INVESTIGATION OF POTENTIAL VIRULENCE FACTORS IN PATHOGENIC MARINE BACTERIA

by

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Abstract

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Coral reefs provide numerous ecological and economic functions and are critical to biodiversity in marine environments. Critical to coral reef health and diversity is the microbial consortium within the coral host along with symbiotic algae. Coral reef invertebrate-microbe interactions within coral reef communities are rapidly shifting out of equilibrium throughout the globe. Higher temperatures are believed to be responsible for this destabilization.

In this study, I assessed the production of potential virulence factors in previously isolated bacteria believed to contribute to disease symptoms in a major reef building coral, *Orbicella faveolata*. I measured the doubling times, respiration rates, biofilm production, serine protease production, and recovery when exposed to reactive oxygen species in potentially pathogenic bacterial strains (*Vibrio splendidus* 1H5, 3F8, 3B7, *Aeromonas trotai* 1A9, 2H12, *Vibrio alginolyticus*, and *Vibrio campbelli* 3A8) associated with Caribbean yellow band disease. The bacterial responses were measured across three different temperatures (26°C, 29°C, and 32°C) corresponding to ambient, elevated, and maximum sea surface temperatures recorded within the coral reef habitat.

There were significant differences in temperature across all bacterial strains. Doubling times were generally higher at 29°C for all *Vibrio* spp. with the exception of *V.*

alginolyticus. Respiration rate trends were similar in all bacterial strains and an elevation in temperature corresponded to an increase in respiration rate. *V. alginolyticus* displayed the fastest respiration rate in comparison to all other bacterial strains. Biofilm production increased as temperature rose in all bacterial strains with the exception of *A. trotai* 1A9 and *V. campbelli* 3A8. Defense against reactive oxygen species showed a general decrease in recovery time as temperature was elevated to 29°C.

These findings suggest that while temperature does play a significant role in the doubling times of these potentially pathogenic species, growth is slowed at elevated temperatures in which Caribbean yellow band disease occurs at. The general pattern of increased respiration as temperature increases suggests that more energy is harnessed when temperatures are elevated. This may indicate that the potential for the synthesis of disease inducing factors increases as sea surface temperatures rise. The pattern of increased biofilm production in the majority of the bacterial strains as temperature is elevated indicates that these microbes may develop a resistance to antimicrobials secreted by the coral host. Further, there may be a dynamic trade off between bacterial strains in savaging the reactive oxygen species potentially secreted by the coral host and algal symbionts under thermal stress that allows for survival of a potentially pathogenic consortium of bacteria.

These findings suggest that there is an allocation of energy between different systems with these bacterial strains may be critical to the progression of Caribbean yellow band disease in *O. faveolata*. The general increase in doubling time at 29°C coupled with increased respiration rates at elevated temperatures suggests that growth alone may not be the key contributing factor in disease progression.

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Chapter 1

Introduction

1.1 Coral Ecology and Disease

Coral reefs are one of the most productive ecosystems on earth and shelter a wide array of biologically diverse organisms. In addition to this high biodiversity, coral reefs provide sustained monetary sustenance via ecotourism and fishing for many coastal communities. In addition to these functions, coral reefs act as a natural shield for coastlines against storm surges and coastal erosion (Ahmed et al. 2007). Novel antimicrobial compounds are found within these systems that provide a means to treat nosocomial infections that are of particular concern in today's age of antibiotic resistance (Nissimov et al. 2009, Thenmozhi et al. 2009*).* These factors make it pertinent to protect these valuable and diverse environments from sustained threats such as global climate change.

The temperature of Earth's oceans has risen steadily over the last few decades (Hoegh-Guldberg et al. 2007, Doney et al. 2012). This has had a profound impact on the decline of coral reefs (Harvell et al. 2004, Harvell et al. 1999, Smith and Smith 2009, Weil et al. 2006, Martin et al. 2010), stimulating many questions on the equilibrium of reef ecosystems. Of particular concern is the disruption of the critical coral-algal symbiotic relationship known as bleaching and may occur as result of temperature and UV stress. Bleaching in corals is a result of the expulsion of their symbiotic algae. The coral animal is a host to endosymbiotic algal cells (*Symbiodinium*) that provide the majority of nutrients to their coral host. In turn, the coral host provides a protective environment for the *Symbiodinium* to grow and reproduce.

One major contributor to the disruption of this equilibrium between the coral host and algae is infection by marine bacteria (Banin et al. 2001). (Rosenberg et al. 2007). In

addition to this disruption in the coral-algal symbiosis, there is also a change in what is known as the surface mucopolysaccharide layer. The surface mucopolysaccharide layer originates from the secretions of glycoproteins from epidermal coral mucus cells (Ducklow and Mitchell 1979) and coats the outer layer of the coral forming an interface between the water column and the actual animal. The microbial community associated with coral surface mucopolysaccharide layer has been shown to switch dramatically with a fluctuation in temperature (Ritchie 2006). This microbial community hosted by the coral is a dynamic network with multiple established niches (Rosenberg et al. 2007). Similar to the biotic imbalance in humans, the disruption in equilibrium between naturally occurring microbial communities within coral mucous layers has been associated with an increased prevalence in disease (Harvell et al. 2007, Mao-Jones et al. 2010, Kvennefors et al. 2012, Cooney et al. 2002).

1.2 Caribbean Yellow Band Disease and Disruption of the Algal-Coral Symbiosis

One particular disease which is prevalent in the Caribbean is Caribbean yellow band disease (CYBD). The disease presents itself as a yellow ring which steadily expands across the surface of the coral due to disruption of the *Symbiodinium* photosystem. This disease has a high prevalence in the important reef building coral *Orbicella faveolata*.(Weil et al. 2009a). CYBD prevalence and severity measured by disease-spread rates has been shown to increase with temperature. For example, the prevalence of CYBD increased from 1% to 55% in the *Montastraea annularis* species complex (of which includes *O. faveolata*) in La Parguera, Puerto Rico after the 2005 bleaching event where recorded temperatures reached their peak over a nine year survey (Weil et al. 2009 b). Although the exact pathogenesis of this disease has yet to be determined, there is a clear microbial shift in diseased colonies versus those of healthy colonies (Cunning et al. 2009).

There have been several attempts to characterize the pathogen(s) associated with CYBD and the genera most common to appear are *Vibrionaceae* and *Alteromonadaceae* (Kimes et al. 2010). *Vibrio* pathogens are prevalent in a multitude of marine species and are known to have the capability of expressing several virulence factors (Xie et al. 2005). There have also been several coral diseases verified to be caused by *Vibrio* pathogens. The microbiota of *Acropora millepora* is known to shift to a community of primarily Vibrios during the progression of disease (Bourne et al. 2007). The bleaching of *Oculina patagonica* and *Pocillopora damicornis* has been shown to be caused by *Vibrio shiloi* and *Vibrio coralliilyticus*, respectively (Kushmaro et al. 1997, Ben-Haim and Rosenberg 2002).

Vibrio proline-rich toxins and zinc-metalloproteases have been found to deactivate photosynthetic systems used by symbiotic algae in several coral species (Sussman et al. 2009, Banin et al. 2001). The deactivation of photosystems (chlorosis) in symbiotic algae by *V. shiloi* is thought to play a role in bleaching episodes in the coral *O. patagonica* (Banin et al. 2001). In the Indo-Pacific coral species *Diploastrea heliopora*, *Symbiodinium* from CYBD infected colonies show significant reduction in photosynthetic pigmentation, a reduced cell number relative to healthy colonies, and distortions within thylakoid membrane components (Cervino et al. 2008). In the Caribbean infections, although the pigmentation in *Symbiodinium* was lowered in CYBD infected tissue, the number of *Symbiodinium* cells within coral host tissue remained similar to that of healthy colonies, suggesting that the *Symbiodinium* cells are not lysed during the course of the disease (Mydlarz et al. 2009). It is currently unknown whether this mechanism is linked to the growth rates of associated pathogens or other virulence factors. In order to better understand the underlying factors which may contribute to the disruption in coral-

symbiodinium relationships it is imperative to ascertain certain fundamental characteristics of potential pathogens.

1.3 The Effects on Temperature on Putative Virulence Factors of CYBD-Associated Pathogens

Among the many factors that may be affecting disease progression in *O. faveolata* is biofilm formation among associated pathogenic bacteria. Biofilm formation is considered a major virulence factor in humans (Waters et al. 2008). In addition to human hosts, this same mechanism of biofilm-mediated resistance may be facilitating the increase in CYBD. In addition to being a potential virulence factor, biofilms are a community of a large microbial consortium existing in the coral reef ecosystem. A common method of antibacterial resistance is through the formation of biofilms from a consortium of species (Burmolle et al. 2006). The secretion of an exopolysaccharide matrix has been shown to aid in the resistance of a bacterial community to antibiotics. This results in a need for an increased concentration of antimicrobial compounds to achieve the same result as that seen in purely planktonic cells (Mah and O'Toole 2001). In addition to providing a physical barrier to halt antimicrobial penetration, biofilms are also characterized by the ability for a single species to take on multiple phenotypes (Sauer et al. 2002). Modification in phenotype typically equates to a change in metabolic activity so that growth characteristics begin to change. Metabolic processes are frequently the targets of antibiotic compounds found in nature. The presence of a biofilm can thus hamper a host's immune system in eradicating an infection, leading to increased damage sustained from the growth of the pathogen. Studies have demonstrated that commensal bacteria residing within the mucosal layer of corals have anti-biofilm activity in human pathogens (Nithyanand et al. 2010) suggesting that the microbial flora in corals have the ability to express antibiofilm compounds for defense.

One of the pathogens associated with black band disease, affecting the Caribbean coral species *Orbicella annularis*, is a member of filamentous cyanobacteria that covers the coral host in a black bacterial mat. A similar phenomenon takes place in corals affected by red band dsease as well (Cooney et al. 2002). There has been relatively little research in bacterial biofilms influencing disease in *Orbicella spp*. to date however, and the details surrounding biofilm colonization within coral mucosal layers are poorly understood (Sweet et al. 2011). There have been studies conducted elucidating the importance of biofilm growth on larval settlement in *Acropora millepora* where temperature appears to be a factor in biofilm production of recruiting factors (Webster et al. 2010, Tebben et al. 2011). In an additional study, it was found that when commensal bacteria isolated from *Oculina patagonica* formed a biofilm, growth of the pathogen *V. shiloi* was inhibited (Nissimov et al. 2009). These studies demonstrate that not only are biofilms essential to coral larva development but that residential flora within the coral mucosal layer provide immunological protection from invading pathogens in the form of biofilm growth.

Proteases are known to be major virulence factors in *Vibrio* spp. There are two main ways that proteases can contribute to disease in a host, by digesting host tissue or by contributing to the synthesis of other pathogenic factors (Shinoda and Miyoshi 2011). *Vibrio* pathogens associated with corals likewise are known to secrete proteases to the detriment of the coral host. This can be illustrated by the interaction between the previously mentioned *V. coralliilyticus* and *P. Damicornis* where an increase in temperature correlates with an increase in extracellular protease activity from *V. coralliilyticus* (Ben-Haim et al. 2003). *V. coralliilyticus* has also been shown to secrete metalloproteinases at elevated sea surface temperatures which attacks coral tissue (Rosenberg and Falkovitz 2004).

In order to combat shifts in the microbial community and presence of virulence factors, the coral host must employ several defense and immune mechanisms. Indeed, there appears to be a dynamic selection process within the mucosal layer of the coral where potentially pathogenic bacteria are selected against by antimicrobial compounds secreted by the coral (Ritchie 2006). In addition to possible antimicrobial compounds being secreted, granular amoebocytes hold acidophilic granules which contain reactive oxygen species. This constitutes a main immunological defense within the coral host which allows for early defense against pathogenic organisms through the use of inhibitory oxygen radicals. Enzymatically released oxidative bursts are also used in attacking invading microbes which provide a brief flux of hydrogen peroxide and other reactive oxygen species (ROS) (Mydlarz and Jacobs 2006).

1.4 Thesis Aims/Objectives

This study examines the growth rates, respiration rates , biofilm production, secretion of proteases, and antioxidant activity of seven potential pathogenic bacteria associated with CYBD, previously isolated from infected *O. faveolata* (Weil et al 2008), under ambient and elevated temperatures. Strains were previously characterized phenotypically using Biolog (Heyward, CA) and identified through16s rRNA sequencing. The seven bacterial strains were most closely related to *Vibrio splendidus* 1H5, *Vibro splendidus* 3F8, *Vibrio splendidus* 3B7, *Aeromonas trotai* 1A9, *Aeromonas trotai* 2H12, *Vibrio alginolyticus*, and *Vibrio campbelli* 3A8. These seven bacterial strains were utilized due to their presence in CYBD infected *O. faveolata* and previously shown association with *O. faveolata* disease when introduced into a healthy host coral. *A. trotai* strains also allow a comparison to be made between *Vibrio* strains which may show differences between unrelated bacteria and suggest new dynamics involved in infection of the host.

I hypothesize that an increase in temperature will lead to not only an increase in growth rates and respiration rates among all bacterial strains but also an increase in biofilm formation, protease production, and a faster recovery from the introduction of reactive oxygen species. I also hypothesize a significant similarity in growth rates and respiration rates between bacterial strains of the same species. This study aims to answer fundamental questions regarding the metabolic activity and virulence factor expression in bacteria associated with CYBD in order to lay a foundation for further investigation of the underlying mechanisms contributing to the decline of *O. faveolata* due to increasing disease prevalence.

Chapter 2

Materials and Methods

2.1 Origin, Isolation, and Maintenance of Bacterial Cultures

All strains were previously isolated from CYBD infected *O. faveolata* colonies near La Paguera, Puerto Rico (Weil et al. 2008). Briefly, samples were collected from surface mucopolysaharride layers using syringes and plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS) and restreaked from isolated colonies using additional TCBS plates and incubated for 24 hr. Strains were previously characterized phenotypically using Biolog (Heyward, CA) and identified through16s rRNA sequencing (Weil et al. 2008). Dr. Kim Ritchie provided the inoculated samples in Luria-Bertani broth supplemented with 2.5 % red sea salt. Each culture was then isolated from the Luria-Bertani inoculated broths using separate TCBS agar plates incubated at 29°C for 24 hr. Isolated colonies from each TCBS agar plate were then used to inoculate separate tryptic soy agar (TSA) plates which were incubated at 29°C for 24 hr and stored at 4°C until needed.

2.2 Doubling Time

The growth rate of each bacterial strain was ascertained through optical density readings by the use of a Synergy 2 Microplate Reader (Biotek Instruments, Winooski, Vermont, USA). Cultures were prepared by inoculating Luria-Bertani broth (Amresco, Solon, Ohio, USA) with an addition of 2.5% Instant Ocean salt mix for a total of 3.5% salt concentration (LB salt) using a separate colony from each TSA plate for each strain in replicates of three. All cultures were incubated over night at 26°C, 29°C, and 32°C in LB salt broth. Each culture was diluted down to $1x10^5$ CFU/mL using LB salt broth of which 200 µL of the diluted bacteria was added to each well in replicates of three for each isolated colony culture. The optical density was then read at 600 nm at intervals of ten

minutes for 24 hr using separate clear round bottom, non-tissue treated 96-well plates (Greiner Bio One, Monroe, North Carolina, USA) at 26°C, 29°C, and 32°C. The doubling time of each bacterial strain was then calculated by taking the slope of the perceived exponential phase and applying the formula for doubling time shown below.

$$\text{Doubling time} = \frac{\text{Time}}{3.3 * \log(\text{Final absorption reading}/\text{Initial absorption reading})}$$

2.3 Alamar Blue Assay

The test used for quantifying respiration rates was achieved through the use of resazurin salt (Alfa Aesar, Ward Hill, USA), commercially known as alamar blue. As per the Invitrogen online manual via TREK Diagnostic Systems, the assay was optimized for use of a 1X10^3 CFU/mL concentration of bacteria (data not shown). Alamar blue was made by combining 5.5 mg of resazurin salt in 50 mL of 0.2 M NaPBS pH 7.6 for an overall concentration of 0.11 mg/ml (O'Brien et al. 2000). Replicates of three for each bacterial strain were grown overnight at 26°C, 29°C, and 32°C in LB salt broth and diluted to 1X10^3 CFU/mL using the same media. 200 µL of the diluted bacteria were then plated and 20 µL of the alamar blue was added to each well of a black, clear flat bottom, non-tissue treated 96-well plate (Greiner Bio One, Monroe, North Carolina, USA). A positive control of 100% reduced alamar blue was made by autoclaving a 10% solution diluted using LB salt broth for 30 min. A standard curve of 100%, 75%, 50%, and 25% reduced alamar blue was also plated with the samples. Photospectrometer parameters were set to 530/25 excitation, 590/35 emission with a sensitivity of 35 for fluorescent reads. Each plate was read for 6 hr at intervals of 10 min with intermittent shaking for 5 seconds between each read. The percent reduction using fluorescent reads was calculated using the formula provided in the Invitrogen online manual. The time point

readings were converted to percent reduction of alamar blue and the time it took each strain at 26°C, 29°C, and 32°C to reach 50% reduction by using Microsoft Excel software.

2.4 Biofilm Production

Biofilm mass was quantified by crystal violet staining using the method in Christensen et al. (1985). Briefly, bacterial cultures were grown overnight at 26°C, 29°C, and 32°C and diluted to $1X10^5$ CFU/mL using LB 2.5% salt media. The bacteria were then plated on a clear round bottom 96 well plate (Greiner Bio One, Monroe, North Carolina, USA) and grown for 24 hr at their respective temperatures in a Synergy 2 Microplate Reader in replicates of nine. After the 24 hr incubation, the plate was taken out and the planktonic bacterial suspension was pipetted out of each well. Each well was then washed three times with DI water and left to dry for 1 hr. 200 µL of 1% crystal violet was then added to each well and allowed to sit for 1 hr. The crystal violet solution was then pipetted out and each well was rinsed with DI water three times. 250 µL of 95% ethanol was added to each well. 150 µL of this solution was then pipetted over to a new 96-well round bottom plate and then put into the Synergy 2 Microplate Reader to be read at 600nm. Crystal violet retained after washing was extracted using 95% ethanol in order to ascertain the non-planktonic biomass adhering to the wall of each well. The crystal violet forms a complex within the peptidoglycan layer of each cell and is retained until extraction with the ethanol. Crystal violet readings provided an indirect measure of the amount of cells present within the biofilm of each experimental well.

2.5 Serine Protease Production

Protease production was assayed through quantification of serine substrate cleavage. 1 mL of supernatant was extracted from 24 hr cultures of each strain at their respective temperatures from 10 mL LB 2.5% salt media after centrifugation 2205 x g for 10 min. The supernatant was then flash frozen in liquid nitrogen and stored at -80˚C until

further use. 10 µL of fluorescein isothiocyanate (FITC) casein substrate (Sigma Aldrich, St. Louis, MO, USA) was added to 20 µL of thawed supernatant extract along with 20 µL of 20 mM PBS (pH 7.6) in a centrifuge tube for 1 hr at 37°C. 150 µL of 10% trichloroacetic acid was added and gently mixed and incubated at 37°C for 30 min. Tubes were then centrifuged for 10 min at 5590 x g. The supernatant of each sample was then read at 485 nm excitation and 535 nm emission wavelength at 535 nm on a Synergy 2 Microplate Reader (Biotek Instruments, Winooski, Vermont, USA).

2.6 Reactive Oxygen Species Recovery

Replicates of three for each bacterial strain were grown overnight at 26°C, 29°C, and 32°C in LB salt broth and diluted to $1X10^5$ CFU/mL. The bacteria were plated in replicates of three for each of three colonies grown from separate strains with 200 µL of bacteria and 20 µL of 500 mM hydrogen peroxide obtained from the dilution of 8,800 M stock solution of hydrogen peroxide in 100 mM of NaPBS buffer. The optical density of each well was read at 600nm at 26°C, 29°C, and 32°C for 24 hr. The recovery time after the addition of hydrogen peroxide was defined as the time point at which each experimental replicate reached an equal optical density as that of the control treatment of 200 µL of bacteria added with 20 µL of 100 mM NaPBS buffer. This was measured to ascertain the ability of each strain to scavenge reactive oxygen species and begin optimal growth as a proxy for defense against reactive oxygen species potentially secreted by the coral host. Time to recovery of the treated bacterial samples was calculated using Microsoft Excel software by identifying intersection points between both the experimental and control growth curves for each replicate.

2.7 Statistical Analysis

The data were analyzed using IBM SPSS Statistical Software version 21.0. Homoscedasticity was confirmed using Levene's test of equality of error variance and

normality was confirmed using the Shapiro-Wilk test. Non-normal data were transformed using the Box-cox method through JMP 10.0 software. One-way ANOVAs were performed for within and among strain effects for each assay performed. Non-parametric tests were performed using Kruskal-Wallis one-way analysis of variance. Tukey-Kramer post-hoc analyses were used to detect differences within and among strains for each factor with normal distributions while Mann-Whitney U post-hoc tests were used for non-parametric data.

Chapter 3

Results

3.1 Growth and Respiration

Doubling time as a measure of bacteria growth followed a general pattern of increasing at 29˚C and dropping at 32˚C. Several strains showed this pattern *V. splendidus* 1H5, 3F8, 3B7, *A. trotai* 1A9, and *V. campbelli* 3A8 respectively (Figure 1, Table 1). Though *V. splendidus* 3B7 shares this general trend, it does not appear to be significant ($F = 4.113$, $p = 0.114$). *A. trotai* 2H12 exhibits the opposite trend, where instead of the doubling time increasing at 29˚C, it decreases indicating an increased rate of growth (Figure 1). In addition, *V. alginolyticus* displays a trend of continually increasing doubling time as temperature increases (Figure 1).

The doubling time did not differ among the bacterial strains at 26°C,or 29°C (Figure 5, Table 5). All strains had a doubling time between 2 and 2.6 hours at these temperatures. At 32˚C, there is a significant difference among strains ($\chi^2 = 2.465$, $p < 0.001$) notably through the significant decrease in the doubling time of *A. trotai* 1A9 (Table 5).

Respiration rates were characterized by the amount of time to reduce resazurin dye by 50% at the 3 temperatures. Within each of the 8 bacterial strains, the respiration rates follows a general pattern of increased respiration at higher temperatures (Figure 2, Table 2).

At 26˚C many of the bacterial strains showed variation in their respiration rates ($\chi^2 = 43.004$, $p < 0.001$, Table 5). Differences can be seen between *V. splendidus* 1H5 and *V. campbelli* 3A8 the grouping of *V. splendidus* 3F8, 3B7, *A. trotai* 1A9, and

2H12, with *V. splendidus* 1H5 and *V. campbelli* 3A8 have lower respiration than the rest. In addition *V. alginolyticus* has an increased respiration rate that differs significantly from all other strains at all temperatures. At 29˚C, all strains with the exception of *V. alginolyticus* behave the same ($\chi^2 = 31.871$, $p < 0.001$) (Table 5) where the time it takes for the bacterial strains to reduce the resazurin salt by 50% measuring approximately 250 min (Figure 6b). Strains assayed at 32˚C displayed similar traits as those at 26˚C in comparison between strains (Table 5) where a similar grouping of *V. splendidus* 3F8, 3B7, *A. trotai* 2H12, and 1A9 were statistically similar while *V. splendidus* 1H5 and *V. campbelli* showed significantly higher times to reduce resazurin compared to the rest of the strains (Figure 6c). *V. alginolyticus*, as with the previous temperature treatments, differed from every other strain in having a decreased time to 50% reduction of resazurin indicating the highest respiration rate of all strains.

3.2 Virulence Factors

Within bacterial strains, biofilm production was correlated to temperature and was increased with increasing temperature (Table 3). *A. trotai* 1A9 was the exception to this trend and biofilm production remained constant until 32˚C where there was a significant decrease in biofilm production (($\chi^2 = 30.899$, $p < 0.001$, Figure 3). Additionally, *V. campbelli* 3A8 exhibited a deviation in this pattern where there was an increase in biofilm production from 26˚C to 29˚C and a significant decrease from 29˚C to 32˚C ($\chi^2 = 96.12$, $p < 0.001$. Figure 3, Table 3).

When comparing biofilm production among strains, there was variation at 26˚C ($\chi^2 = 46.655$, $p < 0.001$, Table 5). A statistically similar trend occurs between all *V. splendidus* strains and *A. trotai* 2H12 at 26˚C where each produces a biofilm measuring absorptions of 1-1.5 optical density units (OD units). By comparison *A. trotai* 1A9 and *V. campbelli* 3A8 both produce a relatively high biofilm mass at this temperature measuring

approximately 2.5 OD units. In contrast to all other strains, *V. alginolyticus* produces a significantly lowered biofilm at 26˚C with an optical density reading of less than 0.5 OD units (Figure 7a). Similarly the same trend occurs at 29˚C ($F = 61.315$, $p = 0.01$) where the clustering of bacterial strains remains the same. *A. trotai* 1A9 and *V. campbelli* 3A8 were found to differ significantly at 29C compared to 26˚C. At 32˚C, all strains behaved differently ($\chi^2 = 41.876$, $p < 0.001$) with optical density readings slightly below 2.0 OD units. *V. alginolyticus* produced the least amount of biofilm regardless of temperature (less than 0.5 OD units) from the other strains (Figure 7c).

Serine protease activity as detected by the fluorescent assay did not show any significant protease activity in any of the bacterial strains at any temperature.

3.3 Reactive Oxygen Recovery

Recovery time from hydrogen peroxide exposure within strains indicated a pattern of faster recovery time at 29˚C versus 26 and 32˚C for *V. splendidus* 1H5, 3B7, *A. trotai* 1A9 and *V. alginolyticus* (Figure 4, Table 4). *V. splendidus* 3F8 and *V. campbelli* 3A8 both display a similar behavior of a sharp increase in recovery time from 26˚C to 29˚C and then a slight decrease at 32˚C (Figure 4). *A. trotai* 2H12 recovered form ROS exposure faster at 32˚C than 26C or 29C ($\chi^2 = 17.967$, $p < 0.001$, Figure 4).

At 26˚C all bacterial strains recovered from ROS exposure at different rates ($\chi^2 = 35.34$, $p < 0.001$). Bacterial strains clustered into 2 groups based on their behavior. *V. splendidus* 3F8, 3B7, *A. trotai* 2H12, *V. alginolyticus*, and *V. campbelli* 3A8 all had an average recovery time of 17 to 20 hours post ROS exposure. In comparison *V. splendidus* 1H5 and *A. trotai* 1A9 had the same recovery time at 15 hours. At 29˚C more pronounced differences were apparent among strains ($F = 43.764$, $p < 0.001$) where all *V. splendidus* strains, *A. trotai* 1A9, *V. campbelli* 3A8 exhibit a recovery time of approximately 5 hours. *A. trotai* 2H12 has a significantly higher recovery time over 15

hours as well as *V. alginolyticus* recovering after approximately 10 hours. At 32˚C bacterial strains behave statistically different from each other ($\chi^2 = 106.52$, $p < 0.001$). *V. splendidus* 378 and *V. campbelli* 3A8 both have decreased recovery times compared with recovery times at 26°C and 29°C. *V. splendidus* 1H5, *V. splendidus* 3B7, *A. trotai* 2H12, and *V. alginolyticus* show similar recovery times around 15 hours while both *A. trotai* strains are statistically similar.

Chapter 4

Discussion

The goals of this experiment were to determine if there was a link between temperature and the doubling time, respiration, biofilm production, serine protease production, and reaction to the presence of hydrogen peroxide in seven strains of potentially pathogenic bacteria associated with Caribbean yellow band disease (CYBD). In addition to determining these effects within individual strains, a comparison among strains was used to see if there were any similarities between individual bacterial strains. These findings showed a significant influence of temperature on doubling time, respiration, biofilm production, and recovery from reactive oxygen species. Doubling times within the majority of the bacterial strains increased when temperature was elevated from 32°C to 29°C and dropped from 29°C to 32°C. All strains behaved the same at 26°C and 29°C however there was variation among strains at 32°C. Respiration rates were consistently increased as temperature was increased for all bacterial strains. *V. alginolyticus* took significantly less time to reduce the resazurin dye to 50% compared to other strains. Biofilm mass increased with elevated temperatures across all bacterial strains with the exceptions of *A. trotai* 1A9 and *V. campbelli* 3A8. Biofilm mass differed significantly among strains where *V. alginolyticus* displayed the least amount of biofilm mass overall. Serine protease production appeared absent in all bacterial strains at all three temperatures. Reactive oxygen species (ROS) recovery generally decreased from 26°C to 29°C and increased from 29°C to 32°C with the notable exceptions of *V. splendidus* 3F8, *A. trotai* 2H12, and *V. campbelli* 3A8. Values of ROS recovery varied significantly among bacterial strains at each temperature.

4.1 Doubling Time

Doubling time between strains at 26°C and 29°C were shown to be statistically similar with the majority of strains doubling every 2 hr at ambient temperatures. This commonality in initial doubling time length suggests that these marine bacteria share a common stable growth pattern under ambient conditions and within the coral mucosal layer, there may exist an equilibrium with surrounding non-opportunistic bacterial floral. The general trend in regards to bacterial growth was found to follow a pattern of increased doubling time when the temperature rose to 29°C. Bacterial strains exhibiting this pattern included all three strains of *V. splendidus*. Though the change in doubling time for *V. splendidus* 3B7 was found to be non-significant, it may be biologically significant indicating that there is indeed a common response in *V. splendidus* strains due to increases in temperature. Similarly this same pattern is found in *A. trotai* 1A9 and *V. campbelli* 3A8 where there is a sharp spike in doubling time at 29°C and a sharp decrease at 32°C. Collectively this suggests that there is an adaptation in the bacteria to begin slowing growth at slightly elevated temperatures.

Interestingly, it has been found in previous studies that a change in coral mucosal flora occurs at different stages of Caribbean yellow band disease (CYBD) that results in a large *Vibrio* and *Altermonas* consortium (Cróquer et al 2013). Additionally, in white band diseased *Acropora cervicornis* potential pathogens are generally found to be present in healthy coral hosts and make up the natural mucosal flora. In certain instances, these potential pathogens play key roles in nutrient cycling and acquisition (Raina 2009). During instances of the microbiota fulfilling these key roles, doubling at an initial rate of approximately every 2 hours may be a result of nutrient availability. It is known that nutrient composition within coral mucosal layers changes during heat stress in *Montipora digitata* (Fairoz 2008). If there is a large alteration in the bacterial

assemblage within the coral mucosal layer, the organisms that are the major occupiers of the environment must be growing faster than those that are being outcompeted. The findings of these growth rate assays though suggest a different scenario may be occurring within the coral host between suspected coral opportunistic pathogens in CYBD and the normal mucosal flora.

Alteration in doubling time can be explained also by the metabolic and anabolic processes occurring within the bacteria. Energy may be being allocated to different pathways resulting in phenotypic variation. Temperature fluctuations could signal certain changes to occur in regards to the regulation of certain aspects of the microorganisms' growth cycle. A similar study on pathogen growth was performed on *Vibrio* AK-1 that infects *Orbicellapatagonica* where a relatively high growth rate (0.5 generations/hr) in *Vibrio* AK-1 at 16°C was not correlated with coral bleaching (Kushmaro et al 1998). These instances may explain situations such the infection process occurring *O. patagonica* by *Vibrio* AK-1 where adhesion factors are known to play a role in recognition of the host tissue by the bacterium (Kushmaro et al 1998). In addition, it has been shown that an increase in growth rate in *Salmonella typhimurium* and in general cultures results in an increase in cell size (Schaecrter et al. 1958). If these bacterial strains do follow the same pattern, cell size would be smaller at 29°C for the majority of the microbes which would correspond to a phenotypically distinct cell type compared to 26°C and 32°C. This may explain significant changes in increased respiration rates as temperature is elevated. Respiration may be a more direct proxy for understanding the complete functioning of bacterial energy allocation during events of above average temperature fluctuations.

4.2 Respiration

Every bacterial strain assayed for rate of respiration at increased temperatures demonstrated a decrease in the amount of time to reduce the total amount of resazurin

salt by 50% of it's maximum reduction potential. The decrease in the amount of time it took to reduce the salt indicates an increase in overall respiration within each bacterium. However there was a significant difference among the rates in the bacterial strains at each temperature. Most notably was the difference seen in *V. alginolyticus* compared with the other bacterial strains. At 26°C it took *V. alginolyticus* on average under 200 min to reduce the resazurin salt by 50% while the other bacterial strains required over 290 min. This significant difference in respiration combined with the increase in doubling time as temperature increased holds a certain amount of ambiguity. *V. alginolyticus* seems to not allocate energy obtained through respiration to growth but rather diverts it presumably to other pathways. This may be explained by the stress from elevated temperatures on the bacterium itself where *V. alginolyticus* may be more sensitive to thermal fluctuations than the other bacterial strains.

The increase in respiration exhibited by all bacterial strains as temperature increased indicates that energy expenditure differs between species in regards to which pathways are upregulated. This suggests that temperature has a definite effect on the microbial consortium occupying the coral host. With an increase in energy generation, there is a high likelihood of potential pathogens adapting to the host's immune responses. In addition the ability of the potential pathogens to combat antimicrobial compounds secreted by the host's flora may be heightened. Additional virulence factors not assayed may also be upregulated through the results of increased respiration.

4.3 Biofilm Mass

A general increase in biofilm mass occurred within the majority of bacterial strains as temperature increased. Interestingly, all three bacterial strains of *V. splendidus* displayed a biologically significant increase in biofilm mass at all three temperatures as temperature was elevated. Biofilm formation in *V. splendidus* has not been studied

extensively however it is known that adhesion to surfaces plays a significant role in *V. cholera* (Vezzulli et al. 2014). In addition, the values of relative biofilm mass obtained from the absorption of crystal violet indicate that each bacterial strain of *V. splendidus* forms the same amount of biofilm at 32°C. Comparisons among strains indicate that this is in fact the case not only among the three *V. splendidus* strains but also those of *A. trotai* 1A9, 2H12, and *V. campbelli* 3A8 at 32°C. Optimal conditions for *Aeromonas caviae* biofilm formation have been previously established to be 28°C when compared at the temperatures 8°C, 28°C, 37°C, and 42°C (Angeles-Morales et al. 2012**).** This suggests that these strains reach an equal allocation of energy to biofilm production or perhaps the biofilm has already been established at the point that attachment and release reaches equilibrium.

The relatively high amount of biofilm formed in *A. trotai* 1A9 and *V. campbelli* 3A8 at 26°C and 32°C could suggest that these bacterial strains colonize the mucosal membrane more swiftly or react to commensal bacterial flora differently that the other bacterial strains. *A. trotai* 1A9 and *V. campbelli* also share a similarity in the pattern of biofilm mass where there is a sharp drop in biofilm mass between 29°C and 32°C. The high amount of initial biofilm mass relative to the other bacterial strains could be the associated with this sharp decline. By synthesizing a protective matrix at a higher rate, detachment may be facilitated much earlier than otherwise would be the case for the other bacterial strains. If assessment of biofilm mass had continued for a longer time period, attachment would occur among all strains. In addition, at these temperatures, the coral host may be secreting less or no defensive responses thus rendering the need for a protective layer unnecessary.

The general increase in biofilm presence at 29°C for all *Vibrio* species coincides with the increase in doubling time seen from 26°C to 29°C. This decrease in growth rate

yields less individual planktonic cells at 29°C. Studies in biofilm development by *Vibrio* spp. indicate that a decrease in cell density corresponds to an increase in biofilm production as a result of the secretion of signaling molecules (Zhu and Mekalanos 2003, Tu et al. 2008). The continual doubling time in *V. alginolyticus* as temperature increased may be an example of this as this coincides with a significant increase in biofilm mass. *V. alginolyticus* has significantly less production of biofilm when compared to all other bacterial strains. This may be explained by the significantly higher respiration rate of *V. alginolyticus* when comparing among bacterial strains. The energy produced from increased respiration may be redirected toward a different system instead of the production of biofilm.

The role of biofilms in coral disease outbreaks has not been extensively studied. To date it is unclear whether the effects of an extracellular polysaccharide matrix secretion contribute to the infection and subsequent exacerbation of disease symptoms within the CYBD infected community. What is known however is that microbial mats do contribute to the decline of *Pachyseris speciosa* and various *Porites* spp. in the form of red and black band disease caused by cyanobacteria (Sussman et al. 2006). In addition, a known coral pathogen (*Serratia marcesens* PDL100) which causes the disease white pox in various *Aiptasia* spp. has been found to have a significant interaction with commensalα-proteobacteria within the host's mucosal layer in the form of biofilm regulation (Alagely et al. 2011). This dynamic between *Serratia marcesens* PDL 100 and commensal bacteria exists in the form of coral microbial flora regulating the production of biofilm components and swarming behavior in the pathogen. It is reasonable to conclude that a similar system of regulation occurs in between commensal bacteria within the mucosal layer of *O. favaeolata* and potential pathogens involved in CYBD.

4.4 Serine Protease Production

The complete absence of serine protease production in all bacterial strains suggests that either I did not have the proper detection system to accurately detect them, or the secretion of serine proteases is not a constitutive response to elevated temperatures. Though proteases have been implicated in the disruption of *Symbiodinium* photosystems (Sussman et al. 2009) these were confirmed to be zinc-metalloproteases and not serine proteases. A direct assay for the presence of zinc-metalloproteases production may have yielded quite different results. Another explanation may be that without the presence of coral immune responses or a similar nutrient environment, secretion of any type of protease would be absent. If this was the case, then a nutrient medium similar to the mucosal environment of the coral host may elicit a response in the bacteria to secrete serine proteases or facilitate the export of metalloproteases.

4.5 Reactive Oxygen Species (ROS) Recovery

Thermal stress is shown to illicit a stress response in the symbiotic algae, which includes a release of harmful oxygen radicals that are not readily scavenged, by the algae or host coral (Lesser 1997, Lesser 2006). Four genes associated with oxidative stress response were upregulated in response to thermal stress in *Acropora millepora* (Csaszar 2009).

V. splendidus 1H5, 3B7, *A. trotai* 1A9, and *V. alginolyticus* followed a general trend of a decrease in recovery time when subjected to hydrogen peroxide as temperature increased. *V. splendidus* 1H5 and 3B7 display the same similar pattern between strains for ROS recovery. Interestingly *V. splendidus* 3F8 does not share the exact same trend as that found in *V. splendidus* 1H5 and 3B7 as has been found in doubling time, respiration rates, and biofilm production though *V. splendidus* 3F8 does share the same trend of a decrease in recovery time as temperature rises from 26°c to

29°C. All bacterial strains essentially share the same pattern of a decrease in the time required to recover from ROS from 26°C to 29°C. This decrease in recovery time may allow these bacterial strains to resist the secretions of reactive oxygen species by the coral host and the *Symbiodinium* elicited from thermal stress in the host.

In *V. splendidus* 3F8, *A. trotai* 2H12, and *V. campbelli* 3A8 there is a consistant decline in the time required to recover from ROS exposure. At 32°C, the time required for these bacterial strains to recover from hydrogen peroxide exposure is the lowest. Interestingly *V. splendidus* 3F8 and *V. campbelli* 3A8 display similar patterns in doubling time changes during increases in temperature with a spike in doubling time occurring at the temperature change that corresponds to the fastest decline in the time required for ROS recovery. This indicates that growth may be slowed in order to provide a preemptive defense against ROS potentially secreted by the coral host or symbiotic algae. *V. splendidus* 3F8 and *V. campbelli* 3A8 also have the fastest recovery time to the addition of hydrogen peroxide at 32°C compared to any other strains.

Differing times to recovery among strains may be explained by the fact that these bacteria are not isolated within the coral mucosal layer. It is indeed possible that the initial secretion of reactive oxygen neutralizing compounds by bacterial strains that begin recovering more rapidly at 29°C actually scavenge enough ROS that the surrounding environment normally does not contain harmful radical products. Other bacterial species would then be unlikely adapted to scavenge large amounts of ROS at this temperature. Simultaneously, those bacterial strains which increase the rate of ROS recovery at 32°C may provide indirectly the same protective influence generated by other species. Working as a consortium may benefit the microbial community and allow for continued colonization of the coral host even during bleaching.

4.6 Concluding Remarks

This investigation of growth, respiration, putative virulence factors, and recovery from reactive oxygen species in seven potential pathogenic bacterial strains associated with Caribbean yellow band disease established a foundational understanding of basic microbial processes that could contribute to the prevalence in disease in *O. faveolata*. Temperature was shown to be a significant contributor to fluctuations in doubling time, respiration, biofilm mass, and reactive oxygen species recovery in all seven bacterial strains. Doubling time was shown to generally increase at 29°C and lower at 26°C and 32°C with variations between strains at 32°C. Respiration rates were found to increase directly with elevated temperature. Biofilm mass increased with increasing temperature with the exception of two bacterial strains. Serine protease production was absent in all bacterial strains at all temperatures. Reactive oxygen species recovery was found to decrease in the time required for bacterial strains to resume normal growth in all strains with variations in behavior at 32°C. Taken together, this information suggests that there is a dynamic change in microbial processes within the mucosal layer of *O. faveolata* during increases in temperature. Overgrowth of *Vibrio* spp. may not be directly responsible for the change in the microbial consortium within the coral host. Rather it seems temperature has a regulatory effect on the production of virulence factors and defenses from host immune responses that may influence the further development of Caribbean yellow band disease (CYBD) in *O. faveolata*. This data supports previous studies finding that increasing temperatures associated with climate change have a direct influence on marine bacteria associated with coral disease.

Further work is required to ascertain the exact causes of Caribbean yellow band disease and the connection bacterial flora have on the development of disease within *O. faveolata*. Additional studies addressing the secretion of zinc metalloproteases and the

genetic basis of regulation controlling the expression of other potential virulence factors may contribute to the recognition of disease causing factors. Also studies aimed at investigating the effects of bacterial growth and secretion of potential toxic compounds on the *Symbiodinium* may be able to shed additional light on the mechanisms involved in disease progression within *O. faveolata*. The understanding of basic microbial responses is critical in designing and implementing protective conservation efforts to alleviate the effects of climate change on coral reef worldwide.

Appendix A

Figures

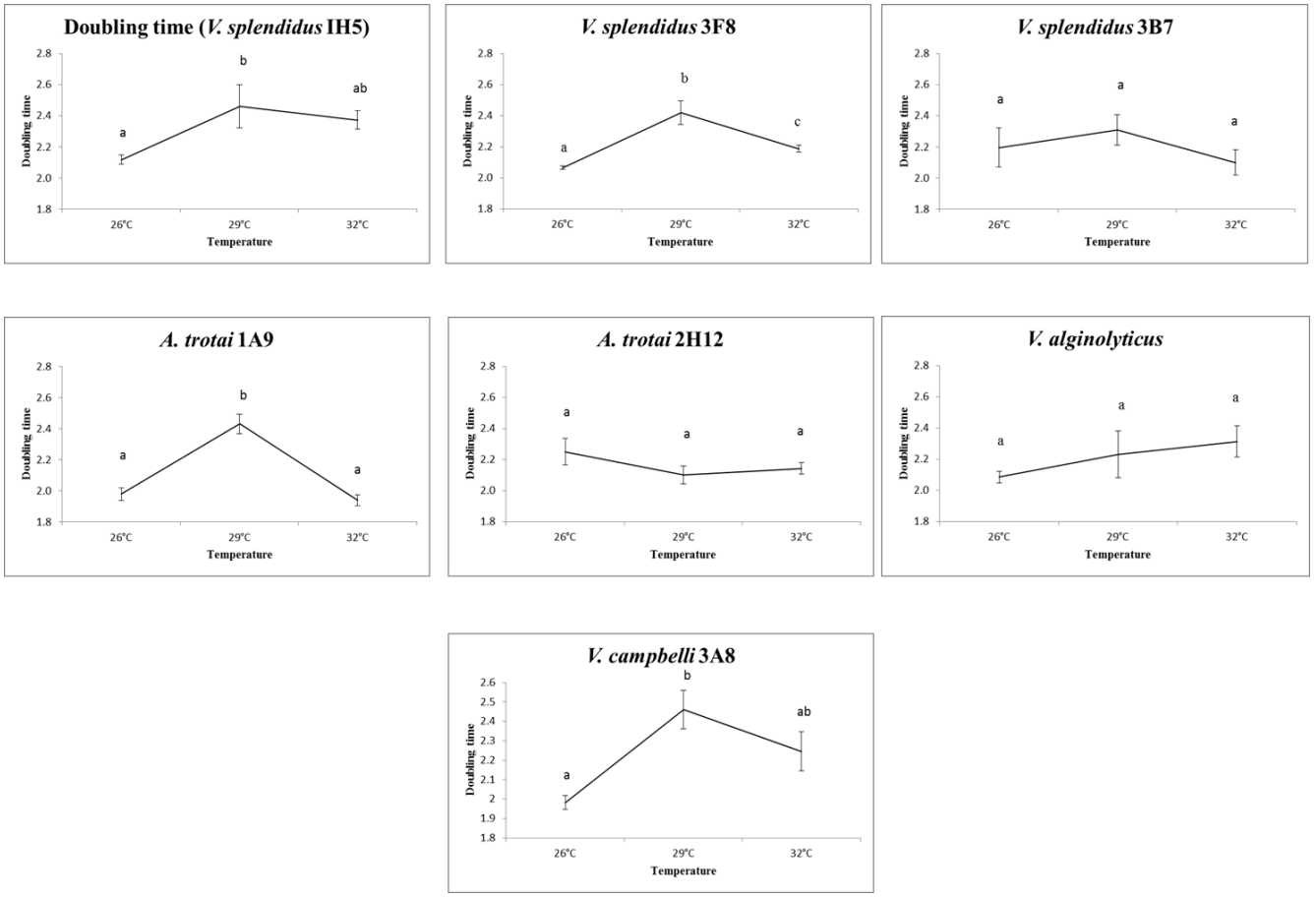

Figure 1: Mean and ± SE doubling time for all bacterial strains at 26°C, 29°C, and 32°C. Doubling times are in units of hours.

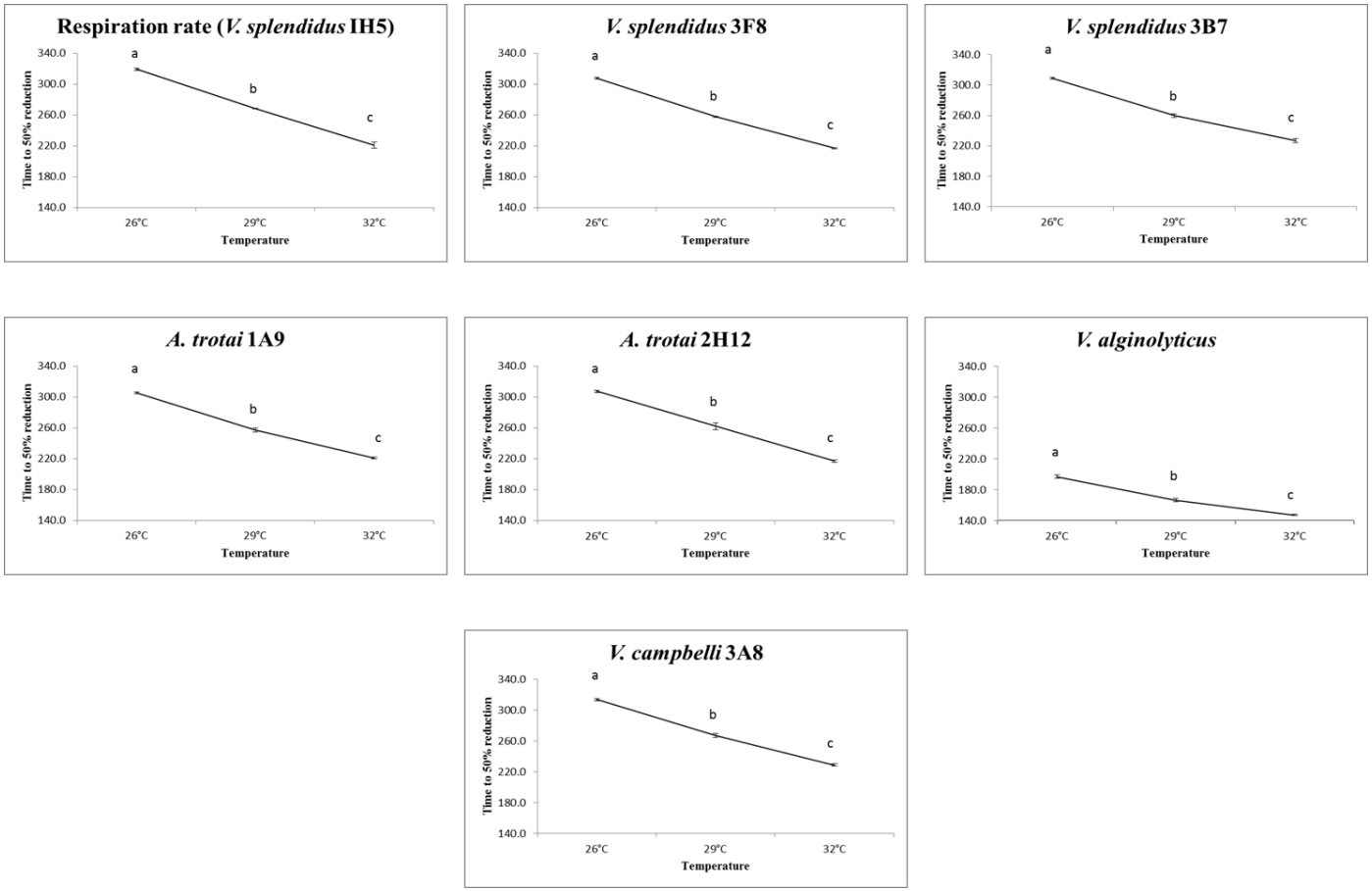

Figure 2: Mean and ± SE respiration rates for all bacterial strains at 26°C, 29°C, 32°C. Respiration times are in minutes required for the reduction of 50% of the resazurin dye.

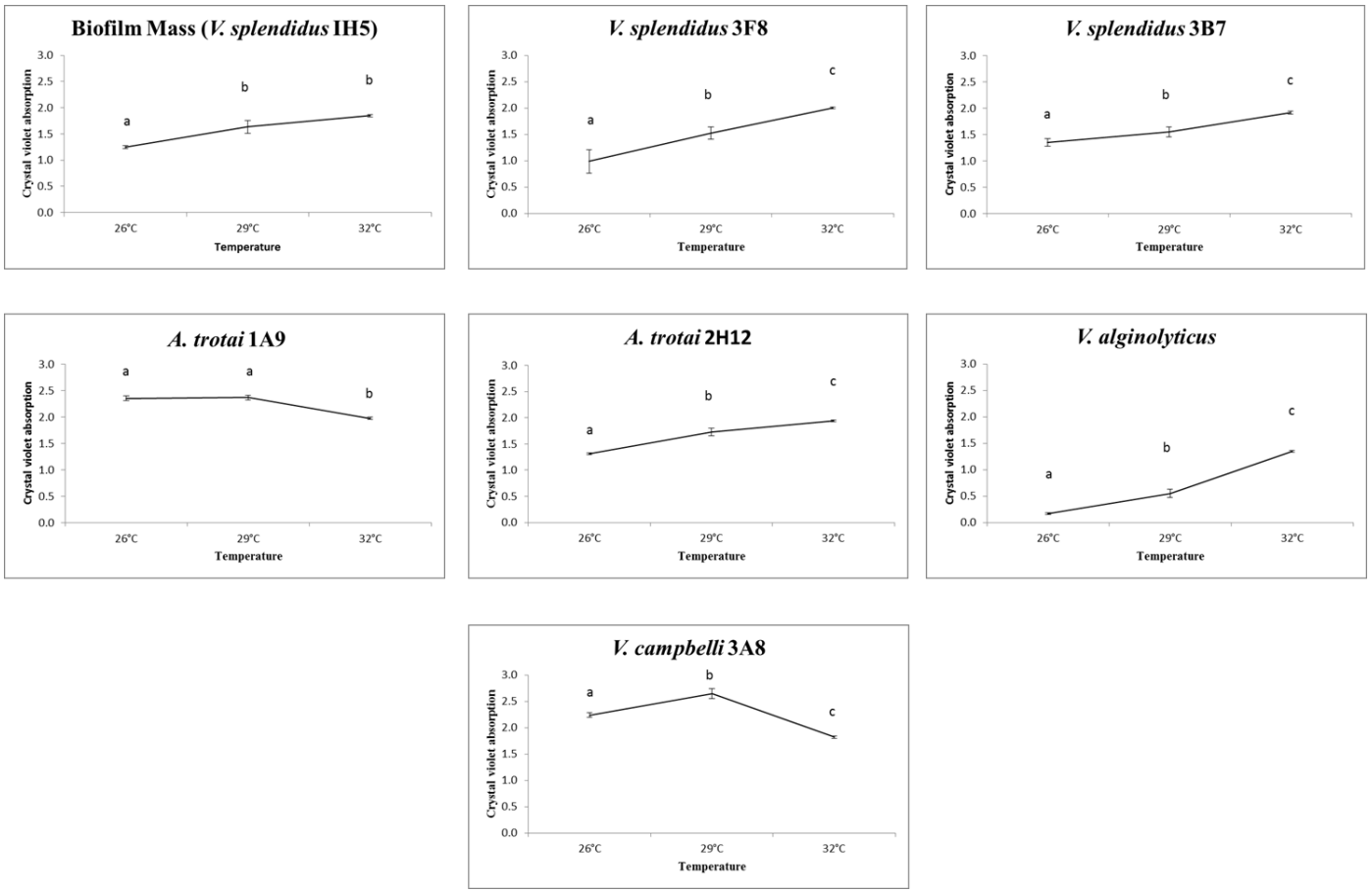

Figure 3: Mean and ± SE biofilm masses for all bacterial strains at 26°C, 29°C, 32°C. Biofilm mass is measured in optical density units.

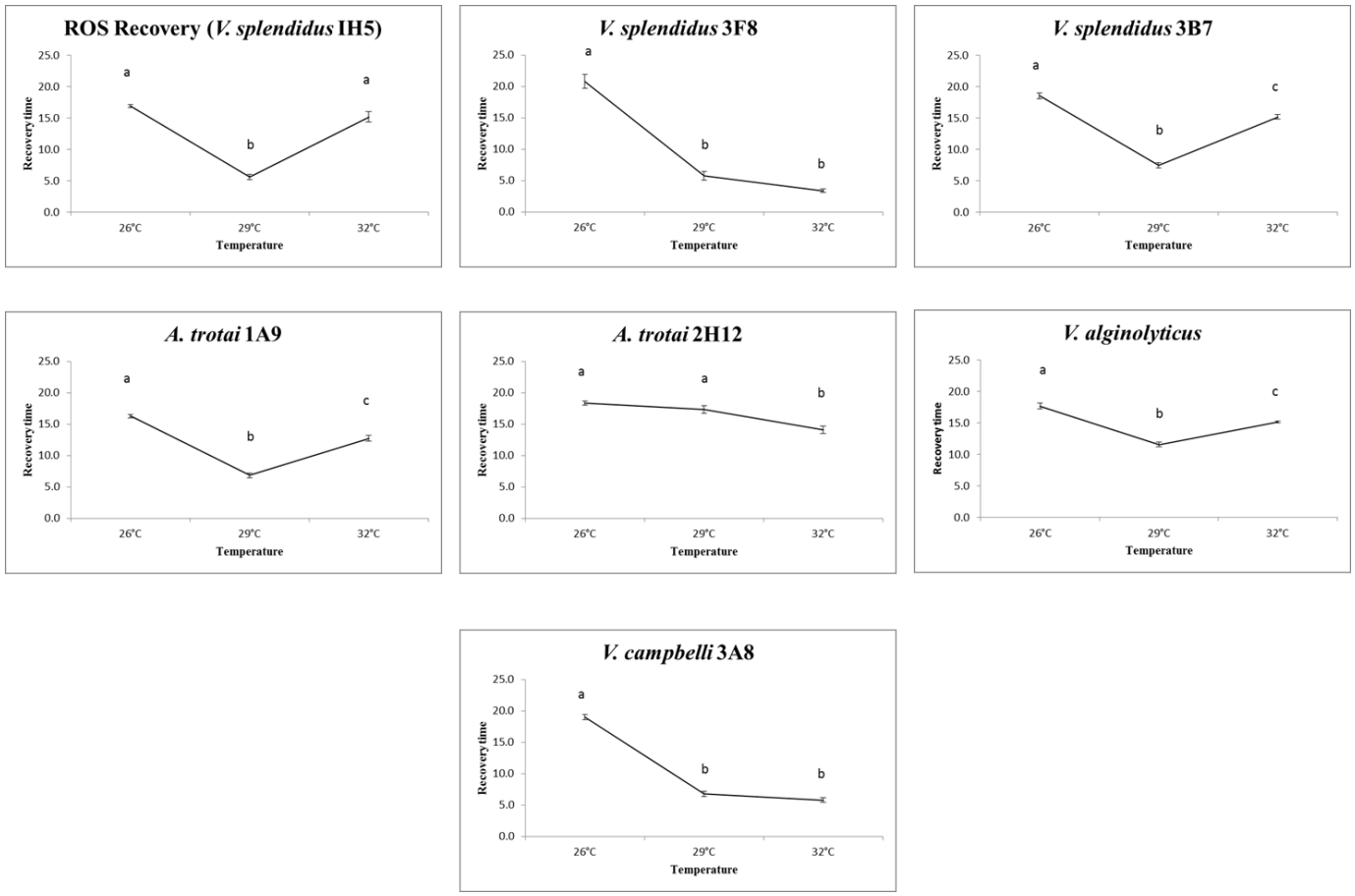

Figure 4: Mean and ± SE ROS recovery times after exposure to hydrogen peroxide for all bacterial strains at 26°C, 29°C, 32°C. Recovery times are measured in hours.

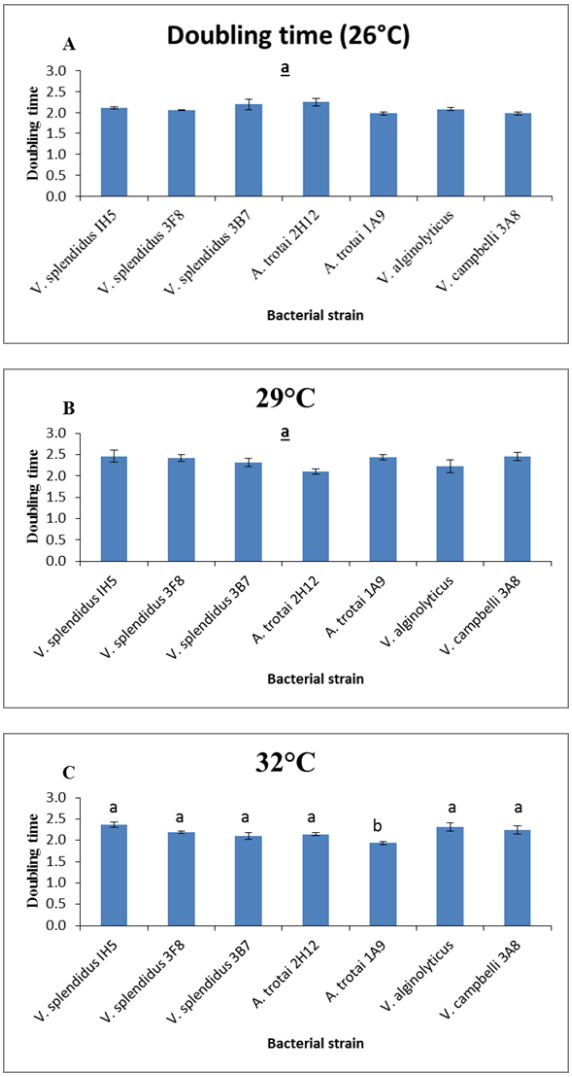

Figure 5: Mean and ± SE doubling time among all bacterial strains at 26°C, 29°C, and 32°C. Doubling times are in units of hours.

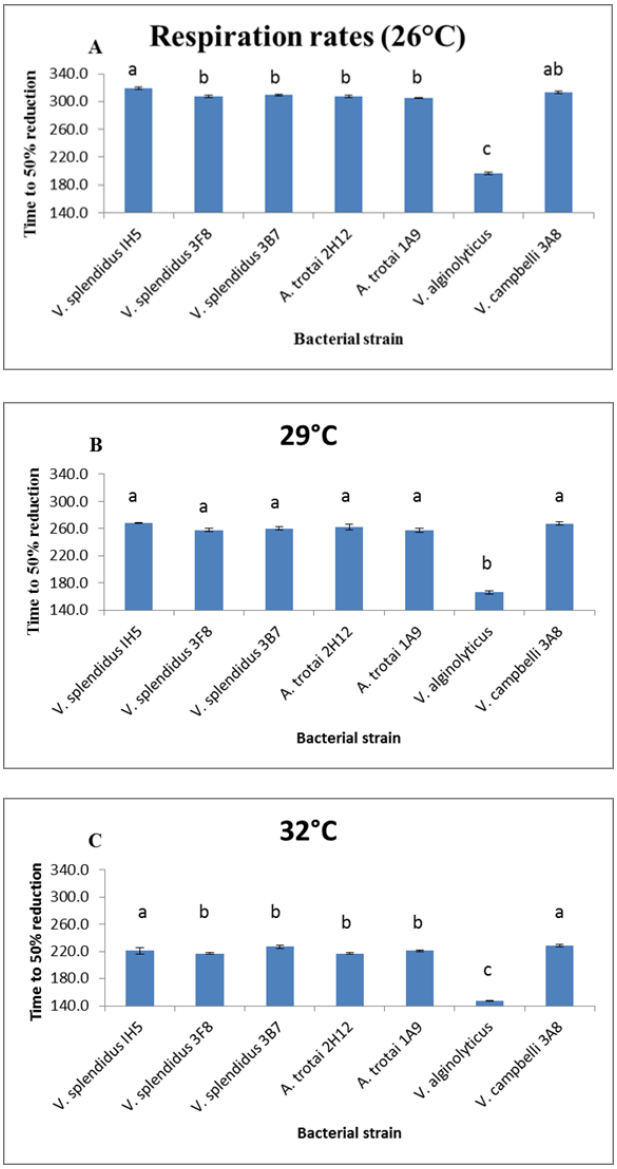

Figure 6: Mean and ± SE respiration rates among all bacterial strains at 26°C, 29°C, and 32°C. Respiration times are in minutes required for the reduction of 50% of the resazurin dye.

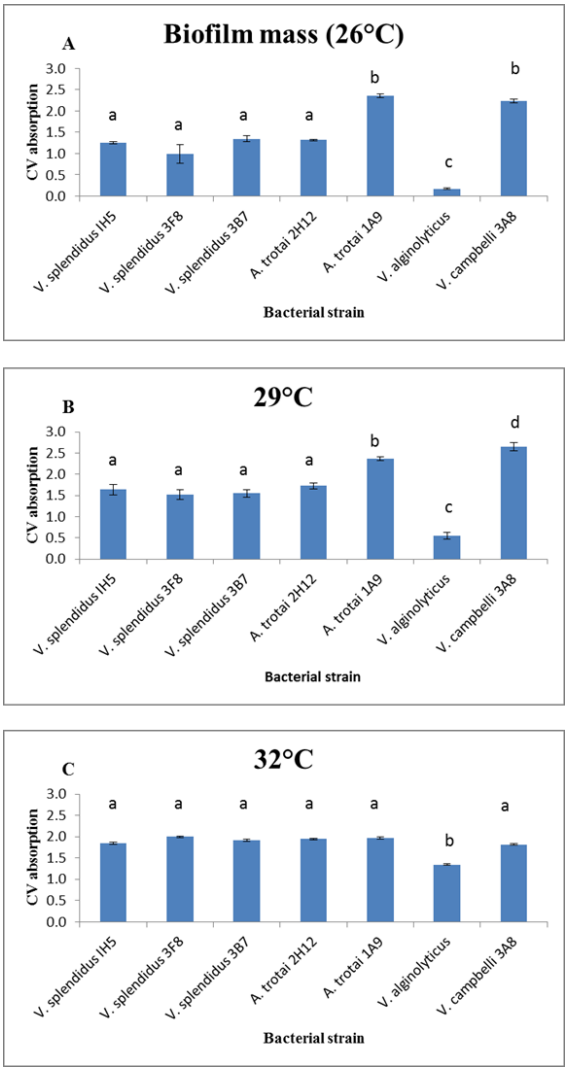

Figure 7: Mean and ± SE biofilm masses among all bacterial strains at 26°C, 29°C, 32°C. Biofilm mass is measured in optical density units.

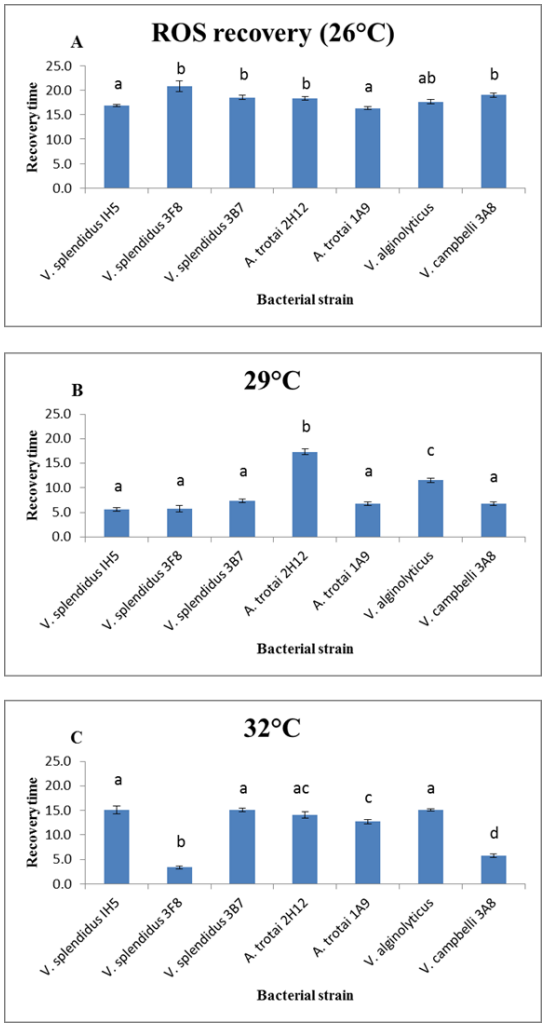

Figure 8: Mean and ± SE ROS recovery times after exposure to hydrogen peroxide among all bacterial strains at 26°C, 29°C, 32°C. Recovery times are measured in hours.

Appendix B

Tables

Table 1: Within strain comparisons of doubling time at 26°C, 29°C, and 32°C utilizing one-way ANOVAs. P-values were compared to an α-value of 0.05 for evaluating statistical significance.

	Doubling time		
Strain	F	d.f.	p
V. splendidus 1H5	4.108	2, 24	**0.029**
V. splendidus 3F8	21.438	2, 24	**<0.001**
V. splendidus 3B7	2.113	2, 23	0.114
A. trotai 2H12	1.464	2, 24	0.251
A. trotai 1A9	0.902	2, 23	**<0.001**
V. alginolyticus	4.096	2, 24	**0.029**
V. campbelli 3A8	1.685	2, 24	**0.008**

Table 2: Within strain comparisons of respiration rates at 26°C, 29°C, and 32°C utilizing Kruskal-Wallis results. P-values for non-parametric tests were compared to adjusted Bonferroni α-values of .016 to determine statistical significance.

	Respiration rate		
Strain	F or $\chi 2$	d.f.	p
V. splendidus 1H5	23.143	2, 24	**<0.001**
V. splendidus 3F8	23.143	2, 24	**<0.001**
V. splendidus 3B7	22.231	2, 24	**<0.001**
A. trotai 2H12	23.143	2, 24	**<0.001**
A. trotai 1A9	22.231	2, 24	**<0.001**
V. alginolyticus	23.143	2, 24	**<0.001**
V. campbelli 3A8	23.143	2, 24	**<0.001**

Table 3: Descriptive statistics and Kruskal-Wallis results for within strain comparisons of biofilm production at 26°C, 29°C, and 32°C. P-values for non-parametric tests were compared to adjusted Bonferroni α-values of 0.016 to determine statistical significances. Comparisons designated with an asterisk were analyzed using parametric one-way ANOVAs using box-cox transformed data.

	Biofilm production		
Strain	F or $\chi 2$	d.f.	p
V. splendidus 1H5	16.541	2, 24	**<0.001**
V. splendidus 3F8	21.438	2, 24	**<0.001**
**V. splendidus* 3B7	15.73	2, 24	**<0.001**
**A. trotai* 2H12	20.031	2, 24	**<0.001**
A. trotai 1A9	30.899	2, 24	**<0.001**
**V. alginolyticus*	23.143	2, 24	**<0.001**
V. campbelli 3A8	96.12	2, 24	**<0.001**

Table 4: Descriptive statistics and Kruskal-Wallis results for within strain comparisons of ROS recovery times at 26°C, 29°C, and 32°C. P-values for non-parametric tests were compared to adjusted Bonferroni α-values of .016 to determine statistical significances. Comparisons designated with an asterisk were analyzed using parametric one-way ANOVAs using box-cox transformed data.

	ROS recovery		
Strain	F or $\chi 2$	d.f.	p
V. splendidus 1H5	18.698	2, 24	**<0.001**
V. splendidus 3F8	21.083	2, 24	**<0.001**
V. splendidus 3B7	23.143	2, 24	**<0.001**
A. trotai 2H12	17.967	2, 24	**<0.001**
**A. trotai* 1A9	0.448	2, 24	0.644
**V. alginolyticus*	75.012	2, 24	**<0.001**
V. campbelli 3A8	18.205	2, 24	**<0.001**

Table 5: Descriptive statistics and Kruskal-Wallis results for among strain comparisons of all treatments at 26°C, 29°C, and 32°C. P-values for non-parametric tests were compared to adjusted Bonferroni α-values of .0024 to determine statistical significances. Comparisons designated with an asterisk were analyzed using parametric one-way ANOVAs using box-cox transformed data.

Comparison	temperature	F or $\chi 2$	d.f.	p
Doubling time				
	26°C	14.903	6, 56	0.021
	*29°C	2.156	6, 56	0.061
	*32°C	2.465	6, 56	**<0.001**
Respiration rate				
	26°C	43.004	6, 56	**<0.001**
	29°C	31.871	6, 56	**<0.001**
	32°C	47.076	6, 56	**<0.001**
Biofilm production				
	26°C	46.655	6, 56	**<0.001**
	*29°C	61.315	6, 56	0.01
	32°C	41.876	6, 56	**<0.001**
ROS recovery				
	26°C	35.34	6, 56	**<0.001**
	*29°C	43.764	6, 56	**<0.001**
	32°C	109.074	6, 56	**<0.001**

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