The effect of environmental CO$_2$-addition on the biofluorescent intensity response of *Lobophyllia hemprichii*

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Abstract

Biofluorescent research has only recently been conducted on coral reef systems. The ecologic importance of this intrinsic quantity is still widely unknown. One possibility is that the intrinsic fluorescence exhibited by coral species is closely related to their corresponding state of health. I investigated the relationship between the large polyp stony coral *Lobophyllia hemprichii*’s fluorescent intensity properties upon environmental CO$_2$-addition. This treatment was designed to mimic the anthropogenic stressor that causes ocean acidification. Fluorescent intensity was measured using confocal microscopy techniques through Cornell University’s Biotechnology Resource Center and analyzed with ImageJ software. IBM SPSS data analysis revealed that there was not a significant difference in the area fraction of fluorescent regions between groups at different pH’s, suggesting that ocean acidification does not lower the fluorescent quantity of *L. hemprichii*’s chromatophores. Future studies should be focused on using a larger sample size, improving the sampling technique, and quantifying spectral shifts.

Introduction

Coral reefs are among the most biologically diverse and economically important ecosystems in the world because they provide valuable ecological services including income via fishing, tourism, and recreation; coastline protection from erosion; marine biodiversity; new sources of medicine; and much more. Despite this global importance, though, anthropogenic stressors continuously threaten the lives of coral reefs. One of the major stressors includes ocean acidification, the lowering of seawater pH due to an increase in atmospheric CO$_2$ levels. Ocean acidification causes coral reefs to undergo catastrophic bleaching events, which is the paling of corals due to severe oxidative stress.
This damages both the coral and its symbiotic dinoflagellate photosynthetic apparatus via zooxanthellae expulsion or algal pigmentation loss. Coral bleaching is not simply a coloration loss. In fact, bleaching quickly leads to coral death if not treated soon enough. Thus, signs of coral bleaching can be used as indicators of coral health (Roth and Deheyn, 2013).

Scientists currently examine the external coloration of coral reefs as an indicator of overall health, and ultimately of bleaching status. Unfortunately, this methodology is effective only after the reefs have begun to experience bleaching. Therefore, a new and noninvasive approach to assessing coral health is in demand.

Fluorescence, the reemission of photons at wavelengths longer than those initially absorbed, has recently been discovered in coral epidermal cells (Treibitz et al. 2013; Mazel, 1995). Chromatophores, light-reflecting and pigment-containing cells, produce large amounts of fluorescent proteins (FP’s) (Roth and Deheyn, 2013). In fact, most of a coral’s overall coloration is due to a family of green fluorescent protein-like proteins (GFP), which fluoresce under visible and UV light (Dove et al. 2000). In certain coral species, these FP’s can sometimes account for as much as 14% of the total soluble protein content (Leutenegger et al. 2007). A relatively new discovery found that healthy fluorescent corals emit wavelengths different from those emitted by bleaching fluorescent corals (Holden et al. 1996). This wavelength distinction suggests a bleaching diagnosis method that could consist of a noninvasive and accurate measure of coral health via in situ fluorescence analysis. However, the specific emission values of are still unknown for numerous coral species.
Although there is a high prevalence of FP’s found within many coral species, little is known about the actual purpose that they may serve (Roth and Deheyn, 2013). One of the main hypotheses scientists have proposed is that FP’s largely affect the overall coral’s health through increasing the photosynthetic potential of zooxanthellae and/or photoprotecting the zooxanthellae during high intensity light events through its dependence of the collective chromatophore fluorescent intensity output. Photoprotection and zooxanthelllar photosynthetic potential are thus related to each other through the intensity of fluorescence in which the epidermal chromatophores emit (Dove et al. 2000; Gilmore et al. 2003). Both cases are similar in that either performance depends on the amount of fluorescent intensity output.

Ocean acidification is known to cause a decrease in coral health by causing corals to undergo bleaching. I propose that ocean acidification disrupts the overall fluorescent intensity output prior to the bleaching that it causes. The purpose of this research is to observe how the large polyp stony coral, *Lobophyllia hemprichii*, fluorescently responds to CO₂-addition (i.e. pH decrease) in hopes of systematically observing the progression of a coral's declining health. The coral's responses of fluorescent intensity will be used as the measure by which the overall health can be determined. I investigated the main hypothesis: Coral fluorescent intensity output is lowered by environmental CO₂-addition. Confocal microscopy was used to image the fluorescent chromatophores over several decreasing pH increments.
Materials and Methods

This research project was conducted in Room 189, Grant Science Center.

Sample Collection, Preparation, and Maintenance

Four 20-gallon marine aquaria, 2 control tanks and 2 experimental tanks, were set up with an undergravel filter, a 12” air stone, a 10-20 gallon tank heater, a LED aquarium light, an additional Aqua Tech side filter, a thermometer, and a mixture of playground sand and Nature’s Ocean live sand. Five 2”-3” red L. hemprichii samples were purchased from Coral Imports and Blue Zoo Aquaria located in California, who harvests the coral from Fiji and the Solomon Islands, respectively.

Prior to beginning the experiment, each tank contained two coral frags. Unfortunately, one coral from each tank bleached before testing began, except for the two frags in Tank 3. Thus, there was one coral frag housed in each tank throughout the experiment, with the exception of Tank 3. The initial freshwater tanks were set up and run for 1 week to ensure proper function of the tank supplies. The freshwater was then transformed into marine water using twenty ½ cup measurements of Instant Ocean. Table 1 lists the marine parameters that each tank were maintained at throughout the experiment.

The parameters that were measured daily included salinity and specific gravity via an Instant Ocean hydrometer, temperature via an on-glass thermometer, pH via a Eutech pH meter, and dKH via an API dKH test kit. All tanks received ~2.5 mL of phytoplankton and zooplankton each three times a week. About 1 mL Reef Trace was added to the tanks every Monday. The corals underwent a 12:12 hour light:dark photoperiod, maintained on Woods mechanical timers, beginning at 11AM and ending at
11PM everyday so that the dark photoperiod took place during the day. This was because *L. hemprichii* feeds during the night. Thus, the corals could be fed during our daytime hours. Garbage bags were draped over each individual tank to keep the insides dark while the laboratory was lit during the day. The control tanks (Tanks 1 and 2) were not subject to CO$_2$-addition, while the experimental tanks (Tanks 3 and 4) were. Carbon dioxide was bubbled into Tanks 3 and 4 until a 0.1 pH decrease was measured and maintained.

**Testing: CO$_2$-addition, Microscopy, and Imaging**

Samples of the corals were removed with forceps. Samples were taken from the coral’s epidermal layer and/or coenosarc, the connective tissue between each polyp. The tissue sample was about the size of the forceps’ tip. Tissue samples were prepared on microscope slides by placing the sample in a drop of seawater under a coverslip. Vacuum grease was used to encircle the sample and reduce physical movement as it was constantly suspended in water. Imaging was performed at the Biotechnology Resource Center, Cornell University. A Zeiss LSM880 Confocal Upright Microscope was used to image throughout the experiment. The C-Apochromat 10x water immersion Korr objective, Argon (488 nm) and DPSS (561 nm) laser excitations, and Zen 2 Software was also used.

Corals were treated with carbon dioxide 24 hours before being imaged. pH measurements were maintained every 4-6 hours to ensure that the amount of added CO$_2$ was consistent. Images were taken at each 0.1 pH decrease, which ranged from 8.4-7.8.

Images were processed in the software ImageJ and analyzed using a One Way Repeated Measures ANOVA. Morphological fluorescence was first measured to
determine how much of the image itself contained chromatophores that fluoresced over a thresholded value. The images were first converted from RGB to 8-bit and the background values were subtracted out from each original image. The image was then thresholded to include only the chromatophores that fluoresced at and above the smallest possible cell:noise ratio. Percent area was automatically calculated by ImageJ and was used as the representative measure of morphological fluorescence. Next, fluorescent intensity was measured from the morphological images’ pixel intensity. Thus, the recently thresholded images presented data on how much of the image contained fluorescent tissue, and then the actual amount of pixel (color) intensity was determined. Images were again converted to 8-bit before a grayscale inversion was applied that highlighted the regions of interest. Images were then thresholded a second time to limit the cell:noise ratio, and the percent area was used as a direct measure of pixel (fluorescent) intensity.

Table 1.

<table>
<thead>
<tr>
<th>Marine Aquarium Parameters</th>
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</tr>
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<tbody>
<tr>
<td>Temperature</td>
<td>72 – 78° F</td>
</tr>
<tr>
<td>Waterflow Rate</td>
<td>80-310 gallons per hour</td>
</tr>
<tr>
<td>dKH</td>
<td>8 – 12 °</td>
</tr>
<tr>
<td>pH</td>
<td>8.1 – 8.4 ± 0.1</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>1.023 – 1.025</td>
</tr>
<tr>
<td>Salinity</td>
<td>31%0 – 34%0</td>
</tr>
</tbody>
</table>

Results

Morphology

The program IBM SPSS was used to perform a One Way Repeated Measures ANOVA on the calculated percent area because each manipulation was not independent of one another. The data failed the Mauchly’s Test of Sphericity, an assumption that
states the variances of the treatment groups are equal. Therefore, statistical values were gathered from the Greenhouse-Geisser correction, which stated that the morphological aspect of the images did not indicate a difference between the area of chromatophores that fluoresced in the carbon dioxide-exposed and control corals \((F = 9.371, \text{df} = 1.359, p > 0.05)\). There was an overall negative trend of morphological fluorescence in the control and experimental tanks (Figure 1).

**Intensity**

IBM SPSS was again used to run a One Way Repeated Measures ANOVA on the data for the same reasons as the morphological data. A Greenhouse-Geisser correction was used since the data failed the Mauchly’s Test of Sphericity, \((F = 5.396, \text{df} = 1.252, p > 0.05)\). The data supports the null hypothesis; Environmental CO\(_2\)-addition does not have an effect on *L. hemprichii*’s fluorescent intensity output. There was an overall negative trend in fluorescent intensity for both the control group and the experimental group (Figure 2).
The percent area of image-containing morphological chromatophores that fluoresced under a limited cell:noise ratio in *L. hemprichii*. Control tank (blue) and experimental tank (red) morphological fluorescence values plotted against their respective time of manipulation. Carbon dioxide-addition occurred for the experimental tanks only through Weeks 1 and 6. A Repeated Measures One-Way ANOVA revealed no significant difference of morphological fluorescence between the corals that received the treatment and those that did not (p > 0.05).
Figure 2
The percent area of chromatophore fluorescent intensity in *L. hemprichii* from the previously thresholded morphological images. ImageJ was used to determine the % area of the pixel count from each image, resulting in a direct measure of pixel intensity. Control tank (blue) and experimental tank (red) fluorescent intensity values plotted against their respective time of manipulation. Carbon dioxide-addition occurred for the experimental tanks only through Weeks 1 and 6. A Repeated Measures One-Way ANOVA revealed no significant difference of morphological fluorescence between the corals that received the treatment and those that did not (p > 0.05).
Discussion

Many coral reefs around the world are experiencing massive bleaching events that are more often than not caused, or at least worsened, by anthropogenically-induced ocean acidification. Data found in this experiment supported the null hypothesis - Coral fluorescent intensity output is not lowered by environmental CO$_2$-addition. Though, interpretations of Figures 1 and 2 indicate that further, sound research should be conducted.

It was expected that in either figure, the control tanks would show no change in fluorescent intensity, while the CO2-exposed tanks would present a steady, decreasing trend of fluorescent intensity. Figures 1 and 2, however, share a common, negatively directed trend in fluorescence in both controls and experimental tanks. The intensity values of each coral in either figure did not read identical for any of the initial values. This poses a problem when trying to analyze data because it becomes difficult to gauge the extent of our treatment. Also, because the fluorescent intensity was interpreted from the percent area of each image, this unexpected result could have been due to an improper sampling technique. For example, a negative trend could have been created by accidentally taking samples, and likewise images, with fewer chromatophores to analyze, resulting in a misinterpreted trend. Though, this would not explain the strikingly similar plotting pattern that both of the control tanks share in each figure. Control tanks in Figures 1 and 2 possess generally high initial values for fluorescence, then drop to a low value at Week (manipulation) 1, and rise to an intensity value near or larger than its original by Week (manipulation) 2, before decreasing for the rest of the manipulations. This suggests that there is a natural fluctuation in the tissues of $L.$ hemprichii’s
fluorescent intensity values, or that the sampling technique posed accidental stressors on the coral itself.

A similar, but not identical, plotting pattern is seen for the experimental tanks. The experimental tanks in both figures begin with Initial values at a high, then drop dramatically at Week (manipulation) 1, and do not dramatically rise at Week (manipulation) 2. Instead, the either values seem to slightly rise at a later week (either W3 or W4). This suggests that the environmental CO₂-addition delayed the response of fluorescent intensity increase, as well as lessened the intensity increase when it did occur.

Figures 1-2 present an interesting trend in general. All of the plotted intensities, except for the control tank in Figure 2, seem to be “climbing” towards its original measured value. That is, while the overall trend is negative, later increases in measured values suggest that a type of adaptive behavior might be occurring. Perhaps it could be said that a coral’s fluorescent intensity is an adaptive trait that actively interacts with its ever-changing environment to eventually reach its original value, or one that is satisfied with its external conditions. This generalization can be strengthened if more samples were taken at later periods of time to observe a more defined pattern.

Hardy et al. (1992) performed research similar to the intent of this project. Pulsed laser light was used to induce fluorescence in 5 difference species of Caribbean coral. Their spectral analysis was used to indicate fluorescent intensity changes with the ultimate goal of determining health status after corals were exposed to temperature-induced stress. They found that each corals’ fluorescence spectra changed prior to visible bleaching events. Thus, pulsed laser light is a potential tool in determining coral health status prior to bleaching events.
Research completed by Salih et al. (1998) succeeded in imaging three-dimensional morphology samples of the fluorescent granules in light- and shade-acclimated corals, showing that the chromatophore location (above or below) with respect to the zooxanthellar symbionts plays an important role in manipulating light to optimize living conditions.

Although this experiment did not find a significant impact of environmental CO₂-addition on coral fluorescent intensity, it is still important to understand that its characteristic biofluorescence is yet to be fully understood. Here, it was not proven that fluorescence can be used as a noninvasive method of assessing coral health prior to bleaching events, however, other experiments show that it can. Further research needs to be conducted on a variety of coral species’ fluorescence values before and after CO₂-addition to truly determine if fluorescence is representative of health status.

Complications were common throughout this project. Preparing a marine tank from artificial materials (Instant Ocean) is not the same as preparing a marine tank from native seawater. The corals were treated for a very limited amount of time before images were taken - perhaps 24-hours is not enough time to allow for a response to occur. Likewise, the overall experimental time period only lasted 7 weeks. Tank 2 was left off for at least 8 hours during week 5, possibly causing the coral to undergo stress due to the temperature drop, light restriction, and stagnant water. Multiple images of each sample were not taken until Week 3, meaning that the data from W1 and W2 are weaker than the averaged data from W3 – W6. The gain of the green channel was only adjusted during the imaging process at and after Week 3, meaning that the gain in W1 and W2 might have been more saturated than in W3 – W6. This does not directly affect the results,
though, since only the images’ red channels were used for analysis. A 10X magnification was used throughout the entire imaging process. A stronger magnification might have been helpful in determining the fluorescent location on the chromatophores. Finally, the tissue sampling technique resulted in morphologically-weak samples. In other words, morphology of the tissue was not well represented as a result of fussing with tweezers.

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