Responses of the reef-building coral *Pocillopora damicornis* to bacterial stress from *Serratia marcescens*

Lauren Knasin¹, Cailey Tone¹, Linzhi Zhu¹
¹Boston University Marine Program, Boston MA 02215

Abstract
Coral reef ecosystems have been extensively affected by the culmination of climate change induced stressors resulting from anthropogenic disturbances. Rises in sea surface temperature specifically have shown to not only increase the spreading of infectious diseases, but also host susceptibility of the already less-resilient corals. Outside of the coral disease hotspots in the Caribbean, there is a lack of research on pathogens originating from outside of the coral microbiome. Here, we studied the susceptibility of 13 different colonies of an Indo-Pacific reef building coral, *Pocillopora damicornis*, when infected with the human-mediated pathogen, *Serratia marcescens*, over a 16-day period. Compared to corals held in the control system, the treatments subjected to the disease experienced an overall greater percentage of affected tissue, increases in photochemical efficiency, and increases in color intensity. Our results indicate that the Indo-Pacific corals were susceptible to the human-induced pathogen exhibiting intraspecific variations in physiological responses.

Introduction
Since 1955, research has shown that the ocean stores more than 90% of the excess heat trapped by greenhouse gases due to the high heat capacity of water (Khilyuk and Chilingar 2004). As a result of this absorption, the oceans are being negatively affected in a multitude of ways including significant changes in storm patterns, reduced pH levels due to rises in CO2 concentrations, and increases in sea surface temperatures (Wright et al., 2019). These climate change driven impacts have become increasingly critical issues and together they threaten coral reef ecosystems globally (Eakin et al. 2008). As storms are increasing in frequency and intensity, corals are at risk of destruction while already compromised from decreases in growth rates and structural integrity due to ocean acidification (Hoegh-Guldberg et al., 2007). While all of these climate change effects negatively impact coral reefs increases in temperature have been shown to be the primary contributor to coral bleaching (Heron et al., 2016). These thermal anomalies cause bleaching, but also have been shown to increase the spread of infectious diseases in corals (Tracy et al., 2019). As temperatures rise, host susceptibility and pathogen abundances increase (Bruno et al., 2007; Maynard et al., 2015) causing severe threats to coral reef ecosystems. In some cases, marine disease has been shown to cause greater coral mortality than bleaching (Maynard et al., 2015; Miller et al., 2009). The culmination of these climate change stressors on corals has been predicted to increase coral disease susceptibility, causing them to be less resilient when fighting off marine pathogens (Maynard et al., 2015), making understanding resilience to pathogens of utmost importance.

The Indo-Pacific reefs contain around 80% of corals worldwide, but not much information is known about the effects of disease on these corals (Willis et al., 2004). Much of the coral disease research has extensively focused on Caribbean corals, suggesting that this area is a coral disease hotspot (Green and Bruckner 2000). Caribbean coral diseases have been shown to originate
from both the coral and human microbiome (Willis et al., 2004). Specifically, elkhorn and staghorn corals of the Caribbean, _Acropora palmata_ and _Acropora cervicornis_, have lost up to 70% of living coral cover as a result of the human-originated pathogen _Serratia marcescens_. This bacteria, an inducer of white pox, causes rapid coral tissue lysis (Patterson, et al., 2002). However, we know very little about how _S. marcescens_, and other human-mediated microbes, influence coral fitness in corals from the Indo-Pacific. Previous studies on Indo-Pacific corals have shown that _Pocillopora damicornis_ and others have been affected by _Vibrio coralliilyticus_, a naturally occurring bacteria in the coral microbiome that becomes toxic in higher concentrations when sea surface temperatures rise (Gibbin et al., 2018). However, the response of _P. damicornis_ to human-related pathogens has not been thoroughly studied.

In order to understand the effect of _S. marcescens_ on an Indo-Pacific coral species, we subjected _P. damicornis_ to infection by _S. marcescens_. This study will determine if _P. damicornis_ is susceptible to a human-induced bacteria and if there is variation among colonies in susceptibility. We hypothesize that after the inoculation of _S. marcescens_, _P. damicornis_ would exhibit moderate signs of infection as seen by loss of coral tissue. This coral is highly abundant throughout the Indo-Pacific, with possible losses being catastrophic to their ecosystems. By understanding specific thresholds to infection, and how these thresholds vary among individuals within a species, we can better predict how these ecosystems will respond to the increasing anthropogenic effects of climate change.

**Methods**

**Coral Collection**

Thirteen colonies of _Pocillopora damicornis_ were purchased from the aquarium trade. These colonies were fragmented, glued to ceramic dishes (4.5 cm diameter), and left to recover for two weeks. Two fragments were then selected from each colony, one for a control system and another for the disease treatment. In total, 26 fragments were studied in this experiment, 13 in each experimental group.

**Experimental Setup and Water Quality**

Two identical systems, control and treatment, were constructed as open water baths to keep a constant temperature of 26°C. Each coral fragment was placed in an individual 16 oz glass jar pre-filled with 300 mL of fresh lab-made seawater and then placed in their respective water baths. Corals of both systems were not fed for the duration of the experiment. Fluorescent UV lights were set on a twelve-hour timer to produce a 12-hour day-night light cycle, with light levels for the control system being maintained at 87.2 ±15.2 µmol m⁻² s⁻¹ and the treatment system at 85.7±17.9 2 µmol m⁻² s⁻¹. Before the experiment began, all corals acclimated for 24 hours in their individual jars without any experimental treatment. Salinity of a randomly selected jar from each system and water temperature of each bath was tested twice each day, in the morning and the afternoon (Table 1). Water changes for each jar occurred daily in the afternoon using fresh seawater that had been placed in water baths each morning to acclimate to 26°C. After each water change, the jars were rotated one position clockwise around the water bath and placed in marked locations to randomize light exposure variation. Experimental instruments used for each system were kept separate to avoid microbe contamination.

**Bacteria Culturing and Dilution**

Culturing- _Serratia marcescens_ from a frozen glycerol stock was streaked on a plate of 1.5% marine agar and left to grow in a 37°C incubator overnight. The following steps were identical for the control and treatment group in all aspects, except the treatment utilized...
bacteria plates while the control utilized marine agar plates as blanks. To obtain liquid cultures of bacteria, 2 mL of marine broth were added to 20 tubes, designating 10 for each experimental treatment. Micropipette tips were used to transfer bacteria to the 10 experimental tubes by poking isolated colonies on the plate, while tips were poked into plain marine agar plates before transferring to the remaining 10 tubes to serve as control media. Once each tube received one micropipette tip, they were placed on an electronic shaker in an incubator operating at 180 rpm in a 37 °C, allowing for bacteria growth for at least 24 hours.

Dilution and Inoculation - After overnight culturing, micropipette tips were removed with sterilized tweezers and cultures were centrifuged at 3000 rpm for 5 minutes. Excess liquid was discarded and a wash of 2 mL of autoclaved seawater (~33 ppt) was added to each tube. Samples were centrifuged again under the same settings with this wash was repeated twice. After the second wash was discarded, 1 mL of seawater was added to each tube and was thoroughly mixed via vortex and micropipetting before being universally combined into two stock solutions, one blank and one bacterial treatment. The concentration of the bacteria solution was obtained using the OD600 of a DeNovix DS-11 with a blank calibration using autoclaved seawater. This stock solution was diluted with seawater until the OD600 read 0.235 ± 0.05 corresponding to ~2.4x10^8 colony forming units (ascertained by streaking dilutions onto marine agar plates grown at 37 °C overnight) with a total volume of at least 39 mL for inoculation into treatment jars.

Following daily water changes, corals received ~3 mL of solution, either blank solution for control corals and bacterial solution for treatment corals at ~4 p.m. each day.

Photochemical Efficiency (Fv/Fm) 

Pulse Amplitude Modulated (PAM) Fluorometry values were obtained on the sixth and fourteenth day of inoculation to measure photochemical activity (Fv/Fm). The Junior-PAM fluorometer, accessed through the Win-Control3 software, was set to the following settings: saturation pulse width- 0.6, saturation light intensity- 12, electronic signal damping-2, electronic signal gain- 4, and measuring light intensity-2. All corals were subjected to at least one hour of dark acclimation before using the Junior-PAM. The system was blanked by pointing the probe away from the corals in the seawater before taking measurements in each system. Measurements for each coral were taken until three values fell within 0.1 of one another, with values above 0.75 omitted. The three accepted values were then averaged.

Color analysis 

Photographs of each coral, placed in the middle of a Coral Health Chart card (CoralWatch) were taken each morning for color and lesion progression analyses. Color analysis was conducted every three days throughout the duration of the experiment. In order to correct for variations in light intensity across images, a white point was calibrated to a value of 255 in Adobe Photoshop, deemed pure white. The white point for every picture was chosen in identical locations on the Coral Health chart across photographs. To quantify color intensity, the macro “AnalyzeIntensity” on MATLAB was utilized (Winters et al. 2009). Ten points were randomly selected for each nubbin, avoiding polyps, and values from the red channel color intensity were collected. The red channel was selected as it is an effective predictor in determining the density of coral symbionts for bleaching. Higher color analysis values are correlated with lower symbiont densities and overall a whiter coral color. Data of the red channel intensity were collected every third day during the
experiment with the first color analysis occurring the day before the first inoculation.

**Cox proportional hazards model**

The effects of the experiment on the survivorship of the nubbins were estimated using the Cox Proportional Hazard model (Cox 1972) visualized in the survival and survminer packages in R (Therneau 2015). Times of affection without recovery for every coral of each system were noted and inputted into this model.

**Percent of affected tissue**

ImageJ (bundled with Java 1.8.0_172) was used for measuring the areas of affected tissue for each applicable coral. The percentage was calculated by dividing the areas of affection by the total tissue cover. The pictures taken on day 16 of the experiment were utilized. Table 3 was created to represent the end phenotypic results for each genet of *P. damicornis* in the control and treatment groups.

**Statistics**

All data were analyzed and visualized using the R environment (R Development Core Team 2016). One-way ANOVAs were conducted to determine any statistical differences between the two systems while paired t-tests were used for comparisons within each system for all experimental factors.

**Results**

**Photochemical Efficiency**

The difference in photochemical efficiency (Fv/Fm) between the two systems was found to be statistically insignificant (p = .966) throughout the course of the experiment (Figure 1A). Corals under treatment exhibited an insignificant (p = .186) increase in Fv/Fm values while the controls showed a significant (p = .031) decrease between the two sampled days, day 6 and 14 of the experiment. The final Fv/Fm measurements for the diseased corals had the largest standard deviation compared to the others (Table 2). The difference between the percent change of PAM values for each system were also found not to be statistically significant (Figure 1B, p = .965).

**Color Analysis**

All experimental samples increased significantly in red channel intensity values over the course of the experiment (treatment- p < .001, control- p = .011) which corresponds to bleaching. Yet, there was no significant difference in these recorded values between the systems (Figure 2A, p = .416). Substantial increases in color intensity for all corals occurred within the first 3 days of the experiment for both systems and maintained color intensity in the next six days. The corals subjected to the disease experienced another loss in color intensity between days 9 and 12 of the experiment. Despite being insignificant, the control and treatment systems share a similar increasing trend of color intensity throughout the experiment, ending at similar mean values (Table 2). The difference in color intensity from the first to last experimental day between the two systems was insignificant (p = .144, Figure 2B). For better visualization, we chose to multiply our data by -1 in figure 2A so decreasing values correspond to bleaching, however increasing color intensity values are still indicative of this color loss.

**Cox-proportional hazard**

Cox-proportional model showed that there was an equal amount of affected corals under both conditions at the end of the experiment (Figure 3, p = .918). Diseased corals did not become affected until day 8 of the experiment while those in the control were impacted as early as day 3.
Percent of affected tissue

Overall, the diseased corals exhibited higher percentages of affected tissue than those of the control (Figure 4). There was an insignificant difference in the percent of affected tissue between the two systems ($p = .0962$). We considered overall bleaching or paling and tissue lesions as indications of affected tissue (Table 3).

Discussion

The aim of this study was to determine if *Pocillopora damicornis* was susceptible to the human-originated pathogen, *Serratia marcescens*, and whether or not the response to the disease would differ among individuals. We had predicted there would be moderate signs of infection from loss of tissue, thus expecting to see an increased percentage of affected tissue, and decreased values of photochemical efficiency ($Fv/Fm$) and color intensity. Our results confirmed increases in the affected tissue area and color intensity. Contrary to our predictions, there was an increase in $Fv/Fm$ values for diseased corals. The experiment also showed variation among colonies subjected to the treatment ranging from sudden tissue lysis to tip and branch lesions, and bleaching and paling.

Effects of Disease on Photochemical Efficiency ($Fv/Fm$)

Higher $Fv/Fm$ values correspond to more efficient photosynthetically active symbionts (Winters et al., 2003). It was expected that by the end of the experiment, lower photochemical efficiency values for the treatment group would show as signs of increasing infection (i.e. tissue lesions and bleaching). Surprisingly, we found that there was an insignificant increase in average $Fv/Fm$ values for the diseased corals (Figure 1A). We hypothesized that these increases in $Fv/Fm$ values were due to the corals feeding on *S. marcescens* receiving excess nutrients to support the productivity of their microbes (Wright et al. 2019). As the corals in both systems were not fed for the duration of the experiment, it was possible that the treatment group utilized nutrients from eating the pathogen to better maintain their microbial environment. Thus, this could explain how symbionts of the treatment were able to keep up and even increase their photochemical efficiency better than those in the control.

The control group experienced a significant decrease in the photochemical efficiency of the symbionts. Corals under experimental conditions experience stress that can cause them to expel some of their symbionts. This would account for the significantly lower recorded $Fv/Fm$ values after the course of the experiment. On day 8 of the experiment, high levels of salinity of $\sim 40$ ppt were recorded in the control system which resulted in the paling of most nubbins. Following this event, control corals maintained higher color intensities for the majority of the experiment until experiencing another increase during day 13. No control coral was able to fully recuperate to beginning color intensity levels possibly resulting in these decreased values.

Overall, the changes that occurred in both experimental groups were not significantly different when compared to each other (Figure 1B). The increase in $Fv/Fm$ values for the treatment group were not significant when compared to the control, thus we are unable to conclude that these results are due to the infection of *S. marcescens*. This experiment should be run for a much longer duration of time in order to make more decisive conclusions, as corals are long-lived species and the effects after 16 days can only produce possible trends.

Effects of Disease on Color Analysis

After the 16-day experiment, corals of both systems showed significant increases in color intensity which corresponds to bleaching (Figure 2A) as predicted. Because there was
not a significant relationship between the percent changes of each system, we cannot conclusively say the diseased corals increased in color intensity due to the pathogen (Figure 2B). Corals of both systems withstood substantial increases in these values within the first 3 experimental days, possibly due to the stress of a new environment. From day 3 to day 9, it appears that the corals of both systems became better acclimated to their environment and maintained their color intensity values. On the eighth day, the control corals suffered from highly saline water, 40 ppt, and increased their values of color intensity once again. As salinity and temperature remained fairly constant after the salinity anomaly, there was a significant increase in color intensity for corals of both systems on day 13. It is possible that the corals had reached a stress threshold to being in an experimental setting in individual jars which didn’t allow for proper water circulation.

Cox-proportional hazards model

At the end of the experiment, both the treatment and control groups experienced an equal percentage of affected corals through tissue lesions, paling, or bleaching (Figure 3). A total of 10 corals exhibited these signs of stress at the end of the experiment, five in each experimental group. The rates at which the corals showed signs of affection were insignificant between the two systems. It is proposed that the insignificant rates and the same number of affected corals are due to the stress of the experimental setting.

ImageJ

The corals under treatment resulted in a higher percentage of total affected tissue compared to those in the control (Figure 4). These areas were measured as regions of lysed tissue and areas of bleaching. Though there was no significant difference between the percent of affected tissue when comparing the two experimental groups. Those that were affected in the control group only showed signs of bleaching. Two of the three treatment corals which were negatively affected by had visible lesions which began at the tips and eventually spread to the entire branch while the others bleached overall. This supports the importance of intraspecific variation amongst corals and their ability to acclimate or overcome certain stressors.

Conclusions

Overall, our findings indicate that *Pocillopora damicornis* showed signs of infection when under the stress of *Serratia marcescens* for some corals. However, across colonies, there were variations in responses ranging from no effect to paling to complete bleaching and branch death. Despite only a small percentage of our subjected corals showing signs of infection, it cannot be ruled out that these corals are resilient to the bacteria as this experiment was confounded by limitations. *S. marcescens* has been studied to have a relatively slow rate of transmission compared to other marine pathogens (McCallum et al., 2003). In addition, prevalent disease activity has only been noted for this disease following the surpassing of maximum mean monthly sea surface temperature (Lesser et al., 2007). Thus, as a result of this relatively short-term experiment and the consistently normal temperature environment, the majority of corals in the treatment system did not exhibit significant signs of disease. In future studies, it would be beneficial to not only increase the longevity of the experiment, but also add the experimental factor of temperature. As sea surface temperatures continue to rise, *S. marcescens* could become a more prevalent threat to corals, making this research essential.
Table 1. Summary of water quality measurements (temperature and salinity) for each system during the 16-day experiment.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Salinity (ppt) ± SD</th>
<th>Temperature (°C) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.31 ± 1.08</td>
<td>25.60 ± 0.50</td>
</tr>
<tr>
<td>Treatment</td>
<td>33.13 ± 1.20</td>
<td>25.10 ± 0.61</td>
</tr>
</tbody>
</table>

Average salinities and temperature with standard deviations over the course of the experiment for each system. Temperatures were taken in the morning and afternoon and salinity was just taken in the afternoon just before water changes.

Table 2. Summary of initial and final measurements of photosynthetic efficiency and color intensity analysis for each system.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Fv/Fm</th>
<th>Final Fv/Fm</th>
<th>Initial Color Intensity</th>
<th>Final Color Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.651 ± 0.022</td>
<td>0.629 ± 0.043</td>
<td>143.09 ± 48.57</td>
<td>183.3 ± 31.44</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.638 ± 0.033</td>
<td>0.643 ± 0.066</td>
<td>126.25 ± 40.60</td>
<td>183.14 ± 10.13</td>
</tr>
</tbody>
</table>

Average Fv/Fm and color intensity values with standard deviations during the course of the 16-day experiment. Initial measurements for Fv/Fm occurred on day 6 of the experiment while final measurements were taken on day 14. A paired t-test determined the change in photosynthetic efficiency for the treatment system was insignificant (p = 0.186) while that of the control system was determined significant (p = 0.031). Color intensity values were taken every 2 days, but only final and initial values are reported in the table. A paired t-test determined the change in color intensity values were significant for both systems (p < .001, control- p = 0.011).

Table 3. Summary of phenotypic responses for 13 colonies of *Pocillopora damicornis* in control and treatment systems.

<table>
<thead>
<tr>
<th>Coral Colony</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Overall bleaching</td>
<td>Overall bleaching</td>
</tr>
<tr>
<td>C2</td>
<td>Overall bleaching</td>
<td>Tip lesions</td>
</tr>
<tr>
<td>EB</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>EG</td>
<td>Tip bleaching</td>
<td>No effect</td>
</tr>
<tr>
<td>EP</td>
<td>Overall bleaching</td>
<td>No effect</td>
</tr>
<tr>
<td>GE</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>OG</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>OM</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>OP</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>P4</td>
<td>Tip paling</td>
<td>Tip paling</td>
</tr>
<tr>
<td>P7</td>
<td>Tip bleaching</td>
<td>Completely bleached</td>
</tr>
<tr>
<td>P8</td>
<td>Overall bleaching</td>
<td>Branch completely bleached</td>
</tr>
<tr>
<td>PG</td>
<td>Overall bleaching</td>
<td>Branch completely bleached, overall bleaching</td>
</tr>
</tbody>
</table>

Two fragments of each colony of *P. damicornis* were subjected to either a control or treatment systems. Using pictures taken each morning throughout the experiment, it was noted the extent to which each coral was affected without recuperation when compared to initial photographs.
Figure 1. Photochemical efficiencies (Fv/Fm) for controlled and treatment Pocillopora damicornis over the 16-day experimental period. Corals under treatment exhibited an insignificant ($p = .186$) increase in Fv/Fm values while the controls showed a significant ($p = .031$) decrease between days 6 and 14 of treatment. Each point is the culmination of the averages of 3 measurements for each coral within their respective treatment, with error bars denoting standard error. (a) The difference in photochemical efficiency (Fv/Fm) between the two systems was found to be statistically insignificant ($p = .966$) throughout the course of the experiment. (b) Percent differences of Fv/Fm values between the 6th and 14th days for control and treatment systems. The change of photochemical efficiencies of the two systems was found non-statistically significant ($p = .965$).

Figure 2. Red channel color intensity analysis for Pocillopora damicornis under bacterial stress from Serratia marcescens over the 16-day experimental period. Significant increases in red channel intensity values for each system were found over the course of the experiment (treatment-$p < .001$, control-$p = .011$). Each point shown in the figure is the culmination of the averages of 10 red channel values for each coral within their respective treatment, with error bars denoting standard error. (a) Red channel color intensities between control and treatment systems. There was no significant difference in these recorded values between the two treatments ($p = .416$) despite the shared trend of increasing color intensity. (b) Percent differences of red channel color intensities between final and initial measurements of each system. The difference in color intensity from the first to last experimental day between the two systems was insignificant ($p = .144$). Color representations of each system, pink for treatment and teal for control, applies to all figures following.
Figure 3. Percent of unaffected *Pocillopora damicornis* nubbins by *Serratia marcescens*. Both control and diseased corals resulted in the same percent of affected corals as seen through tissue loss or bleaching. The rate at which nubbins were affected differed between each system, but at the conclusion of the experiment, equal percentages of treatment and control corals were affected. There was no significant difference at the end between affected corals between systems and throughout with their rates of affection ($p = .918$).

Figure 4. Percent of affected tissue area in *Pocillopora damicornis* under stress from *Serratia marcescens*. Percent of affected tissue was calculated utilizing pictures taken on the last experimental day. Lesions, bleaching, and paling were considered affected, and only corals with these effects were analyzed in this figure (5 corals from each treatment). The affected area of the coral was compared to the total area to yield a percent. The line within the box denotes the median percentage (treatment- 65.1%, control-26.7%). An insignificant difference is shown between the control and treatment systems ($p = .0962$).
References
Katsman CA, van Oldenborgh GJ (2011) Tracing the upper ocean’s “missing heat.” Geophys Res Lett 38:


Cox DH (1972) Regression Models and Life-Tables. Journal of