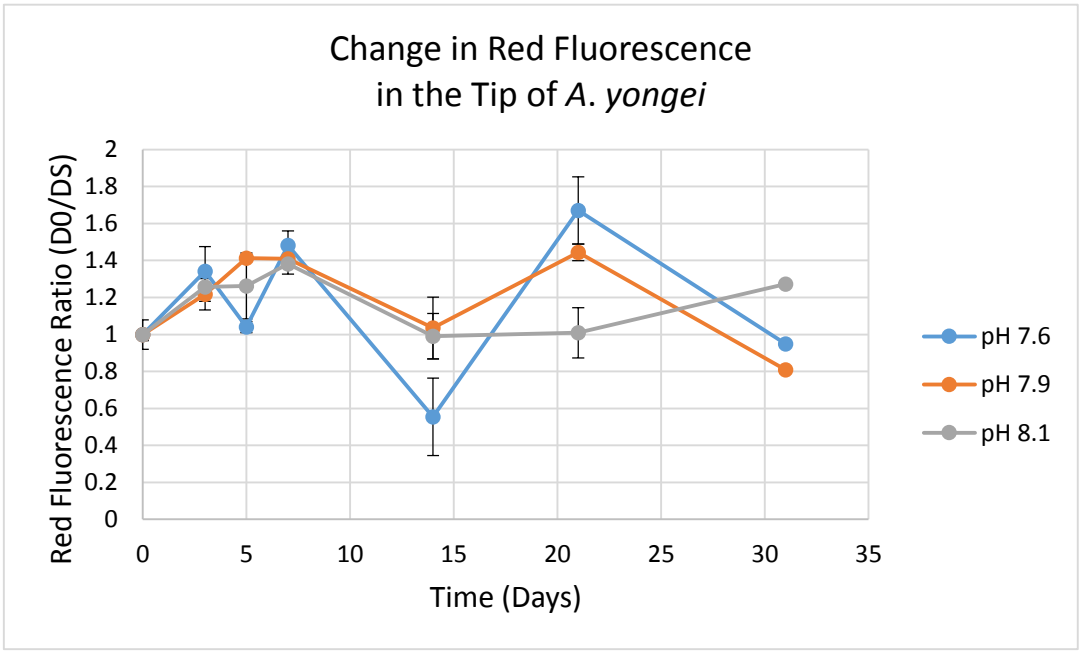
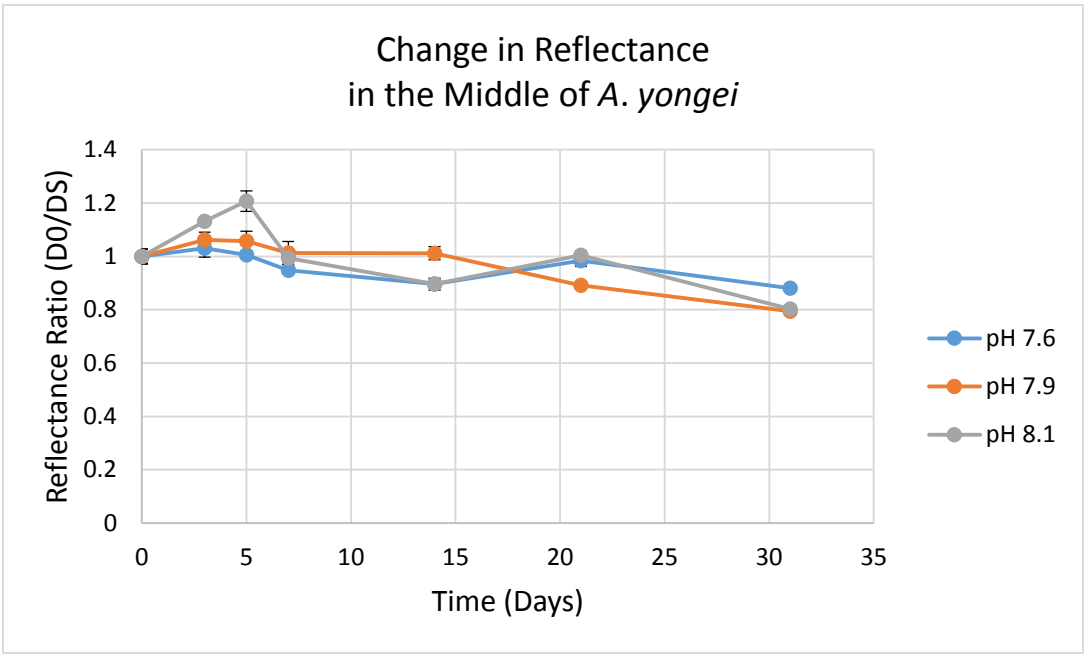


FIGURE 10: HYPERSPECTRAL ANALYSIS OF RED FLUORESCENCE IN CORALS

Figure 10A appears to shows a slight increase in red fluorescence in the OA-treated conditions in the chronic reaction but this change was not found to be statistically significant between pH conditions ($p=0.16$). Instead, time accounted for most of the variance found ($p=0.01$). Figure 10B, again, illustrates the variability in red fluorescence intensity found the tip of the coral (pH $p=0.66$; time $p=<0.0001$). No significant difference was found between the pH 7.6 and pH 7.9 conditions but both were significantly different than the control condition ($p=0.01$ and $p=0.004$, respectively).

A.



B.

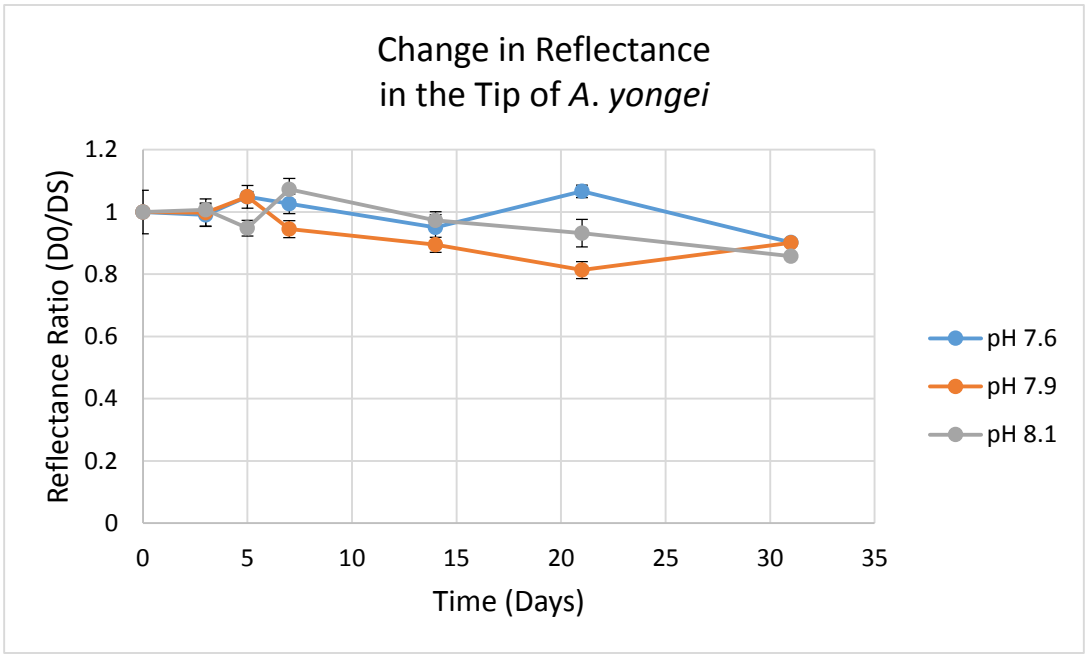


FIGURE 11: HYPERSPECTRAL ANALYSIS OF REFLECTANCE IN CORALS
Figure 11A and 11B show no difference in reflectance between pH conditions across time. Standard deviations are indicated by the bars. In the middle of the coral, time contributed most to variations seen within the treatments ($p=<0.0001$) but no difference was found between treatments ($p=0.12$). The tip of the coral showed a trend between both pH ($p=0.03$) and time ($p=0.0005$), which is likely due to the location of hyperspectral acquisition.

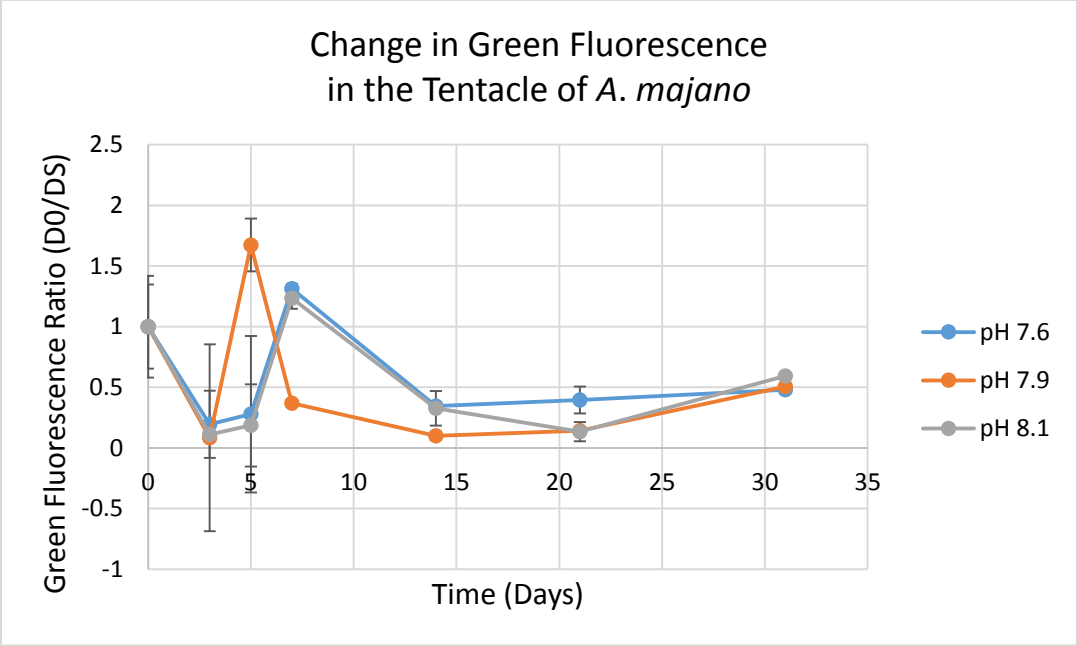


FIGURE 12: HYPERSPECTRAL ANALYSIS OF GREEN FLUORESCENCE IN ANEMONES

Figure 12 shows similar green fluorescence levels between OA conditions as the control conditions in the chronic phase. No significant difference was detected overall across pH ($p=0.59$), only across time ($p=0.04$). Standard deviations are indicated by the bars.

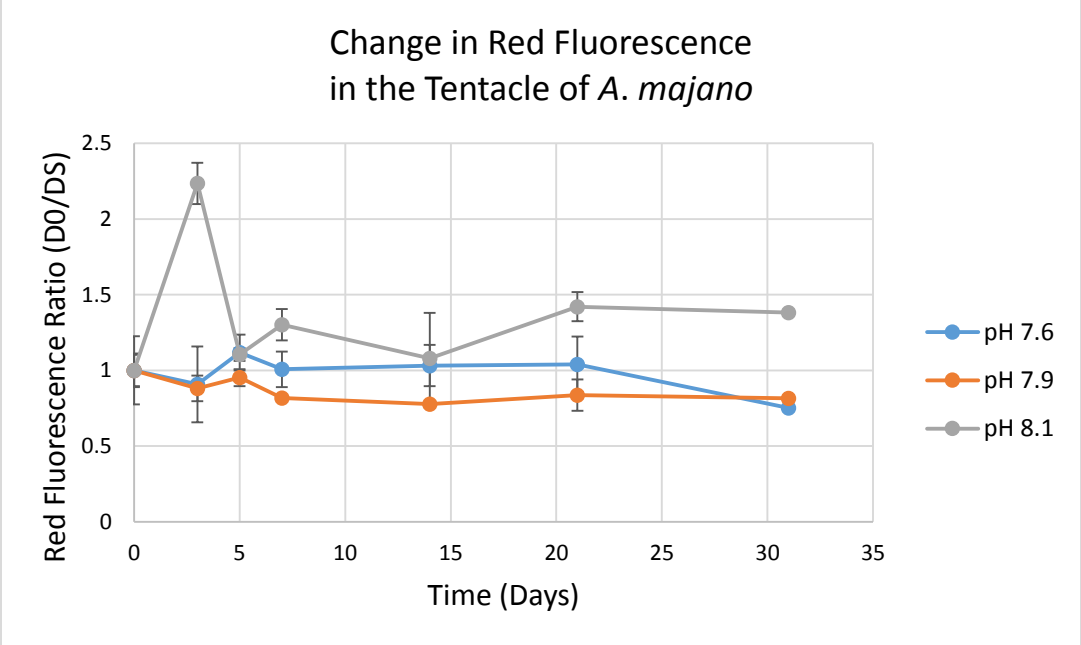


FIGURE 13: HYPERSPECTRAL ANALYSIS OF RED FLUORESCENCE IN ANEMONES

The control corals were able to maintain a higher concentration of zooxanthellae than the corals under OA stress. Significant differences were found between pH conditions ($p=0.0002$) as well as across time ($p=0.04$).

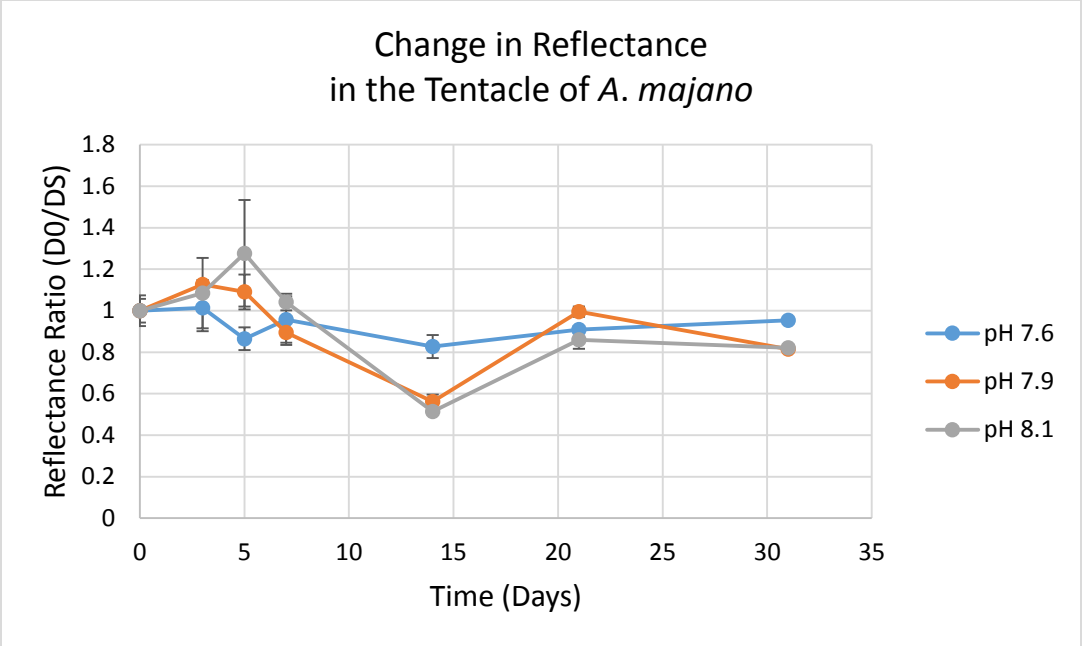


FIGURE 14: HYPERSPECTRAL ANALYSIS OF REFLECTANCE IN ANEMONES
Figure 14 shows no difference in reflectance between pH conditions over time. Standard deviations are indicated by the bars. No significant differences were found regarding the different pH treatments (p=0.72). Time was the factor that accounted for the variance observed (p=0.0002).

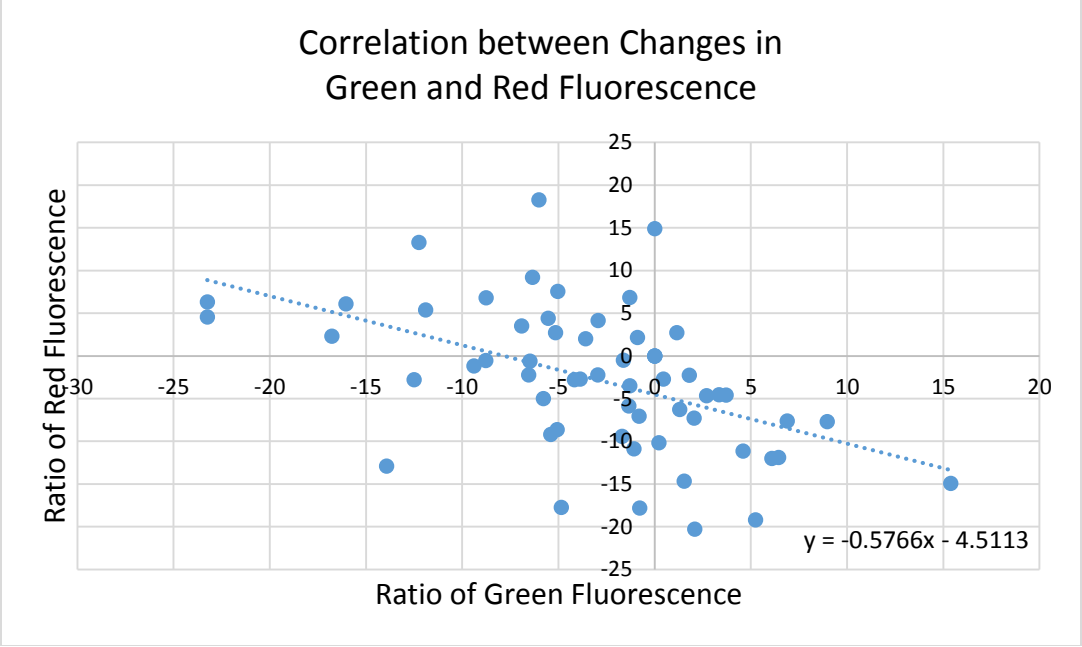
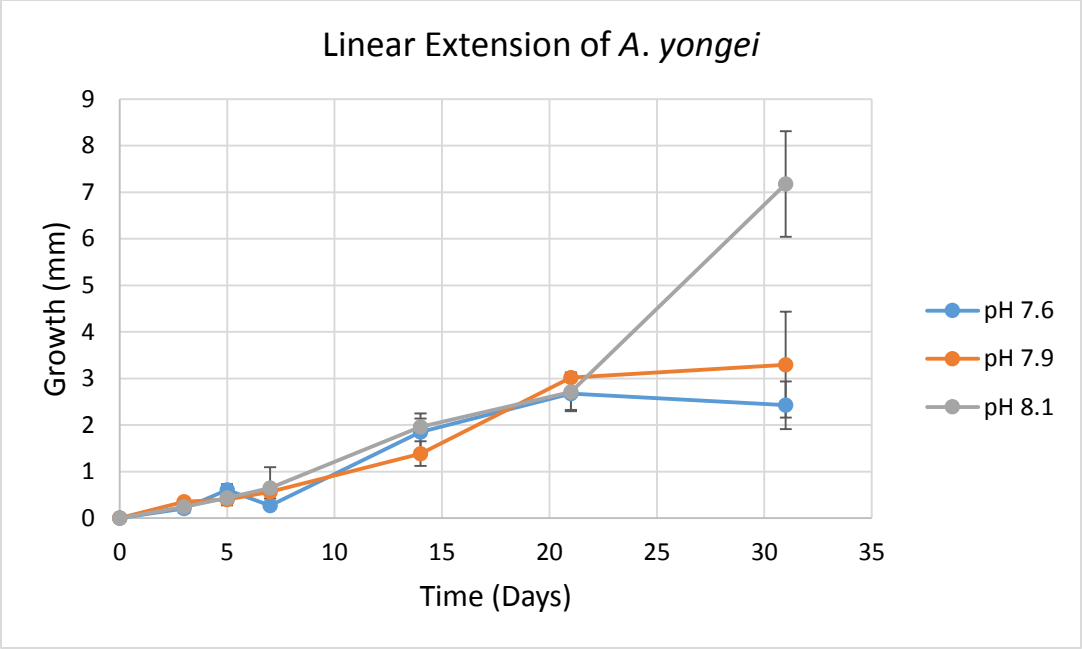


FIGURE 15: GREEN VS RED FLUORECENCE IN CORALS
The correlation between green and red fluorescence in *A. yongei* is highlighted in this graph. The data points have not yet been normalized for the QEN, which can only serve to intensify their relationship.

A.



B.

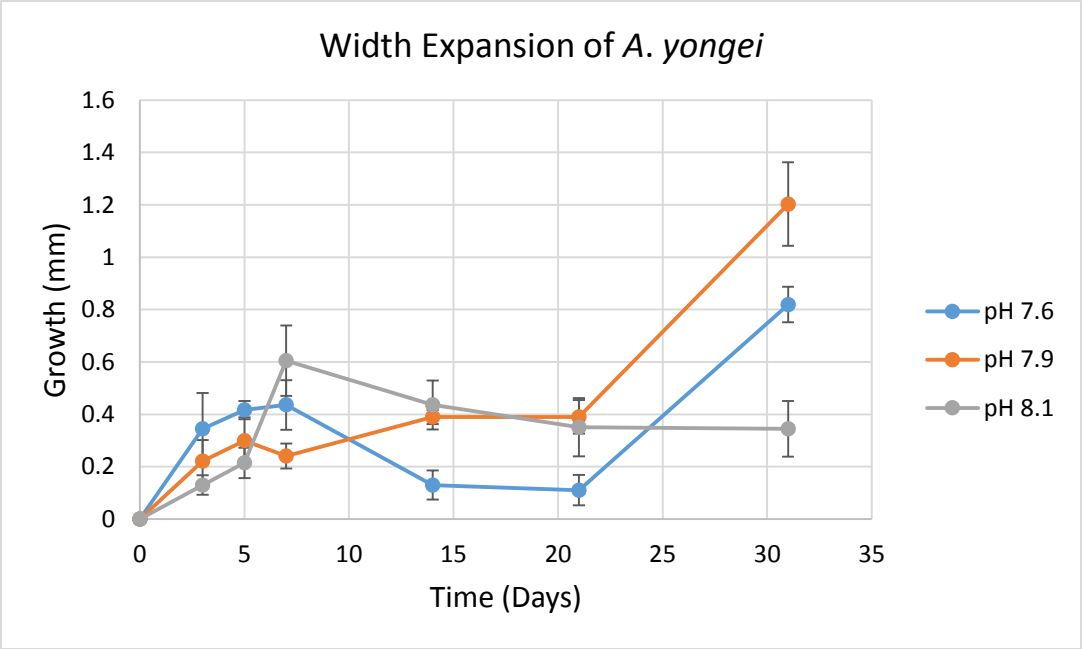


FIGURE 16: CORAL GROWTH TIP ANALYSIS

Figure 16A depicts the ability of the corals in OA conditions to grow just as well as the control corals until prolonged exposure. During the last day of sampling, the growth rate dropped off in the OA-treated corals and a clear distinction can be found between the control corals—who continued to grow at a similar rate—and the corals exposed to OA. However, neither figures 16A nor 16B were found to be overall statistically significant across pH conditions with p values of 0.1820 and 0.6894, respectively. Standard deviations are indicated by the bars.

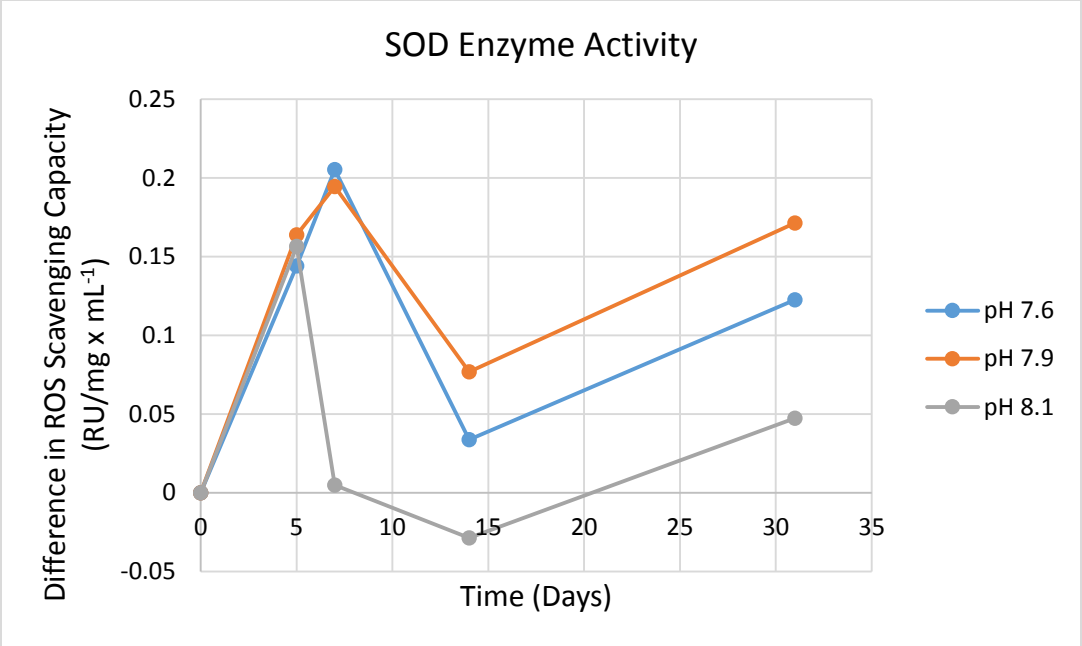


FIGURE 17: REACTIVE OXYGEN SPECIES QUANTIFICATION

Figure 17 depicts the SOD Enzyme Activity (or ROS scavenging capacity) of the coral homogenates. The values have been normalized for protein concentration and against the baseline, set by the day 0 samples. The acidified corals show greater scavenging during the acute reaction and continuously maintain a higher level of ROS scavenging throughout the duration of the experiment compared to the controls.

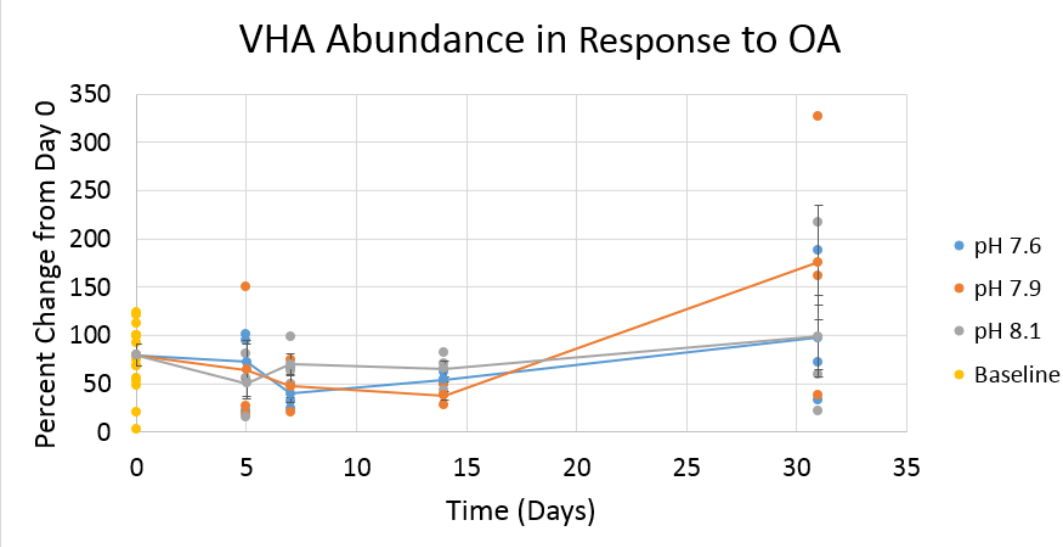


FIGURE 18: CHANGE IN VHA ABUNDANCE OVER TIME IN RESPONSE TO OA CONDITIONS

This graph shows the western blot quantification of VHA abundance over time in response to OA. The graph overlays two charts depicting all the values quantified as well as the average trend over time. No significant difference was found between the three pH treatments ($p=0.84$), indicating that the corals are not changing their VHA abundance in order to maintain their high zooxanthellae content.

TABLES

TABLE 1: DAY/NIGHT WATER CHEMISTRY

During the first round of experimentation, discrete water samples were collected from all three sump tanks, corals tanks, and anemone tanks for chemical analysis. This identified similar pH values across all nine tanks.

It was unsurprising to find that the pH of the seawater is slightly lower that at the end of the night cycle than that of the day cycle. During the night cycle, respiration from both the coral and its symbionts increases the CO_2 in the water, which drives pH down. In contrast, during the day cycle, photosynthesis occurs, which removes CO_2 from the system and thereby increases pH.

End of Night Cycle

Condition	Sump tank	Coral Tank	Anemone Tank
Control (8.05)	8.033	8.000	8.029
7.9	7.850	7.858	7.865
7.6	7.569	7.577	7.581
Median ± Standard Deviation	8.029 ± 0.018009	7.858 ± 0.007506	7.577 ± 0.00611

End of Day Cycle

Condition	Sump tank	Coral Tank	Anemone Tank
Control (8.05)	8.038	8.050	8.059
7.9	7.861	7.888	7.895
7.6	7.593	7.611	7.613
Median ± Standard Deviation	8.050 ± 0.010536	7.888 ± 0.017954	7.611 ± 0.011015

TABLE 2: WATER CHEMISRY SHOWS STABILITY ACROSS TIME
The calculated pHs derived from water chemistry analysis were able to confirm the stability of the pH stat system over the course of the experiment, proving that the optimization prior to the start of experimentation was successful.

Day	**pH 8.1**	**pH 7.9**	**pH 7.6**
0	8.072	7.907	7.657
3	8.078	7.836	7.550
5	8.053	7.880	7.632
14	8.067	7.871	7.583
21	8.047	7.854	7.586
31	8.077	7.828	7.549
Median ± Standard Deviation	8.0695 ± 0.0129	7.8625 ± 0.0294	7.5845 ± 0.0437

DISCUSSION

The original purpose of this study was to identify whether green fluorescence can be used as a better and earlier proxy of health than coral coloration. Analysis was often broken down into an acute (days 3-7) and chronic (days 14-31) reaction to assess whether any fluctuations in fluorescence can be correlated to a physiological change and, thus, act as a proxy to stress levels.

Green fluorescence changes in response to stressors in the literature

In the literature, green fluorescence has been shown to respond to environmental stressors, such as light and temperature (Roth and Deheyn, 2013; Roth et al, 2010). However, the effect of fluorescence and the corresponding physiological changes have never been shown in response to ocean acidification. This is a novel field that has yet to be explored.

Color of green fluorescence in corals remains unchanged in response to ocean acidification conditions

It is important to note that throughout the course of the experiment, the wavelength of green fluorescence did not shift in its spectral signature, meaning that no transition to a different color occurred. Therefore, the green fluorescence always remained green and, consequently, the red fluorescence intensity arose from only that of the zooxanthellae and never from the coral host. Since the two spectrums never merged together, it was possible to identify the endogenous green fluorescence of the coral from

the endogenous red fluorescence of the zooxanthellae. Thus, the only variable that differed between individual organisms was the intensity at which the two proteins fluoresced.

Intensity of green fluorescence in corals changed in response to ocean acidification.

Green fluorescence intensity differed between pH conditions over the course of this month-long acclimation study, while the reflectance did not, indicating that green fluorescence is much more sensitive to the organism's environment and stress levels than coloration. The OA-treated corals did not experience an increase in green fluorescence as the control corals did. In fact, the corals under OA stress maintained approximately the same level of fluorescence during the course of the experiment; for all intents and purposes, the fluorescence decreased with respect to the control corals. This change in green fluorescence was significant across both pH ($p = 0.01$), time ($p = 0.001$), as well as a across a combination of both pH and time ($p = 0.05$). This provides strong evidence of the relationship between the effect of decreased pH and the decrease in fluorescence. It is important to note that no significant differences were found between the two acidification conditions, likely indicating that the pH 7.9 condition is enough to induce maximum stress and that further acidification may not further increase the stress. However, both the pH conditions showed a statistically significant difference when compared to the control. In addition, the acute and the chronic phases were significantly different from one another but sampling between days within each category was not found to be significant.

In the acute phase, both the acidified corals showed a slight decreased in green

fluorescence, yet the control corals also showed such a trend to some extent. This decrease in green fluorescence in the control corals likely resulted from the increase in pH brought on by the red tide that arrived off of the Scripps Pier. This red tide raised the pH of the seawater up to 8.16, as recorded by the IKS-Aquastar Software. Water chemistry analysis provided a calculated pH of 8.19, which is considerably higher than the 8.07 average seen across the experiment. The acidified corals likely did not feel the effects of the red tide as strongly as the control corals because the IKS-Aquastar is a dynamic system that continuously regulates the pH within specified limits and thus would have altered the pH of the seawater accordingly to fit within the range. The control water was not manipulated and thus would have reflected the natural fluctuations of the ocean.

After extended exposure in OA conditions, the OA-treated corals did not maintain the same green fluorescence intensity as the control corals, which continued to get brighter during this time. The intensity of fluorescence between the acidified corals and the control corals started to diverge after day 14 and, during the last two days of sampling (a total time of 17 days in between), fluorescence was considerably lower than that of the control corals. The acidified corals also showed a smaller standard deviation, likely owing to the fact that when energy availability is low, organisms tend to operate within a very narrow range of physiological functions.

Change in fluorescence was not an artifact of accidental fluctuations or shift in pH due to poor regulation by the pH stat system as seawater samples were collected each day a set of organisms were sacrificed. All the pH treatments were confirmed to be both precise and accurate. The deviations between the calculated pH from the designated average pH equate to an additional 3.62% and 8.98% increase in hydrogen ion

concentrations in pH 7.6 and 7.9, respectively, taking into account the logarithmic nature of the pH scale. This is generally considered relatively accurate, considering the many difficulties associated with measuring pH with deviations at such a precise scale and also of that in which the pH changes are ecologically relevant. Outside of the day 7 sampling mentioned earlier, no other abnormalities were found; thus, with the exception of the day 7 control corals for IHC, all the changes observed are valid physiological changes in response to ocean acidification. Chemical analysis of water samples are especially important in confirming the validity of an experiment as inaccurate water chemistry would have led to changes in pH, thereby altering the entire context of the experiment.

The change green fluorescence is correlated to linear elongation of the coral

Previous research in the Deheyn Lab at the Scripps Institution of Oceanography found a positive correlation between green fluorescence and coral growth (Roth and Deheyn, 2013). Because no observable bleaching event occurred during course of the experiment, the increase in green fluorescence can be only attributed to growth. Linear extension analysis was performed to identify whether the lack of increase in green fluorescence in the chronic reaction of the acidified corals correlated to a lack of growth. It was discovered that the pattern in growth was indeed similar to that of green fluorescence. However, in green fluorescence, the divergence between the control and the OA conditions occurred much earlier than in growth. Differentiation between the green fluorescence in the control and the OA conditions started separating as early as day 15, whereas growth did not reflect that same change until after day 21. It took much longer

for growth to reflect the lack of energetic or chemical availability than did the production of green fluorescent proteins.

It can be concluded that our study found similar results to Roth and Deheyn in 2013, with the exception that the relationship between green fluorescence and growth dissociates during times of extended exposure to OA conditions, where one response may lag behind the other. However, the order in which this occurs would be beneficial to the observer (scientist, coastal manager, or policy maker) as a decrease in green fluorescence may indicate a subsequent impending negative physiological change. This may prove to be a useful measure in monitoring and, eventually, restoring coral reefs.

No change in red fluorescence from acidified corals is likely due to increased ROS scavenging protective mechanism by the coral host

Slightly higher red fluorescence, and thus zooxanthellae abundance, was detected between the corals exposed to OA conditions and the corals under control conditions. However, the overall change in red fluorescence was not found to be significant between pH conditions ($p=0.16$). Thus the OA-treated corals were able to maintain the same level of zooxanthellae abundance as did the control corals. This implies that either the symbionts are not sensitive to OA stress or the corals were able to buffer the effects of OA for the zooxanthellae. When zooxanthellae become stressed, they produce high quantities of ROS; when the concentration of ROS exceeds that of the coral's antioxidant capacity, then the coral undergoes oxidative stress—meaning that the ROS become damaging to the coral's proteins, lipids, and nucleic acids, which would eventually lead

to the initiation of bleaching (Smith et al, 2005). Bleaching helps prevent further degradation of coral tissue, where either the corals expel their zooxanthellae or the zooxanthellae leave on own accord due to the hostile environment created by the host (Wooldridge, 2010; Tchernova et al, 2011). This is a risk for the coral, however, who will lose its primary source of energy (zooxanthellae can provide up to 95% it; Muscatine, 1990) and it seems likely, therefore, that the coral would develop molecular and biochemical strategies to best mediate the effects of OA for the zooxanthellae.

Accordingly, it has been shown that green fluorescent proteins have the potential to function as an antioxidant, similar to SOD. Palmer discovered that GFP concentration and antioxidant capacity showed a positive correlation while Bou-Abdallah identified GFP as a mediator of superoxide radicals (Palmer et al, 2011; Bou-Abdallah et al, 2006).

Our study found high SOD enzyme activity, and thus high ROS scavenging capability, during the coral's acute response and decreased scavenging thereafter. These levels were still higher than that of the control corals but pointedly less than the first week. The control corals maintained a low level of ROS scavenging throughout most of the experiment and their enzyme activity hovered around the baseline as set by the day 0 corals.

I hypothesize that the ability of the coral to mediate these additional ROS comes from the ability of GFP and GFP precursors to quench superoxide radicals. Corals have been shown to store approximately a two week's supply of GFP precursors stored in their cells that are ready to be converted into GFP when necessary. The GFP and GFP precursors would have been used to mediate ROS during the acute reaction, which would

result in high scavenging but low fluorescence as the process of absorbing the radical extinguishes fluorescence. This would also explain why the ability to mediate ROS decreases after just two weeks. As a GFP precursor is generated, it is immediately used to quench the ROS, always leaving the coral in short supply of GFP, which is why fluorescence in the acidified corals never quite catches up to the intensity of the control corals. (D'Angelo et al, 2008; D'Angelo et al, 2012)

By looking only at coral coloration, it would not have been apparent that the coral was stressed under OA conditions, which is implied by the increase in ROS scavenging capacity. However, lower levels of fluorescence can be seen throughout the duration of the experiment, which correlates to the high levels of ROS scavenging. This shows that green fluorescence fluctuations allow earlier detection of stress levels than coral coloration, which showed no change between pH conditions.

VHA quantification showed no significant change with OA over time, indicating the corals have already adapted to tolerate fluctuations in external pH

It is apparent that the coral is under stress, as demonstrated by their increased antioxidant capability. The question then proposed is whether the coral is able to maintain their zooxanthellae content by buffering the effects of OA. Does the coral change the abundance of VHA, a proposed carbon-concentrating mechanism that allows the coral to transfer the main compound necessary for photosynthesis to the symbionts? This proposal was tested by performing two different methods: immunohistochemistry and western blots.

Labeling and imaging the symbiosome with immunohistochemistry proved to be a challenge. Staining was very inconsistent, regardless of the consistency in preparation methods, and imaging using the Zeiss microscope with the Axiovision 4.8 program was even more challenging. Often times, staining of the symbiosome could be detected in the binoculars but unable to be imaged by the system. High variability was found between staining in all three pH conditions.

Although the VHA labeling of the symbiosome does not appear well in the immunohistochemistry images in the pH 7.6 conditions, this does not definitively mean that VHA was not present in the symbiosome of these acidified corals. The inability of the symbiosome staining to be photographed could either imply that VHA is present in lower concentrations or it could be present in other location inside the cell where it performs a similar task to maintain intracellular pH. VHA exists within many different compartments; in fact, it was originally known to acidify vacuoles and lysosomes (Finbow and Harrison, 1997). Because symbiosome staining was the primary focus of this study, other locations within the cell were not as well inspected. Further analysis will be necessary to determine where the differences in labeling are significant between conditions and across time points.

Since it was not suitable to quantify the amount of VHA present by the brightness of staining, western blot analysis were performed as an alternative. Six western blots representing different OA treatments were performed on the same day to ensure the same manipulation across samples. While the day 0 samples were never frozen and thawed more than once, they were kept on ice for over 12 hours during the day. This could cause,

and likely did, cause some protein degradation. However, since all the corals were defrosted at the same time and kept on ice until loading, the level of degradation should be equivalent across samples. Although the protein concentration was determined independently at the start of each set of gels, it was found that the protein concentration fluctuated significantly between measurements and thus could lead to any peculiarities, such as additional smaller bands, seen in the blots.

After normalizing all the ImageJ quantification of band intensities, it appeared that the pH 7.6 corals decreased their VHA abundance immediately, while the pH 7.9 corals took slightly longer to do so. It is hypothesized that as the CO_2 concentration in the seawater increases, the coral no longer requires a high abundance of VHA as the CO_2 can then diffuse through the zooxanthellae membrane for use in photosynthesis. Because VHA uses ATP as an energy source, it would not be beneficial for the corals in OA conditions to have high abundance of VHA when the CO_2 content in the seawater is sufficiently transferred to the zooxanthellae for photosynthesis.

It is also possible that the pH 7.9 corals take longer to decrease their VHA concentration because they have already experienced several upwellings off the coast of California during the decade they have been reared in the laboratory. Because they have already become accustomed to these brief acidification conditions, it may suggest that their lack of initial change in physiology could be in anticipation that the acidification will be brief. However, after extended exposure, the corals are forced to change their physiology in order to maintain acid/base regulation. The control corals continuously increased their concentration of VHA during the course of the experiment.

In the chronic reaction (7-31 days), it appeared that the pH 7.9 corals maintained the highest concentration of VHA during the course of the experiment, even though the control corals had the largest growth. Both the control and pH 7.6 corals had roughly the same VHA abundance on the last day of sampling. However, as discussed earlier, VHA is localized to many different compartments within the cell. Because the crude homogenate was loaded into the gel, the western blot quantified the total amount of VHA within the cell, not just the VHA in the symbiosome membrane. Perhaps a decrease of VHA in the symbiosome membrane is counterbalanced by an increase in VHA elsewhere in the host cell or vice versa and thus no change is detected in the overall abundance. This lack of protein abundance can also be due to the preparation of the samples, which could have caused a loss of VHA stability since the protease inhibitor was stored in DMSO, a detergent that disrupts membranes and thus membrane bound proteins.

Although, the overall change in VHA abundance in the coral cells does not appear to change, further analysis using only purified membrane proteins will likely be necessary to give an accurate assessment of whether the presence of VHA in the symbiosome changes in the presence of OA conditions.

Comparison of OA effects on fluorescence of a tropical symbiotic cnidarian failed due to experimental challenges with the organism

I attempted to test a different cnidarian to see whether the change in fluorescence discussed above is constrained to corals or whether the majority of fluorescent proteins react the same way under OA conditions. I was not completely successful in doing so due

to challenges working with the organism.

No significant change was detected between the pH conditions in green fluorescence or in bright field. However, a statistically significant decrease in red fluorescence was found after chronic OA treatment of the anemones. However, this decrease must be analyzed carefully due to the volatile nature of the control anemones, which showed a sharp increase and decrease in the abundance of zooxanthellae during the acute phase.

Sea anemones are most often sessile, however, the species that was tested turned out to be extremely motile and thus required cages to identify one individual from another. While the clear plastic the cages were made from are not known to be toxic to the organisms, it could be likely that either not enough water flow was reaching the cages, the cages did not allow enough light to penetrate for efficient photosynthesis, or, perhaps, the process of removing the anemones from the cages prior to imaging were harmful towards the organism. The plastic used was optically clear and were placed roughly 20 inches underneath the light, which is well within the limits specified by the light system (32 inches). Removal of the anemones from the cages were done with a metal spatula with as much care and tenderness as possible but the physical stimuli still has the potential to act as a additional stressor.

Another problem arose when trying to image the anemones; the anemones are prone to dividing when they are either under duress or when they are content with their environment. In the event an anemone split in two during the course of the experiment, both clones were imaged. Because the anemones are motile and share the same cage, it

was nearly impossible to distinguish the original clone from the one that budded off. The problem lies in the fact that the spectrums obtained were corrected for their exposure time by multiplying by an exposure ratio (lowest overall exposure/the exposure of that acquisition) which was then converted into a ratio to identify the overall change for that individual (normalized intensity at day 0/normalized intensity at sacrificing day). As the size of the anemone's tentacle changes, the exposure must also change accordingly to illuminate the entire organism and to capture the intensity of the organism's fluorescence. This could lead to an increase in the exposure time at sacrificing day since the clones were, more often than not, smaller than the parent.

Because no significant changes in green fluorescence were identified in the anemones, physiological experiments were put on hold due to experimental challenges with this particular species and time constraints.

In conclusion, green fluorescence in corals is an effective proxy of coral stress over long-term OA exposure

Overall, this study determined that green fluorescence is a good measure of coral stress in response to extended exposure to OA; the corals exposed to OA treatment showed a diminished green fluorescence, while reflectance did not change between pH conditions. This decrease in fluorescence was correlated to an increase in ROS scavenging capacity and a decrease in linear elongation. These corals were able to maintain their symbiotic relationship with their zooxanthellae thereby preventing bleaching and it was shown that they likely do this by altering their ROS scavenging

capacity rather than their overall carbon-concentrating enzyme, VHA.

Based on this study, it appears that while coral will not die from the impacts of OA alone, corals experienced many physiological changes. Following climate predictions, the pH of the ocean will decline to the experimental pH 7.6 just after the end of the century. Based on our study, coral reefs may potentially cease to grow and may lose a lot of their beautiful fluorescence as well. If our coral reefs cease to grow, the reefs that become destroyed will mark the definite decrease of coral reef coverage.

However, climate change does not only result in decreased oceanic pH but a wide variety of additional consequences, which may add to the stress that directs the decline of coral reefs, which is the most productive marine ecosystem. To prevent further loss of coral reef coverage and further habitat degradation, we must be able to identify when this system is in distress so that, if possible, we are able to mediate these negative effects. It is imperative that we become cognizant of and re-evaluate our impacts on their environment if we are to prevent further losses of this remarkable ecosystem.

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